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JAPANESE BEETLE (COLEOPTERA: SCARABAEIDAE): Response to Synthetic Sex Attractant Plus Phenethyl Propionate: Eugenol[1,2]

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Abstract—A combination of the synthetic sex attractant (*R,Z*)-5-(1-decenyl) dihydro-2(3*H*)-furanone with a 3:7 mixture of phenethyl propionate (PEP) and eugenol (4-allyl-2-methoxphenol) caught significantly more *Popillia japonica* Newman than either the sex attractant or the mixture did alone. Also, the synthetic sex attractant captured significantly more males than the PEP–eugenol did during the period of heavy adult emergence of the beetles. The two lures were not significantly different in their attractancy to males about a week later and thereafter. A combination of PEP–eugenol and virgin females in the same trap late in the season also significantly increased beetle captures.

Key Words—Japanese beetle, *Popillia japonica*, Coleoptera, Scarabaeidae, sex attractant, survey lure, phenethyl propionate, eugenol, synergism.

INTRODUCTION

Chemical stimuli play an important role in the activities of the Japanese beetle, *Popillia japonica* Newman. The beetles are voracious feeders, and

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[2]Mention of a proprietary product does not constitute an endorsement by the USDA.

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odors are probably the most important factor in the beetle's selection of a host plant (Fleming, 1972). In addition, mate location is greatly enhanced by the presence of a chemical sex attractant produced by the female (Goonewardene et al., 1970; Ladd, 1970).

The beetle is a highly visible pest and is still extending its range in this country. Although effective lures have been developed, there is a continuing need for new attractants and lure combinations for use in programs of survey and pest management. The quest for general lures attracting both males and females began in 1919 (Fleming, 1969) and led to the adoption of the present stardard survey lure, phenethyl propionate (hereafter referred to as PEP) + eugenol (3:7) (Ladd et al., 1976). In addition, Ladd (1970) demonstrated that unmated female beetles emerging from the ground produce a potent, volatile pheromone and could be used to lure large numbers of males to traps. That finding led us to investigations resulting in the elucidation of the natural pheromone and the subsequent development of a powerful synthetic sex attractant, (*R,Z*)-5(1-decenyl) dihydro-2(3*H*)-furanone (hereafter referred to as *R,Z*-furanone) (Tumlinson et al., 1977). Unexpectedly, when virgin females were added as lures to traps baited with the former survey lure PEP–eugenol (7:3), the simultaneous release of the two lures resulted in a significant increase in the captures of females, but did not significantly affect the captures of males (Klein et al., 1973).

Given the availability of two distinct types of synthetic lures and the interaction between virgin females and the former survey lure, our primary objective in this study was to determine the relative attractancy to Japanese beetles of the simultaneous release of the synthetic sex attractant plus the present ratio of PEP–eugenol (3:7) used as a survey lure. Furthermore, since the effectiveness of virgin females and PEP–eugenol changes during the flight season (Klein et al., 1972), we ran our tests at several different times during the life span of the beetles to get information on lure interactions during an extended trapping season. In addition, since we only had information on the simultaneous exposure of PEP lures early in the season (Klein et al., 1973), we included a test using virgin females and PEP–eugenol late in the beetle season to establish their relative attractancy then.

METHODS AND MATERIALS

Tests were conducted in 1976 during June in North Carolina and during July and August in Ohio on golf courses infested with Japanese beetles. Yellow Ellisco® Japanese beetle traps (Ellisco Inc., Philadelphia) baited with PEP–eugenol, synthetic sex attractant, and PEP–eugenol plus sex attractant were exposed at the Buccaneer Country Club, Burgaw, North Carolina, June 24–25, and at the Mohican Hills Golf Club, Jeromesville, Ohio, July 9 and 18

and August 4. Traps baited with PEP–eugenol, virgin females, and the chemical lure plus females were used at the Echo Farms Country Club, Wilmington, North Carolina, June 24–25. Periods of heavy beetle emergence were determined by observations of males searching for females over infested turf. The traps were suspended with the upper edges of the funnels ca. 56 cm above ground on steel trap rods placed 15 m apart and were arranged in a randomized complete block with at least 30 m between the five replicates. Trap canisters were emptied at the end of each day of the tests, and the beetles were counted. Also 100 beetles from each trap were chosen at random from each day's capture, and the sex was determined so we could calculate the number of males and females captured. The data from the two days in each of the North Carolina tests were combined for analysis.

Twenty milliliter of PEP–eugenol (3:7) were placed in glass bottles in the bait well of traps and evaporated from 1.3-cm-diam cotton dental wicks that protruded 2.5 cm above the perforated metal caps. The *R,Z*-furanone was synthesized and purified (99%) at the Insect Attractants, Behavior and Basic Biology Research Laboratory by the method of Tumlinson et al. (1977). The *R,Z*-furanone was exposed by placing a stainless-steel planchet (2.5 cm diam) containing 5 μg of the attractant at the bottom of the bait well when used alone, or on top of the bait bottle next to the wick in tests using combinations of the two lures. The virgin females were collected in the field as 3rd-stage larvae and reared individually at the Japanese Beetle Research Laboratory. Recently emerged females were placed in the bait wells (4/trap) in circular wire cages as described by Klein et al. (1973). The data were analyzed statistically, and mean captures were separated at the 5% level of significance by Duncan's multiple range test.

RESULTS AND DISCUSSION

The average captures of beetles by traps baited with PEP–eugenol, *R,Z*-furanone, and PEP–eugenol/*R,Z*-furanone for a period during heavy emergence of the beetles are given in Table 1. Although the sex attractant was over 3.5 times as attractive to males as PEP–eugenol, simultaneous release of the two lures captured 1.6 times more beetles than the sex attractant alone. In addition, it resulted in a significantly better lure (1.9×) than the PEP–eugenol for capturing females, even though traps baited only with the *R,Z*-furanone captured few females. Furthermore, simultaneous release increased captures of males, females, and total beetles over the combined captures by the two lures separately.

The average numbers of beetles captured after the heaviest emergence (July 18 in Ohio) and very late in the beetle season (June 24–25 in North Carolina and August 4 in Ohio) by the survey lure, the sex attractant, and both

TABLE 1. CAPTURES OF JAPANESE BEETLES IN TRAPS BAITED WITH PEP–EUGENOL, SYNTHETIC SEX ATTRACTANT (*R,Z*-FURANONE), AND BOTH DURING HEAVY BEETLE EMERGENCE (JULY 9, 1976, IN OHIO, 5 REPLICATES)

Lure	Avg. beetle catch/trap[a]		
	Males	Females	Total
PEP–eugenol/ *R,Z*-furanone	1845a	662a	2507a
R,Z-furanone	1122b	64b	1186b
PEP–eugenol	304c	348c	652c

[a]Means in the same columns followed by the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

combined are given in Table 2. Throughout the season, PEP–eugenol/ *R,Z*-furanone was a significantly better lure for both males and females than the two lures used independently. The abilities of sex attractant and PEP–eugenol to attract males about a week after the heavy beetle emergence, late in the season, were not significantly different. The decrease in attractiveness of the synthetic sex attractant after the period of heavy emergence confirms our observations over several years that males are most strongly attracted to virgin females and their extracts during the 1- to 2-week period of maximum beetle emergence.

A stronger response of males to virgin females rather than PEP–eugenol noted in a previous field test (Klein et al., 1972) may have been due to the changes in design (RCB instead of *t* test) used in these experiments, change of placement of traps, use of small quantities of volatile pheromone instead of females, or use in the present test of a more attractive ratio of PEP–eugenol (3:7 rather than 7:3) (Ladd et al. 1976). However, since survey trapping covers an entire season, it is important to note that the presence of *R,Z*-furanone in traps with PEP–eugenol significantly increased captures in tests conducted one month apart. The sex ratios of beetles captured at Burgaw, North Carolina, in traps baited with PEP–eugenol dropped from 1.3:1 (M:F) on June 24 to 0.75:1 (M:F) on June 25. This decrease suggests that a very high percentage of males in the area was captured the first day and that few, if any, additional males moved into the area by the second day. This result may have been caused by male trapping elsewhere on the golf course and natural mortality at the end of the beetle season.

A combination bait of virgin females plus PEP–eugenol late in the beetle season significantly increased captures of males, females, and the total beeetles over the number captured by either lure alone (Table 3). The ability of the virgin females to attract males late in the season was only about 0.6 times as greast as the ability of PEP–eugenol. The lack of attractancy of the females

TABLE 2. CAPTURES OF JAPANESE BEETLES IN TRAPS BAITED WITH PEP-EUGENOL, SYNTHETIC SEX ATTRACTANT (*R,Z*-FURANONE AND BOTH AFTER PERIOD OF MAXIMUM BEETLE EMERGENCE DURING JUNE IN NORTH CAROLINA AND JULY AND AUGUST IN OHIO (5 REPLICATES)

	Avg. beetle catch/trap[a]								
	June 24–25[b]			July 18[c]			August 4[b]		
Lure	Males	Females	Total	Males	Females	Total	Males	Females	Total
PEP-eugenol/*R,Z*-furanone	1420a	745a	2166a	1799a	896a	2695a	508a	341a	849a
PEP-eugenol	474b	385b	859b	732b	400b	1138b	276b	164b	440b
R,Z-furanone	180b	15c	194b	665b	26c	691b	70b	19c	89c

[a]Means in the same columns followed by the same letter are not significantly different at the 5% level (Duncan's new multiple range test).
[b]3–4 weeks after heavy beetle emergence.
[c]Approximately 1 week after heavy beetle emergence.

TABLE 3. CAPTURES OF JAPANESE BEETLES IN TRAPS BAITED WITH PEP–EUGENOL, VIRGIN FEMALES, AND BOTH ON JUNE 24 AND 25, 1976, IN NORTH CAROLINA (5 REPLICATES)

Lure	Avg. beetle catch/trap[a]		
	Males	Females	Total
PEP–eugenol/females	430a	836a	1265a
PEP–eugenol	182b	459b	641b
Females	108b	7c	115c

[a]Means in the same columns followed by the same letter are not significantly different at the 5% level (Duncan's new multiple range test).

(natural sex attractant), together with their ability to improve the PEP–eugenol as a lure, is consistent with what we found for the *R,Z*-furanone (synthetic sex attractant).

The synergism of *R,Z*-furanone and PEP–eugenol will have immediate applications in survey programs for Japanese beetles by providing a much more effective lure throughout the trapping season. In addition, the unique combination of lures for both males and females and the availability of a synthetic sex attractant, *R,Z*-furanone, and an inhibitor, *S,Z*-furanone (Tumlinson et al. 1977), offers a system for studies on control, disruption, attraction, and other aspects of the chemical ecology of the Japanese beetle.

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INTERSPECIFIC EFFECTS OF PHEROMONES ON THE ATTRACTION OF THE BARK BEETLES, *Dendroctonus brevicomis* AND *Ips paraconfusus*[1] IN THE LABORATORY

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Abstract—*Dendroctonus brevicomis* was attracted to a mixture of the *Ips paraconfusus* pheromones, ipsenol, *cis*-verbenol, and ipsdienol at 10^{-9} g each/μl but was not attracted to these pheromones at higher and lower release rates. *I. paraconfusus* was not attracted to the *D. brevicomis* pheromones *exo*-brevicomin, frontalin, and myrcene at any release rate tested. Increased release rates of a mixture of the three pheromones of *I. paraconfusus* inhibited the attraction of *D. brevicomis* to its synthetic pheromones. A mixture of ipsenol + ipsdienol or *cis*-verbenol alone failed to cause inhibition indicating that at least two of the *I. paraconfusus* pheromones are required to inhibit the response of *D. brevicomis*. The pheromones of *D. brevicomis* did not inhibit the attraction of *I. paraconfusus* to its pheromones; however, verbenone was a potent inhibitor.

Key Words—*Dendroctonus brevicomis, Ips paraconfusus,* Coleoptera, Scolytidae, *Pinus ponderosa*, bark beetle, *exo*-brevicomin, frontalin, myrcene, verbenone, ipsenol, ipsdienol, *cis*-verbenol, attractants, inhibition, semiochemicals, pheromones.

INTRODUCTION

In California *D. brevicomis* LeConte and *I. paraconfusus* Lanier colonize the phloem–cambium tissues of ponderosa pine, *Pinus ponderosa* Laws.,

[1] Coleoptera: Scolytidae. This paper is based in part upon a dissertation submitted by J.A.B. to the University of California, Berkeley, in partial fulfillment of the requirements for the Ph.D. degree in entomology, June 1978. These studies were supported by the Rockefeller Foundation; USDA Forest Service; Regional research project W-110, SEA/USDA; and the National Science Foundation and Environmental Protection Agency through a grant (NSF GB-34719/BMS 75-04223) to the University of California. The findings, opinions, and recommendations are not necessarily those of the University of California or the funding agencies.

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during the same period of time. Byers and Wood (1980) found that the attraction of both species to their naturally produced pheromones was inhibited by the presence of the other species feeding in logs of the host tree. The function of this mutual inhibition is believed to reduce interspecific competition for food and space. They showed that the response of *D. brevicomis* to its synthetic pheromones, *exo*-brevicomin (E) and frontalin (F) and the host terpene, myrcene (M) (Silverstein et al., 1968; Kinzer et al., 1969) was inhibited by the presence of logs infested with male *I. paraconfusus*. Byers and Wood (1980) also found that E + F + M had no effect on the attraction of *I. paraconfusus* to its natural pheromone. However, they established that synthetic verbenone inhibited the response of *I. paraconfusus* to its pheromone. Furthermore, they showed that verbenone was present in male *D. brevicomis* feeding in logs cut from pheromone-baited trees that had inhibited the response of *I. paraconfusus* to its pheromone. However, they did not test the pheromones of *I. paraconfusus*, ipsenol (I), *cis*-verbenol (II), and ipsdienol (III) (Silverstein and Rodin, 1965; Silverstein et al., 1966; Wood et al., 1966) as possible inhibitors of the response of *D. brevicomis* to its pheromone or as attractants for *D. brevicomis*, nor did they test for inhibition of *I. paraconfusus* attraction to I + II + III by verbenone. The objectives of this study were to investigate the responses of these beetles to semiochemicals, both intra- and interspecific, under laboratory conditions.

METHODS AND MATERIALS

Both *I. paraconfusus* and *D. brevicomis* were collected from the Sierra National Forest near Bass Lake, Madera County, California, at an approximate elevation of 1000 m. The *D. brevicomis* were obtained by removing bark from naturally attacked ponderosa pines, while *I. paraconfusus* were obtained from infested logging debris. The rearing, preparation, and determination of sex were as described in Byers and Wood (1980).

The responses of *D. brevicomis* and *I. paraconfusus* to semiochemicals were tested in the laboratory olfactometer developed by Browne et al (1974) for *I. pini* Say. However, several modifications were used. The polyurethane foam was removed from the plexiglass manifold to maintain the air speed at 0.9 m/sec at the semiochemical source and 0.6 m/sec where the beetles were released (21 ± 2 cm "downwind"). A positive response was recorded when a beetle arrived within 1 cm of the attractive source in the time required for various mixtures of the semiochemicals in diethyl ether to elute from a 5-μl capillary tube (126 ± 10 sec). The release rate of semiochemicals in the bioassay (g/min) from the 5-μl capillary pipette was estimated to be 2.2 times

the concentration of the starting solution (g/μl), assuming that the compounds were released in proportion to the volume reduction of the solvent. However, the actual release rates of compounds from the pipette probably was not linear due to chemical interactions and different rates of distillation (vapor pressures) of the semiochemicals and the solvent. At least 30 beetles of each sex were tested for each release rate of the compounds. Differences in the percent responding between various release rates were determined by a chi-square test.

Both sexes of *D. brevicomis* and *I. paraconfusus* was tested for interspecific attraction to the appropriate synthetic pheromones: I + II + III (each >98%) (Figure 1) or E (>95%) + F (>95%) + M (>99%) (Figure 2). *I. paraconfusus* were also tested for their response to verbenone (>99.8%, GLC purified) (Figure 2). I, III, E, and F were all racemic, the enantiomeric composition of II was not known, and verbenone was $[\alpha]_{25}^{D} = +90°$. All compounds were obtained from Chemical Samples Co., Cleveland, Ohio.

The three pheromones of each species must be released simultaneously for maximum attraction in the laboratory assay (Wood et al., 1967, 1968, 1976; Silverstein et al., 1968; Wood, 1970; Byers et al., 1979). Each sex of *D. brevicomis* was tested for attraction to E + F + M to determine a release rate that elicited an approximate 50% response (Figure 3). *D. brevicomis* then were tested at this concentration (10^{-9} g E + F + M/μl) for inhibition of their response by increasing the release rate (g/μl) of either I + II + III (Figure 4), I + III, or II (Table 1). Similarly, each sex of *I. paraconfusus* was tested for

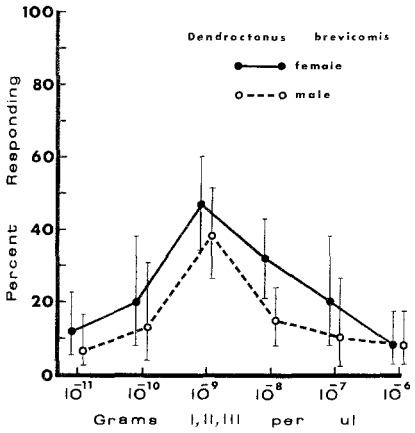

FIG. 1. Response of male and female *Dendroctonus brevicomis* to ipsenol (I), *cis*-verbenol (II), and ipsdienol (III) at 10^{-11} to 10^{-6} g each/μl (October 5–7, 1976). Brackets represent 95% binomial confidence limits.

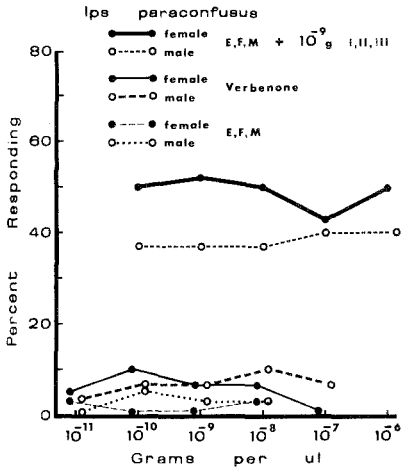

FIG. 2. Response of male and female *Ips paraconfusus* to *exo*-brevicomin (E), frontalin (F), and myrcene (M) at 10^{-11} to 10^{-8} g each/μl; ipsenol (I), *cis*-verbenol (II), and ipsdienol (III) at 10^{-9} g each/μl in mixtures with E, F, M at 10^{-10} to 10^{-6} g each/μl; and verbenone alone at 10^{-11} to 10^{-7} g/μl (July 28-29, 1976).

inhibition of their response to 10^{-9} g I + II + III/μl by increasing the release rate of either E + F + M (Figure 2) or verbenone (Figure 5).

Since verbenone is structurally related to II and thus might compete for acceptor sites of II on the antennae of *I. paraconfusus*, we wanted to know how much II was required to significantly enhance the beetles' attraction to

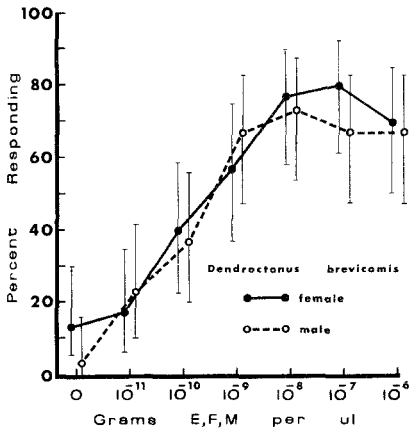

FIG. 3. Response of male and female *Dendroctonus brevicomis* to *exo*-brevicomin (E), frontalin (F), and myrcene (M) at 0 and 10^{-11} to 10^{-6} g each/μl (October 8, 1976). Brackets represent 95% binomial confidence limits.

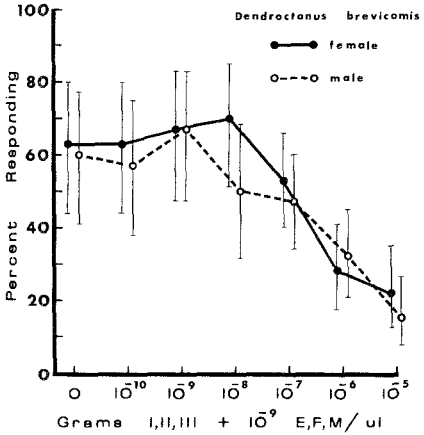

FIG. 4. Response of male and female *Dendroctonus brevicomis* to *exo*-brevicomin (E), frontalin (F), and myrcene (M) at 10^{-9} g each/μl in mixtures with ipsenol (I), *cis*-verbenol (II), and ipsdienol (III) at 0 and 10^{-10} to 10^{-5} g each/μl (October 11–12, 1976). Brackets represent 95% binomial confidence limits.

TABLE 1. RESPONSE OF MALE AND FEMALE *Dendroctonus brevicomis* TO *exo*-BREVICOMIN (E), FRONTALIN (F), AND MYRCENE (M) IN VARIOUS MIXTURES WITH IPSENOL (I), *cis*-VERBENOL (II), AND IPSDIENOL (III) (OCTOBER 16, 1976).

Compounds tested	Dose (g/μl)	Sex	Number tested	Percent responding	Confidence interval (95%)
E, F, M	10^{-9}	♀	90	63	52–73
		♂	120	68	59–76
E, F, M + I, III	10^{-9}	♀	30	87	69–97
	10^{-6}	♂	30	83	64–94
E, F, M + II	10^{-9}	♀	60	53	40–68
	10^{-6}	♂	60	60	46–72
E, F, M + I, II, III	10^{-9}	♀	90	30	20–41[a]
	10^{-6}	♂	90	31	21–42[a]

[a]Significantly different from above treatments ($P < 0.05$).

I + III. This was determined by testing female response to mixtures of a 10-fold concentration series of II from 10^{-13} to 10^{-9} g/μl with 5×10^{-9} g each I + III/μl.

RESULTS

Both sexes of *D. brevicomis* responded in significantly greater proportions to I + II + III at 10^{-9} g each/μl than to these pheromones released at either higher or lower rates (Figure 1). The percent of males responding at 10^{-9} g/μl was different than the percent responding at 10^{-11}, 10^{-7}, or 10^{-6} g I + II + III/μl ($P < 0.05$). The percent of females responding at 10^{-9} g/μl was different than at either 10^{-11}, 10^{-10}, or 10^{-6} g I + II + III/μl ($P < 0.05$). In contrast, *I. paraconfusus* was not attracted to E + F + M or verbenone at any concentration tested ($P > 0.1$) (Figure 2).

Both sexes of *D. brevicomis* responded similarly to each release rate of E + F + M. The percent of males and females responding to 10^{-10} and 10^{-9} g E + F + M/μl, respectively, and all higher rates were significantly greater than the percent responding to solvent controls ($P < 0.05$). There were no significant differences in percent responding between the sexes at any concentration of E + F + M ($P > 0.1$) (Figure 3). The response of both sexes of *D. brevicomis* to E + F + M was inhibited by I + II + III released at 10^{-6} and 10^{-5} g each/μl compared to 0, 10^{-10}, and 10^{-9} g each I + II + III/μl ($P < 0.05$) (Figure 4). Neither I + III nor II inhibited the response of *D. brevicomis* to E + F + M (Table 1) which indicates a mixture of at least two (II + I or II + III) and possibly all three pheromones of *I. paraconfusus* are necessary.

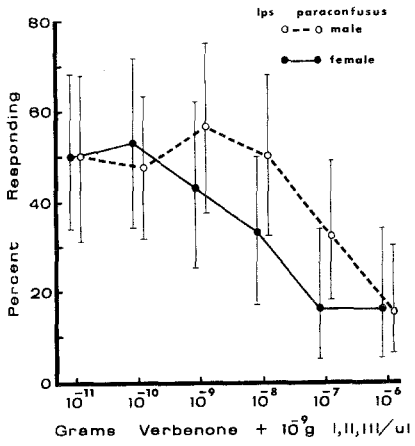

FIG. 5. Response of male and female *Ips paraconfusus* to ipsenol (I), *cis*-verbenol (II), and ipsdienol (III) at 10^{-9} g each/μl in mixtures with verbenone at 10^{-11} to 10^{-6} g/μl (July 27–28, 1976). Brackets represent 95% binomial confidence limits.

The response of either sex of *I. paraconfusus* to 10^{-9} g I + II + III/μl was unaffected by E + F + M at concentrations of 10^{-10} g to 10^{-6} g/μl (Figure 2). However, verbenone at 10^{-7} g to 10^{-6} g/μl was effective in inhibiting the response of both sexes of *I. paraconfusus* to I + II + III (Figure 5). The percent of males responding to I + II + III at 10^{-6} g verbenone/μl was significantly less than the percent responding at 10^{-11} or 10^{-10} g/μl ($P < 0.05$). The percent of females responding to I + II + III at 10^{-7} or 10^{-6} g verbenone/μl was significantly less than the percent responding at 10^{-11} or 10^{-10} g/μl ($P < 0.05$) (Figure 5).

The response of female *I. paraconfusus* to 5×10^{-9} g each I + III/μl was increased from 25% (95% binomial confidence limits, BCL, 16% and 38%) to 57% (BCL 38% and 75%) (different at $P < 0.05$) by adding as little as 10^{-10} g II/μl to the I + III mixture.

DISCUSSION

Dethier (1947) and Dethier et al. (1952) described a phenomenon in which a gustatory response to an optimum dosage of a stimulant could be lowered by either increasing or decreasing the dosage. A similar relationship has been shown for *D. frontalis* Zimm. and *D. pseudotsugae* Hopk., where response to a certain release rate of a pheromone was lowered by both increasing and decreasing the release rate of the intraspecific compounds, verbenone or 3-methyl-2-cyclohexen-1-one (MCH), for each species, respectively (Rudinsky 1973a,b). We report the first instance of this type of response curve for interspecifically active compounds, i.e., I + II + III at 10^{-9} g each/μl was attractive to *D. brevicomis* but either lower or higher release rates were not (Figure 1). Further experiments with various mixtures of I, II, and III are needed to elucidate which compounds are involved. In contrast, *I. paraconfusus* was not attracted to either E + F + M or to verbenone (Figure 2). There have been no reports of *I. paraconfusus* trapped in the field at sources of naturally produced or synthetic pheromones of *D. brevicomis*. The attraction of *D. brevicomis* to I + II + III in our laboratory studies, but apparently not to natural or synthetic pheromones in the field (Wood et al. 1968, 1976), may be explained by a high release rate of the pheromones thus inhibiting the beetle before encountering the trap or by differences in the walking and flight response to I + II + III.

Struble and Hall (1955) and Miller and Keen (1960) have summarized several reports that *I. paraconfusus* may precede *D. brevicomis* in the successful colonization of a ponderosa pine. The attraction of *D. brevicomis* to trees that were top-killed by *I. paraconfusus* also appeared to be greater than to trees that had their tops removed (Miller and Keen, 1960). *D. brevicomis* may exploit weakened and more susceptible hosts by responding to the pheromone produced by *I. paraconfusus* which is believed to be a less aggressive tree-killer. However, field experiments utilizing various release

rates of synthetic and naturally produced pheromones are required before this sequence of host selection can be attributed to interspecific attraction.

The proportion of *D. brevicomis* responding to a constant dose of E + F + M was reduced as the release rate of I + II + III was increased (Figure 4). The inhibition occured only at the two higher release rates tested, which suggests that *D. brevicomis* would be inhibited in the field only at close range to substrates containing a mixture of these species. The inhibition of attraction of *D. brevicomis* to naturally infested substrates and synthetic pheromones in the field by logs infested with *I. paraconfusus* males (Byers and Wood, 1980) may be due, at least in part, to the release of I + II + III from these logs. In this regard, the laboratory assay has provided the first evidence that a mixture of at least two compounds is necessary to cause this interspecific inhibition.

The response of *I. paraconfusus* to male-produced pheromone was not inhibited by E + F + M in the laboratory. This supports the field observation where E + F + M did not inhibit the response of *I. paraconfusus* to male-infested logs (Byers and Wood, 1980). However, in the present study, verbenone at relatively high release rates inhibited the response of *I. paraconfusus* to I + II + III (Figure 5), which complements the field results where verbenone inhibited the attraction of *I. paraconfusus* to naturally produced pheromone (Byers and Wood, 1980). *I. paraconfusus* probably would be attracted in flight to a tree under colonization by both species but inhibition of *I. paraconfusus* would increase as the beetles approached the *D. brevicomis*-infested areas where the concentration of verbenone is highest.

Certain sensory cells on the antennae of *I. paraconfusus* and *I. pini* are known to be sensitive to several monoterpenes (verbenone, II, and *trans*-verbenol) that are structurally related (Mustaparta, 1979). It is possible that less evolutionary change in *D. brevicomis* and *I. paraconfusus* would be necessary to acquire sensory systems responsive to interspecific compounds, if each species utilized compounds structurally similar to their own pheromones. Thus, the acceptor sites for myrcene (2-methyl-6-methylene-2,7-octadiene) on the antennae of *D. brevicomis* may have evolved into new sites capable of accepting the structurally similar I (2-methyl-6-methylene-7-octene-4-ol) and/or III (2-methyl-6-methylene-2,7-octadiene-4-ol). Similarly, the *D. brevicomis* acceptor site for the intraspecific inhibitor verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) (Bedard et al., 1980) may have evolved into new sites capable of accepting structurally similar II (*cis*-verbenol = 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol). *I. paraconfusus* may have evolved acceptor sites for verbenone from acceptor sites for II.

On the other hand, verbenone may compete with II for the acceptor sites of II in *I. paraconfusus* so that the beetle effectively can respond only to I + III which has been shown to be essentially unattractive in the field (Wood et al., 1967, 1968). However, in competitive interactions verbenone would probably have to have a much stronger affinity for the acceptor site than II to

cause the observed inhibition of the behavioral response since as little as 10^{-10} g II/μl significantly increased the response of female *I. paraconfusus* to 5×10^{-9} g/μl each of I + III. Further, the enhancement of attraction by adding II to I + III does not support the hypothesis of Kikuchi and Ogura (1976), based on molecular binding-site models, that II interacts with acceptor sites in a similar conformational way as III or by the conversion of III into II.

Verbenone primarily from male *D. brevicomis* (Renwick, 1967; Byers and Wood, 1980), I + II + III from male, and II from female (Renwick et al., 1976) *I. paraconfusus* appear to cause, at least in part, the observed interspecific inhibition of the responses of these species to their pheromones. We do not know how closely the release rates of I, II, III, and verbenone reported in our study represent the release rates from infested pine substrates in nature. However, Browne et al. (1979) have quantified the release rates of E, F, and M per beetle per day in a tree as 4.1×10^{-6} g E, 8.6×10^{-7} g F, and 4.1×10^{-4} g M. Our release rates were estimated to range from 3×10^{-8} to 3×10^{-3} g/day (Figure 3). Inhibition of the response of *D. brevicomis* to naturally produced pheromone by I + II + III has not been tested in the laboratory or field, nor has the test of inhibition of response to E + F + M by I + II + III been conducted in the field. Further work would be necessary to elucidate the role or each semiochemical at the enantiomeric level (Wood et al., 1976; Borden et al., 1976). Verbenone and I + II + III may prove useful in inhibiting aggregation and host colonization by *I. paraconfusus* and *D. brevicomis* and thus function to reduce tree mortality caused by these bark beetles.

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A CROSS-CULTURAL STUDY ON THE ATTITUDE TOWARDS PERSONAL ODORS

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Abstract—Human axillary odor was used in testing the ability of male and female subjects to distinguish between gender and individuals. The subjects also gave a qualitative evaluation of the odors. The tests were carried out in Japan, Italy, and Germany. Of all three cultures, 80% of the participants could significantly distinguish among the odor of individuals; 50% could identify the person correctly to whom the recognized odor belonged. Discrimination between male and female odor was significantly shown by 20% of Italian, 30% of German, and 60% of Japanese subjects. The qualitative evaluation of male and female odor was the same in the three cultures: male odor was classified more unpleasant and less pleasant than female odor. Men classified their own odor more unpleasant than women did with their own. A cultural difference was found concerning partner's odor: though men classified it alike (predominantly pleasant), women differed. Japanese and Italian women classified their partner's odor predominantly unpleasant, German women predominantly pleasant. In general the Japanese subjects classified the odors less often pleasant than the Italian and German subjects did.

Key Words—Axillary odor, nonverbal communication, human body odor.

INTRODUCTION

Due to the belief that the sense of smell doesn't play an important role in human nonverbal communication, research in this field has been neglected. Although nonhuman primates can recognize conspecific individuals by odor (Holst and Lesk, 1975, for *Tupaia;* Mertl, 1975, for lemurs; Kaplan, 1977, for saimiris), little data are available for humans. We know that blind and deaf-blind children can distinguish familiar persons from unfamiliar ones on the

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basis of smell (Eibl-Eibesfeldt, 1973) and that newborns can distinguish the smell of their mothers from the smell of other women (McFarlane, 1975). Similar abilities have been observed in kindergarten age children (Montagner and Henry, 1974). In adults the capacity to distinguish individuals by their personal odor also appears to be present, at least in American and German samples (Russell, 1976; Hold and Schleidt, 1977; McBurney et al., 1977). In these experiments axillary odor has been used, this being the main source of personal odor.

Hold and Schleidt (1977) found women to be more able to recognize conspecifics by olfaction than men, perhaps reflecting their relatively lower olfactory threshold (Mattei, 1901; Elsberg, 1937; Le Magnen, 1948; Moncrieff, 1951; Koelega and Köster, 1974).

Subjects classified male odor more often unpleasant and female odor more often pleasant (Hold and Schleidt, 1977). The attitude towards one's own odor was similar: men judged their own odor mainly unpleasant, while women judged theirs mainly pleasant. This possibly reflects the fact that women have on the average smaller apocrine glands than men, and therefore produce less intense body odor (Doty, 1977; Hurley and Shelley, 1960; Shehadah and Kligman, 1963).

Doty et al. (1978) demonstrated that male axillary odor is classified by subjects as more intense and stronger than female axillary odor. McBurney et al. (1977) found a positive correlation between axillary odors classified as unpleasant and a description of the likely personality of the donors with masculine as well as undesirable traits.

The unpleasantness evaluation of male odor may be analogous to findings in nonverbal communication studies that men keep a greater distance and have less eye contact with each other than do women (Baxter, 1970; Exline, 1963; Mehrabian, 1972). Obviously men prefer a greater personal distance.

As the qualitative evaluation of personal odor seems to parallel other social signals in nonverbal communication, the question also arose whether contact cultures differ from noncontact cultures in respect to dealing with personal odors. According to several authors (Hall, 1966; Altmann, 1977; Watson, 1970) noncontact cultures are characterized by relatively low sensory involvement, reflected by greater interpersonal distance, minimal eye contact, little touching, and a lower direct body orientation than contact cultures. The latter are to be found in southern Europe, Latin America, and the Arab world, the former in North America, northern Europe, and Asia. As it is likely that odor is a stimulus in nonverbal communication, like eye contact or direction of body axis, we undertook odor experiments in Japan (noncontact culture) and Italy (contact culture). Both were compared with earlier results from Germany (Hold and Schleidt, 1977).

Another question is whether there might be differences in odor perception and qualitative evaluation between ethnic groups which have apocrine glands of different size. According to Adachi (1903) and Hurley and Shelley (1960) Japanese have smaller apocrine glands and less odor than Europeans.

METHODS AND MATERIALS

Twenty-four German and 25 Italian couples took part in the investigation. In Japan we were only able to find 7 cooperative couples and therefore added 15 female and 15 male single students. The age of the subjects were: German, between 20 and 50; Italian, between 30 and 40; Japan, between 20 and 30. The German subjects had been married for 2–25 years, Italian subjects had been married more than 10 years. The experiment usually took place in groups of 10 subjects (5 couples or 5 single men and 5 single women). The married Japanese subjects were tested in one group of 4 couples and one of 3 couples.

To best guarantee a constant procedure for the experiment and a comparison with the earlier German results, one or both of the German authors was present at the tests in Italy and Japan. Each participant wore the same type of cotton shirt for seven consecutive nights. Subjects were given children's soap (Penaten) to wash with, and they were asked to use no perfume and no deodorant. For the experiment the ten shirts of one group were numbered, folded, and laid out in a separate room. Each subject was asked to take the shirts and sniff them for as long and often as wished, and to complete the following questionnaire: (1) Which shirt has your own smell? (2) Which shirt has your partner's smell? (3) Which shirts smell male? (4) Which shirts smell female? (5) Which shirts smell pleasant, which indifferent, which unpleasant?

About an hour later the subjects were asked to reassess the shirts, the shirts being renumbered. In all, there were three experiments with each subject.

There are several factors known to influence both body odor and the ability to smell it. The olfactory threshold in women oscillates with the menstrual cycle (Le Magnen, 1952; Schneider, 1974). It is said that smoking habits might influence the ability to smell; illnesses as well as certain drugs or food intake are known to alter personal body odor. Therefore the participants were asked to complete a questionnaire about menstruation, smoking habits, illnesses, drug use, consumption of garlic. They were also asked to give information about their sleeping and washing habits.

The data were statistically evaluated as follows: a particular odor was

said to be significantly recognized by a person when this person chose in at least two of the three tests the same shirt (binomial test, $P < 0.03$). A significant difference between male and female odor was—separately for each subject—calculated with the chi-square test ($P < 0.01$). In the emotional classification (pleasant, indifferent, unpleasant), the difference between the clusters (e.g., 'own odor' and 'supposed own odor' as defined in Figure 1) was calculated with the chi-square test (P at least < 0.05).

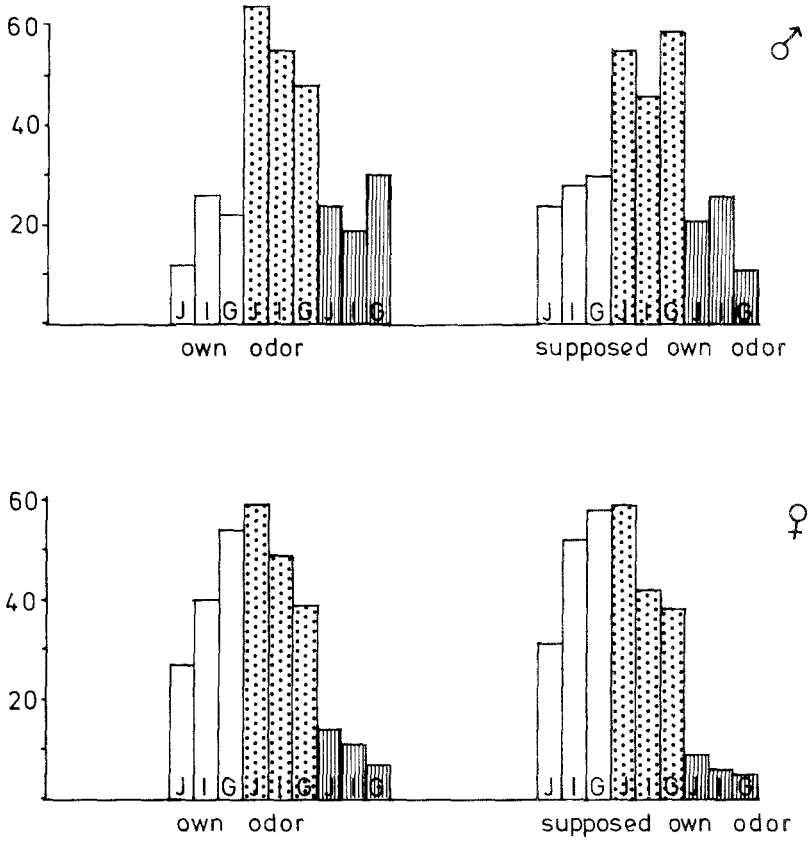

FIG. 1. Classification of own odor. Ordinate: percent responses. Each cluster of bars shows the classification of odors as pleasant = white; indifferent = stippled; unpleasant = striped. Subjects from different ethnic groups are marked: J = Japan; I = Italy; G = Germany. The three bars of each ethnic group add up to 100%. The clusters on the abscissa represent the following categories of odor: own odor = real own odor of subjects, whether correctly recognized or not; supposed own odor = odor which the subjects assume to be their own, whether they are right or wrong; ♂ = classification given by male subjects; ♀ = classification given by female subjects.

TABLE 1. RECOGNITION OF INDIVIDUAL ODOR[a]

	Germany $N = 45$	Italy $N = 50$	Japan (singles) $N = 44$	Japan (couples) $N = 14$
	%	%	%	%
1. Subjects recognize own odor	31	38	25	
2. Subjects recognize partner's odor	33	30		21
3. Subjects recognize own or partner's odor	49	52		29
4. Subjects mistook odor of another person as own odor	29	34	41	
5. Subjects mistook odor of another person as partner's odor	24	30		57
6. Subjects could do (1) or (2) or (4) or (5)	82	88	79	

[a]The table contains the significant ($P < 0.03$) recognition of each subject.

RESULTS

Recognition of Individual Odor. Table 1 gives a summary on the recognition (for both sexes) of own odor, of odor of the partner, and of those cases in which the odor of a certain person was erroneously taken for the odor of the partner or of oneself. Nearly 80% of the participants (Table 1,6.) in all three cultures distinguished between the odor of individuals; 50% could identify the person correctly, at least in one case, as partner's or own odor (Table 1,3.). Only 16.5% (23 persons of 139 tested persons) failed to distinguish between any individual odor, i.e., in all three test rounds they chose different shirts.

In all three cultures women were better able to identify the odors than men, which may be due to their lower olfactory threshold. This ability was not correlated with the estrogen cycle.

A nearly equal percentage of subjects in the three cultures significantly mistook the odor of a certain person for the odor of the partner or for own odor (Table 1,4.5.). This means that at a subconscious level these subjects had identified a certain person by his odor. This finding fits the understanding of Cain (1978), who states that the connection between odor and language appears to be relatively weak; his subjects had difficulties in associating the appropriate label with an odorant. He writes that man is equipped to fix the identity of objects by smell at a perverbal level, and that perverbal identification is perhaps most appropriately viewed as a kind of recognition.

TABLE 2. DISCRIMINATION BETWEEN MALE AND FEMALE ODOR[a]

	Germany $N = 50$	Italy $N = 50$	Japan $N = 44$
	%	%	%
Men	20	16	50
Women	44	24	82
Sexes together	32	20	64

[a]The table contains significant ($P < 0.01$) discrimination of each subject.

Recognition of Sexual Odor. Table 2 shows how many subjects of the three ethnic groups could discriminate significantly between male and female odor. As can be seen, far more women than men could do this.

According to their statements the participants seem to have estimated the stronger and/or more pungent and/or more unpleasant odor to be male. In Russell's (1976) study the male odors were usually classified as musky and the female odors as sweat. So there seems to be a qualitative difference between these two types of odor that may be due to different composition of hormones and other substances. Fiedler (1955) found a higher percentage of lactic acid in male sweat compared to female, and androstenon is found in axillary sweat of men (Brooksbank et al., 1974: Claus and Alsing, 1976), although not in women (Claus, personal communication).

On the other hand one should not exclude the possibility that a solely quantitative difference exists between male and female odor. The statements of our subjects saying that men smell stronger than women point in this direction. Also in the studies of Doty et al. (1977, 1978), the subjects labeled strong smells more often as male and unpleasant, while less strong odors were labeled as female and pleasant. The hypothesis, that men in general smell stronger (although qualitatively men and women have the same smell) and therefore the difference between male and female odor is only a quantitative one, is further supported by the fact that there exists a large difference between individuals: in nearly every group we tested, some male odors were significantly assigned to be female by the whole group and some female odors to be male. This also corresponds to Doty's findings that given samples of only male or only female odors his subjects assigned odors to both sexes.

Qualitative Evaluation of Individual Odor. The results of the qualitative evaluation of subject's own odor is summarized in Figure 1. Women classified their own odor to be more pleasant than unpleasant in contrast to men who classified their own odor less pleasant and usually more often unpleasant (Significance levels of the different evaluation of own odor by men and women: German subjects, $P < 0.001$, Japanese, $P < 0.02$, Italian, $P < 0.05$). The "supposed own odor" (see definition in Figure 1) is classified by women as more pleasant and less unpleasant as own odor, the same is the case with

German and Japanese men's classification. Italian men have no different classification between own and supposed own odor. One could hypothesize an underlying norm that everybody "should not smell bad."

The largest number in all the men's judgement of their own and supposed own odor fell into the class "indifferent." It seems that men cannot easily decide concerning their own odor.

Figure 2 gives the result of the qualitative evaluation of partner's odor. In women's classification there appears to be a difference between cultures. Contrary to the findings in Germany, Japanese and Italian women classify

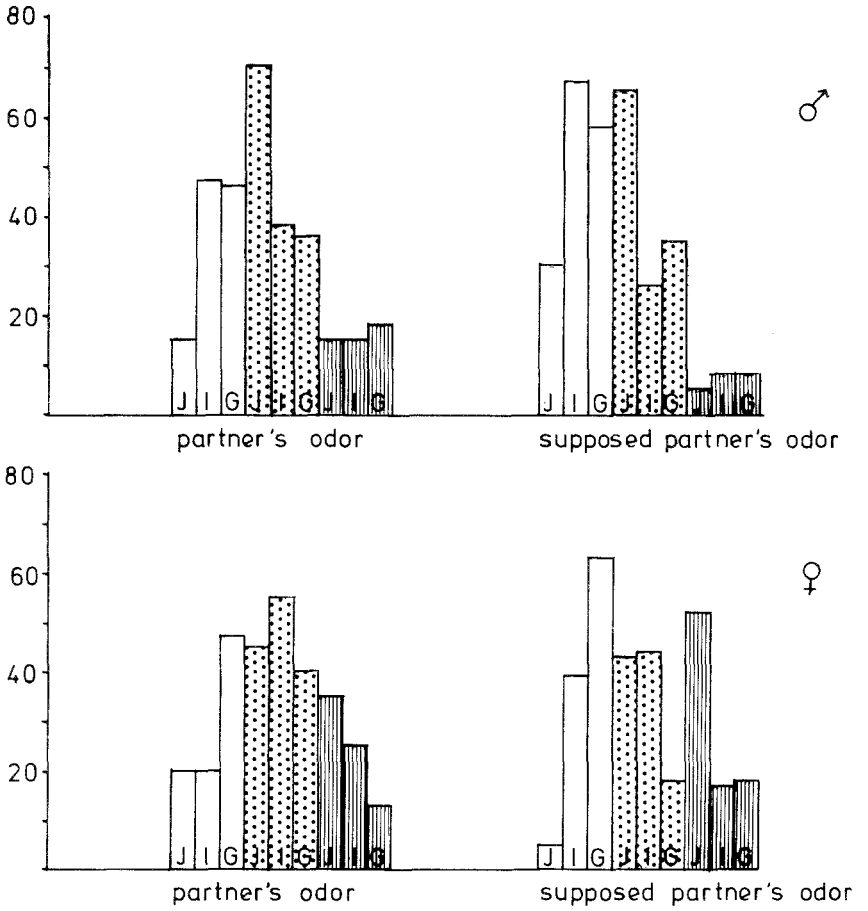

FIG. 2. Classification of partner's odor. Bar designation and explanation of symbols as in Figure 1. The clusters on the abscissa represent the following categories of odor: partner's odor = real odor of partner, whether correctly recognized or not; supposed partner's odor = odor which the subjects assume to be their partner's odor, whether they are right or wrong.

their partner's odor more often unpleasant than pleasant ($P < 0.01$). The odor of the supposed partner is classified by Italian women in the same manner as by German women: predominantly pleasant. The difference in Italian women's attitude to partner's and supposed partner's odor is significant ($P < 0.001$). Japanese women, however, classify the odor of partner and supposed partner alike: predominantly unpleasant.

In men's classification there is no significant difference between cultures. Italian and German men judge their partner's odor predominantly pleasant; Japanese men judge it pleasant and unpleasant equally often. All three groups judge the supposed partner's odor predominantly pleasant. The Japanese men show a large amount of "indifferent"; it seems not easy for them to decide.

The classification of "supposed partner" compared to "partner" (with the exception of Japanese women) again brings forward a speculation about a possible underlying norm saying: "my partner smells good."

Qualitative Evaluation of Sexual Odor. Figure 3 contains the classification of male odors and Figure 4 female odors. It is remarkable that the Japanese participants classify odors in all groups less often as "pleasant" than the Italian and German subjects, and in most groups more often as "unpleasant." That is to say, on the whole, they give a less positive evaluation of the odors.

In all three ethnic groups men and women judge male odor different from female odor, the latter more pleasant ($P < 0.001$). Furthermore they classify the groups "significant male odor" and "supposed male odor" more unpleasant than "male odor." That means an odor is classified most often as unpleasant (and least often as pleasant) in two cases: when the group of tested subjects agrees that an odor smells male (significant male odor) and when the single subject's decision is that an odor smells male (supposed male odor). This parallels the findings of Doty (1978) of a positive correlation between the ratings of male and unpleasant. Female odors are classified vice versa: in both cases, by the group and by the individual, when an odor is classified as female, it is also classified most often as pleasant (and least often unpleasant).

Influence of Other Factors. In analyzing the questionnaire containing the personal data of the Italian and Japanese subjects, we found, as earlier for the German sample, no influence of further factors on the results. Neither medicine intake, garlic consumption, smoking habits, women's menstrual cycle, duration of marriage or association, sleeping or washing habits, nor the age of the subjects showed any correlation to the olfactory abilities of the subjects or to their qualitative evaluation of the body odors.

DISCUSSION

The results of our study show that 80% of the participants, regardless of their cultural and racial differences, could differentiate significantly between

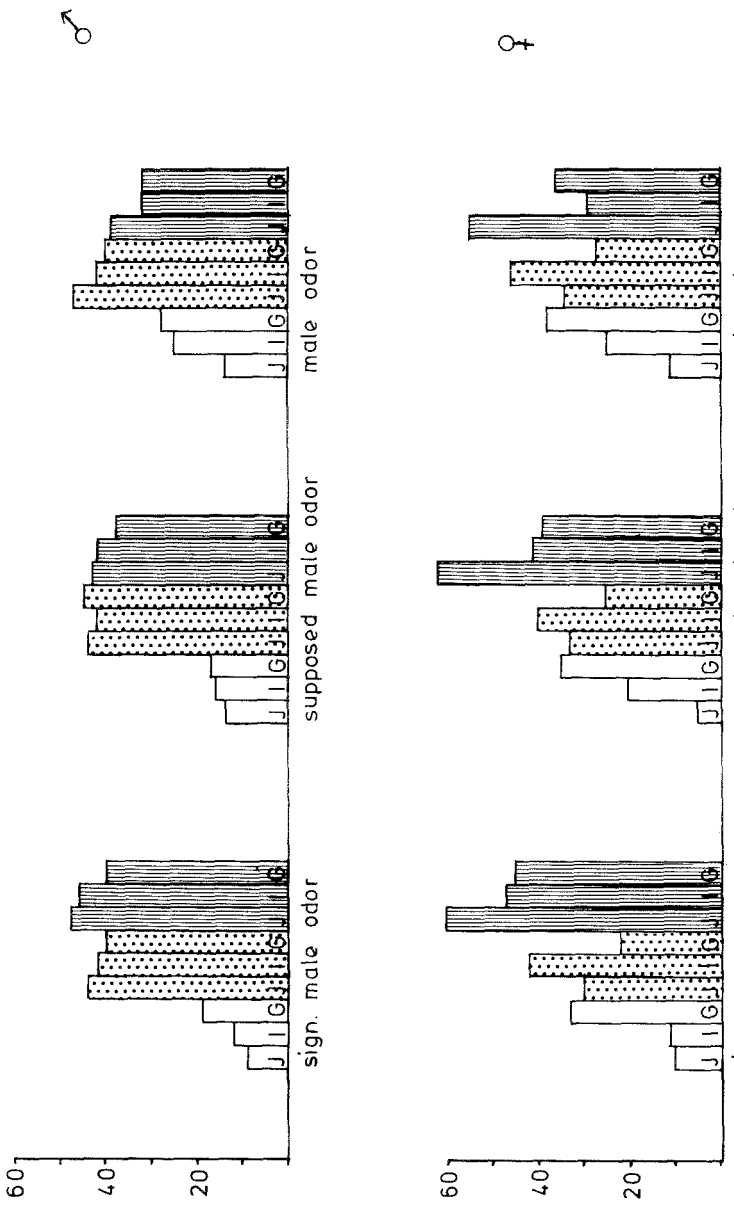

FIG. 3. Classification of male odors. Bar designation and explanation of symbols as in Figure 1. The clusters of the abscissa represent the following categories of odor: male odor = real odor of males, whether correctly recognized or not; supposed male odor = odor which the participants assume to be male, whether they are right or wrong; significant male odor = odor which was classified as male by the majority of the group.

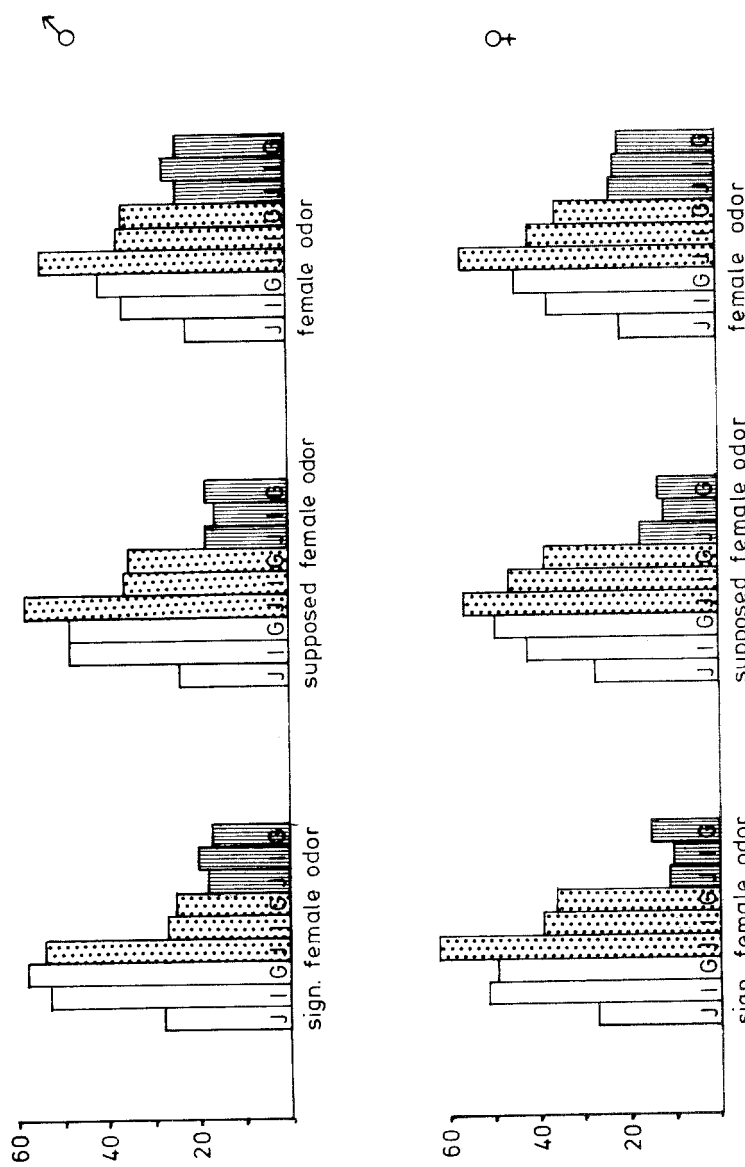

FIG 4. Classification of female odors. Bar designation and explanation of symbols as in Figure 1. The clusters on the abscissa represent the following categories of odor: female odor = real odor of females, whether correctly recognized or not; supposed female odor = odor which the participants assume to be female, whether they are right or wrong; significant female odor = odor which was classified as female by the majority of the group.

individual odors; 50% could correctly identify the person to whom the recognized odor belongs. Of our subjects 20–60% were able to distinguish significantly between the odor of men and women. Noteworthy is the fact that Japanese people tend to have smaller apocrine axillary glands than Europeans and presumably have less personal odor (Adachi, 1903; "the yellow race does not smell at all"). Nevertheless the same test method produced similar results in Japan as in Europe. So it seems that in Japan less intense body odors may be matched by a lower detection threshold.

In the qualitative evaluation of the odors the different odor categories (male, female, own, partner) are classified nearly alike in the three ethnic groups, with one exception: judgement of partner's odor by women. Italian and Japanese women classified it predominantly unpleasant; in contrast German women classified it predominantly pleasant. Although we have to be cautious with the Japanese findings in this respect because of the small sample, it is possible that this result reflects the role women play in these different societies.

Perhaps personal odor is a relevant factor in sexual attraction and is one of the variables which plays a role in the choice of a partner. Then judging the partner's odor as predominantly unpleasant could indicate a different strategy of choosing a partner compared to when a partner's odor is judged predominantly pleasant. Maybe the women's choice of a partner in Italy and Japan, in contrast to Germany, is more influenced by economic, social, and educational factors, than by sexual attraction (represented by olfactory attraction in this study).

In contrast men of the three ethnic groups judge their partner's odor predominantly pleasant. Besides the observation that women's odor is in general judged in this way, one could at the same time hypothesize that men choose their partner more on the basis of sexual attraction than do women.

A cultural difference is to be found between Japan, on the one hand, and Italy and Germany, on the other, concerning the overall judgement of personal odors. Men and women in Japan judge the odors in general less positively, i.e., they classify all odor categories less often as pleasant and more often as unpleasant. This might reflect a more negative attitude towards personal odors in Japanese society (Adachi, 1903). In Japan there is a strong cultural pressure to suppress obvious body odors. This attitude is also reflected by the old tradition of taking a ritual bath almost every day.

If we consider Italian people as Mediterraneans typically belonging to a contact culture and Japanese people as Asians typically to a noncontact culture, we find a parallel to the different proxemic behavior in the evaluation of personal odor. Our Japanese subjects show perhaps a preference for a more distant contact—supposing odor to be a variable in proxemic behavior.

Assuming that body odor plays an important role in sexual attraction, the more negative attitude towards personal odors in Japan may be

interpreted as reflecting a more negative attitude towards sexuality as compared to the situation in Italy and Germany. This hypothesis is supported by the findings of Iwawaki and Eysenck (1978) on the sexual attitudes among British and Japanese students, saying that Japan "is still very Victorian (nonpermissive) in its outlook on sex" (p. 289).

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BEHAVIOR OF THE LESSER GRAIN BORER *Rhyzopertha dominica* (COLEOPTERA: BOSTRICHIDAE) Male-Produced Aggregation Pheromone Attracts Both Sexes[1]

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Abstract—Adult males of the lesser grain borer, *Rhyzopertha dominica* (F.), produce a pheromone that attracts both sexes. The volatiles from males collected on filter paper or Porapak-Q were attractive in two laboratory bioassays. The mating behavior is described and the function of the pheromone is discussed. The pheromone is responsible for the characteristic sweetish odor of grain infested with the borer.

Key Words—Coleoptera, Bostrichidae, *Rhyzopertha dominica*, aggregation pheromone, lesser grain borer, grain insect pest.

INTRODUCTION

The lesser grain borer, *Rhyzopertha dominica* (F.) is one of the most resistant of the stored-product insect species to gamma radiation (Tilton et al., 1966), to infrared treatment (Tilton and Schroeder, 1963), and to a variety of pesticides (Champ, 1979). This species gained much prominence during World War I, when shipments of Australian wheat developed heavy infestations because of delay by submarine warfare (Monroe, 1969). At the time it was introduced into the southern United States via California, it was known as the Australian wheat weevil. The larvae of *R. dominica* can feed both inside the grain and on grain dust produced by the boring adults. As this species is able to attack

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sound, dry grain in storage, it must be regarded as a primary pest. *Rhyzopertha dominica* and the *Sitophilus* spp. grain weevils are the major insect pests of grain throughout the world.

Crombie (1941) studied oviposition, olfactory conditioning, and host selection in *R. dominica.* In preliminary studies on recognition of the opposite sex he reported that in an olfactometer males are not attracted by smell to a "bait" of 20 females. He also lacquered either the legs or antennae of both males and females and concluded from his experimental data that olfactory receptors for recognizing the opposite sex as well as food are to be found on these organs. As a result of his observations of mating in dishes, he concluded that female odor neither arouses the male to go searching nor elicits the mating reflex and that there is no attraction between the sexes until they meet, and then they recognize each other by smell. He also used a Y-tube olfactometer to test the attraction of each sex towards the opposite sex. Out of 253 males, 124 (49% ± SE 3.14%) went to the arm containing the females, while out of 258 females 137 (53.1% ± SE 3.11%) went towards the males. He concluded that there was no attraction between the sexes in an olfactometer and that they could be used together in host selection experiments.

Results of the present investigation of mating behavior and sex attraction in *R. dominica* may result in more effective and desirable ways to detect and control this pest.

METHODS AND MATERIALS

Rhyzopertha dominica pupae and adults used in this study were from a stock culture reared on whole grain wheat. Rearing of stock cultures was improved by adding whole wheat flour with 5% brewer's yeast to the whole grain wheat in the ratio of 1:3. The jars were each stocked with 400 adults. To obtain pupae for testing, only whole wheat flour with 5% brewer's yeast was used. The pupae were screened from the flour with a 20-mesh screen. The cultures, pupae and adults used for testing were held at 27 ± 1°C and 60 ± 10% relative humidity with 16:8 light–dark photoperiod. Rearing procedures followed those reported by Cline (1973). Pupae were removed from the culture and separated by sex according to Halstead (1963) and placed in petri dishes. In mating behavior studies, males and females were paired in filter paper-lined 9-cm petri dishes for observation.

To obtain the attractive material, the adults emerging within a 24- or 48-hr period were placed in shell vials (15 × 45 mm) with a highly absorbent paper disk (12.7-mm-diam antibacterial assay disk, Schleicher and Schuell, Keene, New Hampshire) and food (cracked wheat) on the bottom. The vials were closed with caps and returned to the rearing chamber (the males and females were held in separate chambers). Control vials without insects were

set up at the same time and handled in the same manner as those with insects. Adults of the same age as those used in the shell vials were held in filterpaper-lined petri dishes for bioassay. These tests were carried out in three-choice and multichoice olfactometers described by Burkholder and Dicke (1966) and Burkholder (1970), respectively.

In the first series, the bioassays were made after the insects had been held on the paper disks 8–10 days. This duration was selected because preliminary tests indicated that attractant material was released within 7 days after the emergence of the male. The tests were usually conducted in quadruplicate, between 1 and 3 PM and under indirect artificial light. Disks from vials containing four insects were used in each test. The treated disks and controls were each bioassayed with groups of 10 males or females 8–10 days old.

The insects' response, as indicated by attraction to, and aggregation near, the test disks of the three-choice olfactometer or in the air stream of the multichoice olfactometer, was recorded at 1-min intervals for 5 and 10 min, respectively. The bioassays were conducted 6–8 hr after the onset of light. Percentage response was based on four replicates.

In the second series, the volatiles were collected from unmated males and females (60 insects/jar). They were maintained on food (whole wheat flour and cracked wheat) in a 2-oz jar, while air was drawn over them by vacuum applied downstream of the Porapak-Q holder.

The Porapak-Q was prepared by the same procedures reported by Cross et al. (1976). The Porapak-Q holder consisted of a 1-ml glass disposable pipette with the tip removed. Glass wool was packed in one end and approximately 0.7 ml Porapak-Q was drawn into the tube by vacuum and then held in place with glass wool. The tubes were washed with 10–15 ml of diethyl ether, followed with 10–15 ml *n*-hexane, and were dried with N_2 for 10 min prior to attaching to the aeration chamber.

The air (at 2 liters/min) was drawn through charcoal, then through the aeration jar, and into the Porapak-Q holder for 2 days. During the aeration, all insects were maintained at 27 ± 1° C and on a 16:8 light–dark photoperiod. After aeration, the Porapak samples were extracted first with 4 ml redistilled *n*-hexane and then with 3 ml diethyl ether. The combined solvent washes were concentrated to 1 ml by evaporating the solvent in a nitrogen stream. For bioassay, we used the same olfactometers used in the previous experiments. Twenty μl (2.4 beetle days) or 40 μl (4.8 beetle days) of extracts were applied to filter paper disks for the bioassays.

RESULTS AND DISCUSSION

Results of pairing males and females confirmed the results of Crombie (1941) that the female does not apparently arouse the male to vigorously

search for her. Both males and females were observed to walk around the observation chamber with their antennae extended and, on contact with the opposite or same sex, would actively palpate the insect, especially in the abdominal area. It appears there is a palp-mediated mating response by a male when it contacts the female and sometimes when it contacts another male. Our results confirmed those of Crombie (1941), in which males attempted to mate with either females or males. Females did not make mating attempts.

The male usually mounts the female after he touches the tips of her elytra with his maxillary palpi. In mounting, he climbs on her posterior dorsum and, walking forward, taps the top of her elytra and thorax lightly with his palpi. Then he lowers his last sternite and extrudes his aedeagus. Copulation is achieved with the male firmly mounted and having his ventor on her dorsum. His head is bent down with his palpi lightly tapping the dorsal surface of the prothorax.

An attractive material which elicited a behavioral response in males and females was obtained from the disks that were in the vials with *R. dominica* male adults for 8–10 days. The behavioral response by both males and females was characterized by an excited and rapid walking movement, with the head, thorax, and antennae extended forward and upward, and by aggregations near the pheromone source.

The male and female responses to disks exposed directly to either males or females are shown in Figure 1. The response by both males and females to the male-treated disks in each type of olfactometer was approximately 60%. Few males or females responded to the female-treated disks or the disks from food-only controls.

The male or female responses to disks treated with *n*-hexane and diethyl ether extracts of male or female aerated Porapak-Q was nearly identical to that shown in Figure 1. The response in the three-choice olfactometer to the 2.4 beetle-day extract and in the multichoice olfactometer to the 4.8 beetle-day extract was approximately 50% for the male extract. Few males or females responded to the extracts from female-treated or food-only-treated Porapak-Q.

During the study it was noticed that only males produced the characteristic sweetish odor that is commonly associated with culture or infestations of *R. dominica*. The culture odor from male containers was similar to that of the mixed male and female containers. Bioassay results of tests with volatiles collected on Porapak-Q from mixed females and males were similar to those of tests with male-only extracts.

The male-produced attractant appears to serve as a population aggregating pheromone that brings the sexes together. The insects are often found breeding deep within bins of grain. It is of interest that there is virtually no competition from other bostrichid insects in temperate areas of the world; however, they may compete with certain other grain storage insects. The

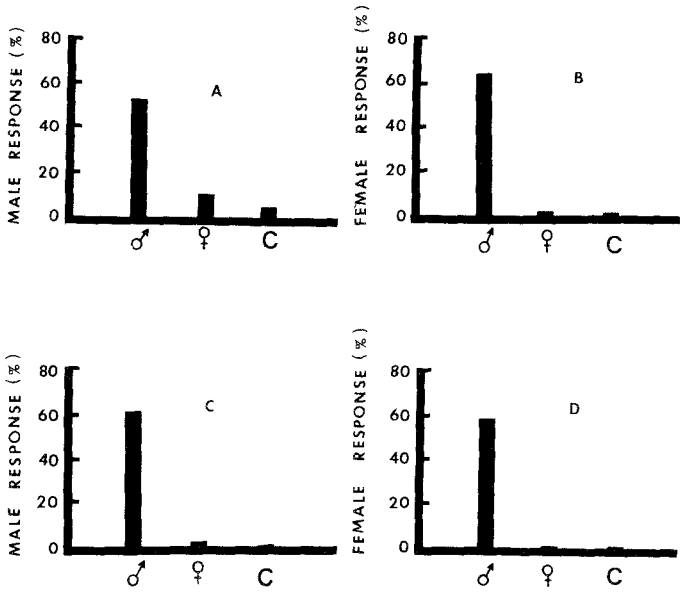

FIG. 1. Percentage of *Rhyzopertha dominica*, males and females 8–10 days old, attracted to paper disks from vials that contained 4 virgin males or females 8–10 days old. Percentage calculated from the response of 10 males or females during five observations at 1-min intervals by using the three-choice olfactometer (A and B) or of 10 males or females during 10 observations at 1-min intervals by using a multichoice olfactometer (C and D); average of four replicates.

insects are excellent fliers and we have observed that they will fly to a light source as well as a pheromone source and that light appears to enhance attraction to the pheromone. The use of the pheromone as a monitoring and/or control tool appears promising. Studies on the chemistry of the pheromone will be reported separately (Williams et al., 1981).

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INSECT FEEDING AND OVIPOSITION DETERRENTS FROM WESTERN RED CEDAR FOLIAGE[1]

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Abstract—The feeding deterrent activity of fractions from the foliage of western red cedar, *Thuja plicata* Donn, was studied in laboratory bioassays using the white pine weevil, *Pissodes strobi* Peck, as a test insect. The most active fraction was the volatile mixture that comprises the leaf oil of this tree species. Further fractionation of the leaf oil indicated feeding deterrent activity in the monoterpene hydrocarbon, thujone, and terpene alcohol fractions. When tested alone, both (−)-3-isothujone and (+)-3-thujone, which made up 75–88% and 5–10% of the leaf oil, respectively, deterred feeding by the weevils. Western red cedar leaf oil also showed antifeedant activity with the alder flea beetle, *Altica ambiens* (Le Conte), and served as an oviposition deterrent for the onion root maggot, *Hylemya antiqua* Meigen. The leaf oil, however, had no inhibitory effect on the feeding of the leaf roller, *Epinotia solandriana* L., and the red-backed sawfly, *Eriocampa ovata* L.

Key Words—Insect feeding deterrents, antifeedants, *Pissodes strobi* Peck, Coleoptera, Curculionidae, *Thuja plicata* Donn.

INTRODUCTION

The wood of western red cedar, *Thuja plicata* Donn, is resistant to fungal decay due to toxic substances in the heartwood (Rennerfelt, 1948; Erdtmann

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and Gripenberg, 1948; Roff and Whittaker, 1959; Van Der Kamp, 1975). Insect resistance, however, has been less studied, but Wellington (1969) and Barton et al. (1972) have shown that western red cedar wood contains compounds with juvenile hormone activity. Hach and McDonald (1971) found that the wood extractive thujic acid had a repellent effect on mosquitoes.

Little is known about the susceptibility of *T. plicata* to attack by defoliating insects in its natural habitat. As fewer insects are reported on western red cedar[4] than on other tree species, its foliage may contain chemical feeding deterrents, repellents, or substances with insecticidal effect. VanderSar and Borden (1977), investigating discrimination by the white pine weevil, *Pissodes strobi* Peck, between sympatric host species, demonstrated that the weevil would not feed on twigs of its normal host, Sitka spruce, *Picea sitchensis* (Bong.) Carr., if they had been soaked in a crude extract of western red cedar branchlets. There results were corroborated by Alfaro et al. (1979) with *T. plicata* leaf oil tested in a feeding choice bioassay.

This paper reports a series of experiments designed to elucidate the chemical nature of the antifeedant activity of western red cedar foliage using *P. strobi* as a test insect.

METHODS AND MATERIALS

Instrumentation. A Varian 2100 chromatograph modified for use with glass capillary columns and equipped with a Spectra-Physics Autolab Minigrator was employed for analytical gas–liquid partition chromatography (GLC). Analyses were carried out on the following capillaries: a 33-m × 0.25-mm-ID column coated with OV-101, a 23.4-m × 0.27-mm-ID whisker-walled column coated with Silar-10C and a 23.2-m × 0.28-mm-ID whisker-walled column coated with Carbowax 20M (Schieke et al., 1975; Sandra and Verzele, 1977). A Varian 700 was used for preparative GLC, and separations were performed on a 152-cm × 6.35-mm-OD stainless-steel column packed with 25% Carbowax 1540 on Chromosorb A 60/80, at 133°C. Combined gas chromatography–mass spectroscopy (GC-MS) was carried out with a HP5985/MS/DS mass spectrometer interfaced through a Pt-Ir capillary to a 30-m × 0.25-mm-ID glass capillary column coated with SP-1000 (J & W Scientific, Inc., available from Supelco). Optical rotations of neat liquids were measured with a Rudolph polarimeter.

[4]Data collected in British Columbia by the Forest Insect and Disease Survey, Canadian Forestry Service, indicates that between 1972 and 1978, only 65 different insect species were collected from *T. plicata* compared to 144 from *Tsuga heterophylla* (Raf.) Sarg. and 235 from *Pseudotsuga menziesii* (Mirbel) Franco (J.W.E. Harris, personal communication, Pacific Forest Research Centre, Canadian Forestry Service, Victoria, B.C.).

Extraction of Branchlets and Preparation of Western Red Cedar Leaf Oil. Western red cedar foliage was extracted as indicated in Figure 1. Cedar branchlets (400 g) were macerated with 50% aqueous alcohol in a Waring blender. The syrupy pulp was allowed to stand overnight at 7°C, filtered through cheesecloth, and the residue discarded. The aqueous alcoholic solvent in the filtrate was removed on a rotary evaporator. A portion of the residue left in the rotary evaporator was separated for testing (fraction A); the

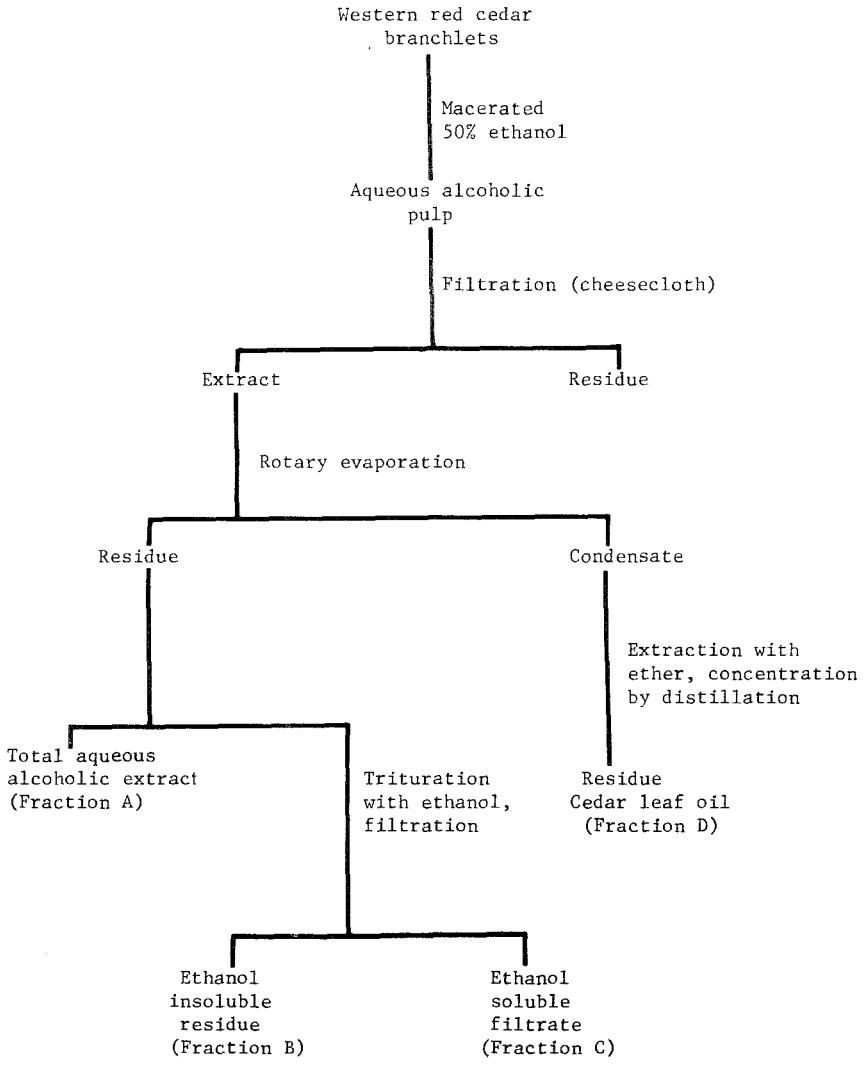

FIG. 1. Flow chart of aqueous alcoholic extraction of western red cedar foliage.

rest was triturated with ethanol and filtrated under suction to give an ethanol insoluble and soluble fraction (B and C, respectively). The condensate in the receiver of the rotary evaporator was diluted with water and extracted several times with ether. The combined ethereal extracts were washed, dried ($MgSO_4$), and concentrated by distillation. The residual oil (hereafter referred to as cedar leaf oil) was redistilled in a microdistillation apparatus at reduced pressure (approx. 0.5 mm) to give 0.55 g of a colorless liquid (fraction D).

Fractionation of Cedar Leaf Oil. Cedar leaf oil obtained from fresh cedar branchlets by steam distillation (von Rudloff, 1962) was separated by preparative GLC into fractions as indicated in the analytical chromatogram in Figure 2. Analysis of the leaf oil by GC-MS (temp. program, 60–160° at 4° C/min) indicated that fraction I contained, inter alia, α-pinene, myrcene, limonene, γ-terpinene and p-myrcene. Fraction II comprised (−)-3-isothujone and (+)-3-thujone (M^+ 152). Terpinen-4-ol was the major component of fraction III. (−)-3-Isothujone was isolated from redistilled cedar leaf oil by preparative GLC, and was 97% pure by analysis on the OV-101 glass capillary column (temp. program, 85° C at 4° C/min). (+)-3-Thujone was prepared by

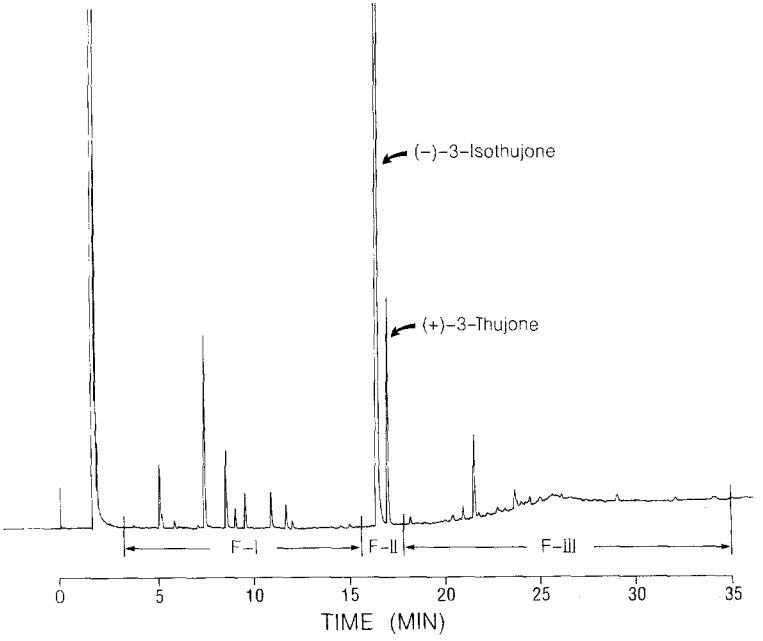

FIG. 2. Gas chromatogram of cedar leaf oil on the Carbowax 20 M capillary column. Temperature program: 60° C until elution of solvent, then 4° C/min to 160° C. fraction I (F-I): monoterpene hydrocarbons; fraction II (F-11): thujones; fraction III (F-III): major peak is terpinen-4-ol.

the procedure of Hach et al. (1971) and exhibited only one peak on the Silar-10C capillary column (temp. program, 60–200° C at 6° C/min): $[\alpha]_D^{22}$ = +78.6 (Literature $[\alpha]_D^{25}$ = 78.8) (Hach et al., 1971). The extracts, fractions, and compounds tested for antifeedant activity are indicated in Table 3.

Bioassays with P. strobi. Weevils for use in experiments were collected on Vancouver Island, B.C., as mature larvae infesting terminal leaders of Sitka spruce. They were allowed to complete their development in screened cages at room temperature. After emergence, they were held in a refrigerator at 4° C, on a modified diet (Zerillo and Odell, 1973) that contained Sitka spruce bark.

Most bioassays were conducted with the paired agar disk procedure described by Alfaro et al (1979). One percent Sitka spruce bark (dried and finely powdered) from lateral branches was incorporated into the agar as a feeding stimulant. Candidate antifeedant stimuli were applied in solvent to the lens paper covering one of the paired agar disks while the other served as a solvent control. Except where otherwise indicated, experiments were run at 20–21° C on a laboratory counter top with a natural photoperiod.

In all experiments, feeding deterrency was calculated as a percentage by subtracting the amount feeding on the treated disks from the feeding on the control and dividing the difference by the feeding on the control.

An experiment was conducted to determine whether the fractions obtained in the aqueous alcoholic extraction of cedar branchlets exhibited antifeedant activity. Extracts were tested at concentrations equivalent to 0.5 mg of *T. plicata* foliage. The solvents used were: 50% ethanol for the total extract (fraction A); distilled water for the ethanol-insoluble fraction (B); pure ethanol for the ethanol soluble fraction (C); and pentane for the volatile fraction (D). The amount of feeding was evaluated by counting the number of feeding punctures made in 24 hr by two weevils into the surface of the treated and untreated discs. The first three fractions were tested using 20 replicates, and the fourth included 15 replicates.

The deterrent effect of cedar leaf oil on feeding of *P. strobi* was tested using Sitka spruce twigs as a feeding substrate. The experimental unit consisted of 2 Sitka spruce twigs (2 cm long), from lateral branches, connected end to end by means of a headless entomological pin inserted through the pith. One of the twigs in each couple was dipped for 2 sec in a 1% pentane solution of cedar leaf oil. The second, control twig, was dipped in pure pentane. Each replicate consisted of one pair of connected twigs placed with a single weevil under an inverted glass jar (448 ml) over a filter paper floor. The experiment lasted for 24 hr and was evaluated by counting the number of feeding punctures made by each weevil on the twigs. The number of replicates for each sex was 16.

Another experiment studied deterrency to *P. strobi* of cedar leaf oil and its components when tested at similar concentrations to those which occur in nature (Table 3). In western red cedar, the leaf oil accounts for about 5.5% of

the dry weight of the foliage (von Rudloff, 1962). In our experiment we applied 100 μg of cedar leaf oil to the paper surface of agar–spruce bark disks. Since the weight of this paper surface is about 2.4 mg, the resulting concentration on the paper was about 4% of its dry weight. The experiment included 15 replicates per treatment and was run for 24 hr in a growth chamber at 24° C. Due to the large size of the experiment and numerous observations that male and female *P. strobi* respond similarly to feeding deterrents and stimulants (VanderSar and Borden, 1977; Alfaro et al., 1979), the sexes of *P. strobi* were not separated.

Effect of Cedar Leaf Oil on Feeding and Oviposition of Other Insects. Insects in three species, the alder flea beetle, *Altica ambiens* (Le Conte) (Coleoptera: Chrysomelidae); a leaf roller, *Epinotia solandriana* L. (Lepidoptera: Olethreutidae); and the red-backed sawfly, *Eriocampa ovata* L. (Hymenoptera: Tenthredinidae), were collected on Burnaby Mountain, B.C., where they defoliate red alder, *Alnus rubra* Bong., a species which is sympatric with *T. plicata.* The effect of cedar leaf oil on the feeding of these phytophagous insects was tested by giving the insects a choice between one half of an alder leaf dipped for 2 sec in a pentane solution of cedar leaf oil and a solvent-treated half-leaf. The two halves were placed on moist filter paper in a 10-cm glass petri dish. Four concentrations of cedar leaf oil (0.01%, 0.1%, 1%, and 2%) were tested.

The experiment with adult *A. ambiens* included five replicates with seven insects each. Feeding was evaluated by counting the number of feeding holes made by the insects in 12 hr. *E. solandriana* and *E. ovata* larvae were tested in separate, five-replicate experiments using five insects per replicate. Feeding was evaluated with the aid of a camera lucida, by drawing on paper the area eaten in 24 hr. The drawings were then cut and weighed on a balance.

Cedar leaf oil was tested as an oviposition deterrent for the onion root maggot, *Hylemya antigua* Meigen (Diptera: Anthomyiidae), using the oviposition bioassay of Vernon et al. (1977), in which *H. antigua* is induced to oviposit into notches cut around the lip of an inverted Nalgene beaker containing a suspended onion slice. In the oviposition deterrency test reported here, 300 μg of cedar leaf oil, dissolved in pentane, were applied to the filter paper under each notch. The control papers received only pentane. Two treated and two control sets of bioassay apparatus were introduced into each of three screen mesh cages that contained 25 gravid female flies. The number of eggs laid under each beaker after 48 hr was counted. The experiment was repeated three times with different groups of insects.

RESULTS AND DISCUSSION

The total aqueous alcoholic extract of western red cedar branchlets has no significant antifeedant effect on *P. strobi* (Table 1). Surprisingly, the

TABLE 1. TOTAL[a] NUMBER OF FEEDING PUNCTURES MADE BY *P. strobi* ON AGAR-BARK DISKS TREATED AND UNTREATED WITH FRACTIONS OF WESTERN RED CEDAR FOLIAGE TESTED AT CONCENTRATIONS EQUIVALENT TO 0.5 MG OF FOLIAGE

	Males			Females		
Stimulus description	Control disk	Treated disk[b]	Percent deterrency	Control disk	Treated disk[b]	Percent deterrency
Total extract	60	57	5.0	85	63	25.8
50% aqueous ethanol soluble	27	72**	—	55	86	—
50% aqueous ethanol insoluble	54	87	—	57	120*	—
Volatile	20	1**	95.0	20	2**	90.0

[a] Each total includes 20 replicates (two weevils each), except for the volatile fraction which included only 15 replicates.
[b] Student's *t* test on difference between treated and control indicated by: * $P < 0.05$, ** $P < 0.01$.

ethanol-soluble and -insoluble fractions (fractions C and B, respectively) were stimulatory. Only the fraction containing the volatile leaf oil showed significant feeding deterrent activity (Table 1). Analysis by gas chromatography indicated that (−)-3-isothujone and (+)-3-thujone accounted for more than 80% of the leaf oil, similar to results reported by von Rudloff (1962). The experiment in which Sitka spruce twigs were dipped in a cedar leaf oil solution showed that cedar leaf oil depressed significantly the feeding of *P. strobi* (Table 2).

Table 3 indicates the relative composition of the components of cedar leaf oil. When tested at similar relative concentrations, all fractions that comprise the leaf oil of western red cedar had a deterrent effect on the feeding of

TABLE 2. MEAN NUMBER OF FEEDING PUNCTURES MADE BY ONE *P. strobi* ON SITKA SPRUCE TWIGS DIPPED FOR 2 SEC IN 1% PENTANE SOLUTION OF CEDAR LEAF OIL[a]

Sex tested	Control twig	Treated twig[b]	Percent deterrency
Males	2.5	0.5**	80.0
Females	2.3	1.2*	47.8

[a] Control twigs were dipped in pure pentane. N = 16 replicates for each sex.
[b] Student's *t* test on difference between treated and control indicated by: * $P < 0.05$, ** $P < 0.01$.

TABLE 3. MEAN NUMBER OF FEEDING PUNCTURES MADE BY 3 *P. strobi* ON AGAR-BARK DISKS TREATED WITH PENTANE SOLUTION OF CEDAR LEAF OIL OR ITS COMPONENTS (N = 15 REPLICATES PER STIMULUS)

Stimulus description	Proportion of cedar leaf oil (%)	Amount used in bioassay (μg)	Control disk	Treated disk[a]	Percent deterrency
Unfractionated cedar leaf oil	100	100	14.3	7.7**	46.1
Fraction I, monoterpene hydrocarbons	15	15	33.7	16.4**	51.3
Fraction II, (−)-3-isothujone and (+)-3-thujone	80–90	80	28.2	14.1**	50.0
Fraction III, terpene alcohols	5	5	30.0	20.5*	31.7
(−)-3-Isothujone	75–88	75	21.4	16.3	23.7
(+)-3-Thujone	5–10	5	28.7	18.7*	34.8
Fractions I + II + III combined	100	100	26.7	5.1**	80.2

[a]Student's *t*-test on difference between treated and control indicated by * $P < 0.05$, ** $P < 0.01$.

P. strobi. (+)-3-Thujone caused significant inhibition at very low concentrations (5 μg), whereas the inhibition caused by (−)-3-isothujone was lower and not significant. However, additional testing showed that when the concentration of (−)-3-isothujone was increased to 100 μg/disk, feeding inhibition was highly significant (*t* test, $P < 0.01$).

Alder flea beetles fed significantly more on solvent-treated alder leaves than on leaves dipped in the cedar leaf oil solution (Table 4). The feeding

TABLE 4. MEAN NUMBER OF FEEDING HOLES MADE BY *A. ambiens* ON RED ALDER HALF-LEAVES TREATED AND UNTREATED WITH CEDAR LEAF OIL

Stimulus concentration (%)	Control leaf	Treated leaf[a]	Percent deterrency
0.01	17.7	6.0*	66.1
0.10	26.6	14.4*	45.9
1.00	26.2	10.4*	60.3
2.00	26.2	7.0*	73.3

[a]Student's *t*-test on difference between treated and control indicated by: * $P < 0.05$, 88 $P < 0.01$.

TABLE 5. TOTAL NUMBER OF EGGS LAID BY 25 *H. antiqua* ON CEDAR LEAF OIL-TREATED AND -UNTREATED OVIPOSITION STATIONS (N = 3 REPLICATES PER EXPERIMENT)

Experiment No.	Control stations	Treated stations[a]	Percent deterrency
1	1660	186*	88.8
2	965	367*	62.0
3	1763	162**	90.8
Total	4388	715	83.7

[a] Student's *t*-test on difference between treated and control indicated by: * $P < 0.05$, ** $P < 0.01$.

deterrency did not increase proportionally with increasing stimulus concentration, suggesting that the lowest concentration tested may have been above the threshold for deterrency; thus the difference between treatments is probably due to random variation. Cedar leaf oil had no effect on the feeding activity of *E. solandriana* and *E. ovata* larvae, which fed equally on treated and untreated leaves. The leaf rollers (*E. solandriana*) exhibited their normal leaf rolling behavior on the treated leaves before starting to feed. Oviposition by the onion root maggot was highly inhibited by the presence of cedar leaf oil in the treated oviposition stations (Table 5).

Our experiments indicate that cedar leaf oil inhibits the feeding of at least two phytophagous insects. These insects, *P. strobi* and *A. ambiens*, must discriminate between *T. plicata* and their normal hosts in nature. However, other host discrimination mechanisms must exist for insects such as *E. solandriana* and *E. ovata*, which are not inhibited by cedar leaf oil but which do not feed on *T. plicata* in nature. Feeding (and possibly oviposition) deterrency may partially account for the observed resistance of western red cedar to foliage feeders in nature. However, the relatively high concentrations required to elicit feeding inhibition and the lack of absolute deterrency (Tables 3–5) suggest that applications of cedar leaf oil or its components as commercial feeding deterrents are unlikely.

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HOST SELECTION BEHAVIOR OF BARK BEETLES (COLEOPTERA: SCOLYTIDAE) ATTACKING *Pinus ponderosa*, WITH SPECIAL EMPHASIS ON THE WESTERN PINE BEETLE, *Dendroctonus brevicomis*[1]

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Abstract—Detection of weakened hosts from a distance by bark beetles through olfaction was investigated in field experiments. No significant numbers of Scolytidae were attracted to anaerobically treated pine bolts, stem disks, or sugar and ponderosa pine bark including phloem. Treatment of living trees with cacodylic acid induced attacks by *Dendroctonus brevicomis*, *D. ponderosae*, *Ips latidens*, *Gnathotrichus retusus*, and *Pityophthorus scalptor*, beginning two weeks after treatment. There was no significant difference between landing rates of *D. brevicomis* and *D. ponderosae* on screened treated trees and screened controls. There was a significant increase in landing rates of *G. retusus* and *I. latidens*, because both species had penetrated the screen and produced pheromones. Tree frilling alone did not increase the landing rate of bark beetles. Freezing of the lower trunk with dry ice did not increase significantly the landing rate of *D. brevicomis*, *D. ponderosae*, *G. retusus*, or *I. latidens* on screened trees, whereas unscreened frozen trees were attacked by all four species. There was no significantly higher landing rate by *D. brevicomis*, *D. ponderosae*, *I.*

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paraconfusus, *I. latidens*, *G. retusus*, or *Hylurgops subcostulatus* on screened trees evidencing symptoms of severe infection by the root pathogen *Verticicladiella wagenerii*, than on symptomless trees. These experiments show that *D. brevicomis*, *D. ponderosae*, *I. paraconfusus*, *I. latidens*, and *G. retusus* land, apparently indiscriminately, on healthy and stressed hosts. Thus, in these species host discrimination must occur after landing and prior to sustained feeding.

Key Words—Primary attraction, tree predisposition, *Dendroctonus*, *Ips*, *Gnathotrichus*, *Pityophthorus*, Coleoptera, Scolytidae, Buprestidae, *Verticicladiella*, *Pinus ponderosa*.

INTRODUCTION

Bark and ambrosia beetles have been shown to be selective in their colonization of host plants, both with respect to the tree species and the physiological condition of the tree. With few exceptions (Rudinsky, 1962) bark beetles infest trees that have been weakened by biotic or abiotic agents such as drought (Blackman, 1924; Craighead, 1925; Ferrell, 1978; Hall, 1958; Kalkstein, 1976; King, 1972; Merker, 1952; St. George, 1929, 1930; Thomas, 1957), excess rainfall or flooding (Hetrick, 1949; Kalkstein, 1976; King, 1972; Lorio and Hodges, 1968), winter injury (Walters, 1955), lightning (Anderson and Anderson, 1968; Hodges and Pickard, 1971; Johnson, 1966a,b; Lorio and Yandle, 1978; Schmitz and Taylor, 1969; St. George, 1930), smog (Stark et al., 1968), mechanical injury (Bennett, 1965; Gara and Holsten, 1975; Hines and Heikkenen, 1977; St. George, 1930; Thatcher, 1960), fire (Furniss, 1965; Hall and Eaton, 1961; Miller and Patterson, 1927; Swain, 1968), competition (Clements, 1953; Coulson et al., 1974; Sartwell and Stevens, 1975; Thomas, 1957), defoliation (Dewey et al., 1974; Drouin and Turnock, 1967; Schultz and Allen, 1977; Wickman, 1963; Wright, 1976), diseases (Bega et al., 1966; Cobb et al., 1974; Davidson, 1964; Felix et al., 1971; Ferrell, 1974; Ferrell and Smith, 1976; Goheen, 1976; Hertert et al., 1975; Hetrick, 1949; Jørgensen and Petersen, 1951; Lorio, 1966; Merker, 1952; Nuorteva and Laine, 1968; Partridge and Miller, 1972; Stark and Cobb, 1969; Wagener and Cave, 1946; Wagener and Mielke, 1961; Wright et al., 1956), and herbicides (Chansler et al., 1970; Drew, 1977; Goldman et al., 1978; McGhehey and Nagel, 1967; Newton and Holt, 1971; Oliver, 1970; Švihra, 1974). Time of tree felling influences the subsequent incidence or severity of attacks by ambrosia beetles (Annila, 1975; Dyer, 1967; Dyer and Chapman, 1965) and other insects (Johnson, 1964; Johnson and Zingg, 1969; Morley, 1939; Pfeffer, 1957). The trap tree method of bark beetle control is based upon the preference of various beetle species for trees treated in various ways, such as felling in sun or shade, girdling, and injection of the herbicide, cacodylic acid (Buffam, 1971; Buffam et al., 1973; Buffam and Yasinski, 1971; Frye and Wygant, 1971; Massey and

Wygant, 1954; Nagel et al., 1957; Sedlaczek, 1921; Stelzer, 1970; Švihra, 1968; Whitten and Baker, 1939). Tree risk-rating systems were based on the observation that trees of a certain age, vigor, and appearance were preferentially killed by bark beetles (Johnson, 1972; Keen, 1936, 1943, 1946; Keen and Salman, 1942; Mogren, 1955; Person, 1928; Salman and Bongberg, 1942; Schenk and Benjamin, 1964; Struble, 1965).

The mechanism of the above selectivity has long been postulated to be initiated by odors emanating from the host which the insects recognize and follow to the source. This has been termed "primary attraction," and efforts of many researchers have been directed toward the isolation and identification of these attractants (Adlung, 1958, 1960; Chararas, 1959; Dässler and Henker, 1959; Heikkenen and Hrutfiord, 1965; Kangas et al., 1967; Merker, 1955; Rudinsky, 1966; Yasunaga, 1962). In most cases, however, there is a lack of experimental evidence that primary attraction was occurring in the field, as opposed to the alternative hypothesis of initial random landing of beetles on various hosts and nonhosts, followed by sustained feeding on suitable hosts (Wood, 1972, and references cited therein). The primary attraction picture was further complicated by the discovery that many scolytid species produce powerful attractant pheromones (Borden et al., 1975). This makes the interpretation of earlier studies difficult, since it is not known whether strict precautions were taken to keep bark beetles from producing pheromones in trees or logs evaluated for primary attraction.

Primary attraction has been demonstrated in the ambrosia beetles *Trypodendron lineatum* (Olivier) (Borden et al., 1968; Chapman, 1962, 1966; Francia and Graham, 1967; Francke and Heeman, 1974; Graham, 1968; Moeck, 1970b), *Xyloterus* (*Trypodendron*) *domesticus* L. (Kerck, 1972), *Xyleborus saxeseni* (Ratzeburg) (Souto, 1974), *Gnathotrichus sulcatus* (LeConte) (Borden and Stokkink, 1973; Cade et al., 1970; Chapman, 1966), and *G. retusus* (LeConte) (Chapman, 1966), as well as the bark beetles *Hylastes nigrinus* (Mannerheim) (Chapman, 1966; Rudinsky and Zethner-Møller, 1967; Souto, 1974), *Dryocoetes autographus* (Ratzeburg) (Chapman, 1966), *Pseudohylesinus nebulosus* (LeConte) (Rudinsky, 1966; Souto, 1974; Stoszek, 1973), *P. grandis* Swaine (Rudinsky, 1966), *P. granulatus* (LeConte) (Souto, 1974), *Leperisinus fraxini* Pz. (Schönherr, 1970), *Scolytus multistriatus* (Marsham) (Peacock et al., 1971), *S. quadrispinosus* Say (Goeden and Norris, 1964), *Ips typographus* L. (Rudinsky et al., 1971), *Dendroctonus pseudotsugae* Hopkins (Chapman, 1964; Jantz and Rudinsky, 1966; Rudinsky, 1966), and *D. rufipennis* (Kirby) (Moeck, 1978). In all of the above studies, cut host material was used.

Further, attractant compounds have been identified for *T. lineatum* (Bauer and Vité, 1975; Moeck, 1970b, 1971; Nijholt and Schönherr, 1976), *X. domesticus* (Kerck, 1972), and *G. sulcatus* (Cade et al., 1970).

Conversely, other field experiments in which cut host material was

presented have failed to demonstrate primary attraction for *Dendroctonus brevicomis* LeConte (Vité and Gara, 1962), *D. frontalis* Zimm. (Vité and Pitman, 1968), *D. rufipennis* (Gara and Holsten, 1975), *Ips paraconfusus* Lanier (Wood, 1963; Wood and Vité; 1961), *I. calligraphus* Germ. (Wilkinson, 1964), *I. avulsus* Eichh. (Vité et al., 1964), *I. grandicollis* Eichh. (Vité et al., 1964; Wilkinson, 1964), *I. pini* (Say) (Anderson, 1948), *I. acuminatus* Gyll. (Bakke, 1967), *I. borealis* Swaine, and *Polygraphus rufipennis* (Kirby) (Gara and Holsten, 1975). Furthermore, the only studies to date which utilized standing trees naturally predisposed to bark beetle attack have also failed to demonstrate primary attraction for *Dendroctonus ponderosae* Hopkins and *D. brevicomis* (Wood, 1972, 1976).

From the above, it would appear that some scolytid species are capable of orienting to weakened hosts through primary attraction, whereas other species either do not have or use this capability, at least as far as can be determined with field methods used to date. However, Person (1931) and others (Miller and Keen, 1960) reported that *D. brevicomis* was attracted to fermenting pine phloem in laboratory experiments. Also, preliminary tests using olfactometers designed by Moeck (1970a) and Wood and Bushing (1963) indicated that some individuals of *D. brevicomis*, *D. ponderosae*, and *I. pini* responded positively to odors of *Pinus ponderosa* Laws. phloem treated anaerobically for approximately 7 hr by the method of Graham (1968) (Moeck, unpublished). It could therefore be argued that since these bark beetle species appear to be capable of responding to host odors in the laboratory, the negative field results were due to the lack of attractants in the cut host material or trees at the time they were presented, or beetles were not flying at the time of the test. In bolts, the age since cutting may have been too little or too great. Living trees, although in a class shown to be highly susceptible to bark beetle infestation (Wood, 1972, 1976), may not have been attractive at the time (both within and between seasons) the studies were conducted. The following field experiments on primary attraction were designed to test these possibilities by anaerobic treatment of host material (Graham, 1968), artificial predisposition to bark beetle attack of living, apparently healthy trees, and selection of severely diseased trees that had a high probability of being attacked by bark beetles during the current season (Goheen, 1976).

METHODS AND MATERIALS

Experiment 1

Treatment 1, Aug. 17–20, 1971. A living *P. ponderosa*, 25 cm diameter at breast height (dbh), was felled and cut into 75-cm-long bolts. Five bolts were treated anaerobically in the following manner: the bolts were placed into a

clean 55-gal steel drum, which was evacuated with two small compressors in series on the vacuum mode to a pressure of approximately 70 mm Hg and left outside for 12 hr overnight. Five bolts were placed in a coldroom at approximately 4°C for the same period to serve as untreated controls. In order to monitor for flight activity of *D. brevicomis*, two bolts were prepared by manually infesting them with 25 females each (Bedard et al., 1969). All bolts were covered with 1.6 × 1.4-mm mesh aluminum screen to prevent volunteer attacks.

The bolts were placed upright on 10-gal drums used as support platforms and surrounded by 1 m^2 of Stikem-Special®-coated 6.4-mm mesh hardware cloth (Bedard and Browne, 1969). The bolts were deployed approximately 90 m apart in two lines on ridges about 0.4 km apart near Bass Lake, Madera County, California. All insects longer than 1 mm were picked from the traps after 18 and 42 hr.

Treatment 2, Aug. 25–Sept. 8, 1971. A living *P. ponderosa*, 23 cm dbh, was felled and cut into seven 70-cm-long bolts. Five bolts were treated anaerobically as in treatment 1 for 24 hr. Two *D. brevicomis* monitor bolts were prepared as above. The next day another living *P. ponderosa* of similar diameter was cut into five bolts to serve as untreated controls. All bolts were screened to keep out volunteers. The bolts were placed upright on the ground or on old stumps at the forest edges, Blodgett Forest Research Station, El Dorado County, California, and surrounded with 0.74 m^2 of Stikem-coated hardware cloth. All insects longer than 1 mm were picked from the traps after 1, 2, 5, 7, and 13 days.

Treatment 3, Sept. 1–15, 1971. A living sugar pine (*Pinus lambertiana* Dougl.), 30 cm dbh, was cut and all the bark, including phloem, from the stem below the live crown was removed.

Half of the approximately 7 m^2 of bark was treated anaerobically as in treatment 1 for 21 hr, and the remaining half was left in a screened box as the control. The treated and untreated bark was stacked on the ground in a forest opening at Blodgett Forest in the vicinity of logging slash containing the brood of *Ips* spp. and other bark beetle species. The bark was covered with 0.71 × 0.71 × 0.91-m screen cages, and one empty cage was used as another control. The sides and top of each cage were covered with 2.5 m^2 of Stikem-coated hardware cloth. The cages were placed at the vertices of an equilateral triangle with 46-m sides. After 13 days, the insects were washed from the traps with hot kerosene (Browne, 1978).

Treatment 4, Sept. 14–24, 1971. A living *P. ponderosa*, 30 cm dbh, was felled, and the bark, including phloem, was removed. Half of the approximately 6 m^2 of bark was treated anaerobically as in treatment 1 for 20 hr, and the remaining half was left in a screened box as a control. The treated and untreated bark was stacked inside 0.36 × 0.74 × 0.74-m cardboard boxes, the tops of which were covered with 1.6 × 1.4-mm mesh aluminum screen; one

empty box served as additional control. One square meter of Stikem-coated hardware cloth was placed in triangular fashion on top of each box. The boxes were placed at the vertices of an equilateral triangle with 30-m sides immediately inside the forest edge at Blodgett Forest. After 9 days, the insects were washed from the traps with hot kerosene. Samples of fresh bark and that treated anaerobically in the drum and separately in a desiccator in the laboratory for the same time period were frozen for subsequent gas-chromatographic analysis of ethanol content (Moeck, 1970).

Treatment 5, Oct. 8–14, 1971. A living *P. ponderosa*, 36 cm dbh, was felled; part of the lower stem was cut into 5-cm-thick disks and part of the upper stem was cut into four bolts 0.6 m long. Half of the disks were treated anaerobically as in treatment 1 for 20 hr and the remaining half was left as a control. Two *D. brevicomis* and two *I. paraconfusus* monitor bolts were prepared as in treatment 1, with 30 females and 20 males each, respectively. The bolts were screened to keep out volunteers. Untreated and treated disks were placed in four screened boxes as in treatment 4 and, with two empty control boxes, were arranged in two triangular groups in the Pilgrim Creek area of the McCloud Flats near McCloud, Siskiyou County, California. The monitor bolts were placed upright on the ground. Bolts and boxes were each provided with 0.5 m^2 of Stikem-coated hardware cloth. To monitor for ambrosia beetles, six traps with 950 ml of 10% ethanol in water and six water control traps, each with 0.25 m^2 of Stikem-coated hardware cloth, were deployed in the same area, 30–60 m from the boxes. At each site, a test and a control trap were placed approximately 5 m apart on 30-cm squares of corrugated cardboard on the ground (Moeck, 1971). After 5 days, the insects were washed from the traps with hot kerosene. One fresh and one anaerobically treated disk were frozen for subsequent gas-chromatographic analysis of ethanol content.

Gas Chromatography. Wood and bark samples frozen in experiment 1, treatments 4 and 5, were freeze-dried (Moeck, 1970), and the condensates obtained were analyzed on a Varian Aerograph model 2700, using a 6.35-mm-OD × 1.52-m copper column packed with 100/120 mesh Porapak Q®. Nitrogen carrier gas was applied at a pressure of 17 psi; air and hydrogen flow rates to the flame-ionization detector were 300 and 30 ml/min, respectively. Injector temperature was 200°C, column temperature was 120°C, and detector temperature was 202°C; sample size was 5 μl. Ethanol at 1%, 0.1%, 0.01% and 0.001% in water (v/v) were used as standards.

Experiment 2

July 19–Oct. 27, 1972. This study was conducted in flat terrain (McCloud Flats) near McCloud, California, in young densely stocked *P. ponderosa* stands containing high-level populations of *D. brevicomis* in some areas. Five

plots were selected, each with the following treatments: (1) an untreated tree; (2) an untreated tree with the bole screened to the base of live crown with 1.6 × 1.4-mm mesh aluminum screen; (3) a tree with an axe frill at the base; (4) a tree treated with 150–200 ml of undiluted Silvisar® 510 (cacodylic acid; several times the recommended dose was used to ensure rapid tree kill; Oliver, 1970) in an axe frill at the base; (5) a tree treated with cacodylic acid as in 4 and the bole screened; (6) a tree with the lower stem packed with dry ice to interrupt the upward flow of water and thus stimulate drought stress; (7) a tree with the lower stem packed with dry ice as in 6 and the bole screened.

Trees were screened as follows (Figure 1): the trees were climbed with the aid of Swedish cone-picking ladders (with the pointed spacers padded to prevent damage to the bole or screen) and pruned of dead branches to the base of the live crown. The outer bark of the stem just below the first live branch was then shaved smooth with a drawknife and wrapped with two layers of 5-cm-wide heating duct tape. The aluminum screen (purchased in 1.22 × 30.5-m rolls) was cut to proper length, with one edge tapered to fit the tree, and then stapled at 30-cm intervals along one inside edge to lath strips. The screen was hoisted to the top with braided nylon line looped over a branch and tacked to the tree at the proper height (Wood, 1976). The ladders were then removed, the screen wrapped around the tree and the ladders repositioned. The screen was then stapled to the tree at the prepared top strip and to the lath strips at 2- to 3-cm intervals down the length of the tree. Alternatively, after the ladders were removed, the stapling was done by a man controlling his own descent in a harness attached to a rope looped around the stem and over a live branch. At the base, the screen was stapled and taped to the smoothed bark.

Lower stem freezing was accomplished as follows: most of the outer bark was removed with a drawknife to a height of about 0.6 m. A corrugated cardboard sleeve, 15 cm greater in diameter than the stem and 30 cm long, was then stapled, tied, and taped to the tree just above the root collar and covered with 5-cm-thick foil-faced fibreglass building insulation. Approximately 30 kg crushed dry ice were placed in the sleeve which was then tied with a drawstring. The dry ice was replenished after 1- to 3-day intervals. Tree moisture stress was monitored with a pressure bomb (PMS Instrument Co., Corvallis, Oregon); readings were taken on previous years' needle fascicles (Johnson and Nielsen, 1969) from branch tips cut with pole pruners or shot down with a .222-caliber rifle equipped with a telescopic sight. Moisture stress was determined at dawn, and on Aug. 21, at 1000, 1200, 1400, and 1600 hr (PDT). Comparison readings were taken on nearby healthy untreated trees.

All trees were provided with 6 Stikem-coated hardware cloth traps, each 30.5 × 61 cm, two each at 2 m, midbole and base of live crown; middle and upper traps were raised and lowered with braided nylon line (after the method developed by Bedard, unpublished). Total trapping surface was 1.12 m^2/tree. Traps were placed on the trees on July 28 (plot 5) and July 31 (plots 1–4).

FIG. 1. Screened diseased ponderosa pine, experiment 3, Blodgett Forest, California, 1973.

Cacodylic acid treatment and frilling treatment were applied on Aug. 8 (plot 5) and Aug. 9 (plots 1–4). Freezing treatment was applied discontinuously between Aug. 15 and Sept. 29. Scolytidae, Cleridae, Trogositidae (Coleoptera), and Siricidae (Hymenoptera) were picked from the traps after 9, 17, 22, 39, and 53 days and all insects were washed from the traps with hot kerosene after 88 days. In August 1973, all cacodylic acid-treated and frozen trees were cut, and the status of insect infestations beneath the bark was determined. All other trees were checked for phloem and crown condition.

Trap catch data were analyzed as follows: pairwise comparisons under the different treatment regimes were made with the Wilcoxon signed rank test for matched pairs (Lehmann, 1975, Chap. 3). Matching was based on trapping period and plot, i.e., comparison pairs consisted of trap catches during the same period on trees in the same plot. A large number of ties and zeros occurred in the data, necessitating the use of midranks. Since the published tables for the signed rank test are generally incorrect when midranks are used, a computer program was developed to calculate the exact significance probabilities of test results.[6]

Experiment 3

April 26–Sept. 14, 1973. This study was conducted at the University of California Blodgett Forest Research Station in the central Sierra Nevada mountains in the Gaddis Creek basin (elev. 1300 m). Forest cover is a mixed conifer type, with *P. ponderosa* 50–80 years old predominating. A high population of *D. brevicomis* has persisted in this area since at least 1955 and appears to be intimately associated with a spreading infection of the root pathogen, *Verticicladiella wagenerii* Kendrick (Cobb, et al., 1974; Stark and Dahlsten, 1970). A 4-year study by Goheen (1976) has indicated that a tree with 50% or more of its circumference at the root collar stained by *V. wagenerii*, as determined by arch punch sampling early in the season, had a 0.45 probability of being attacked by bark beetles in the same year. This method was therefore chosen to select trees naturally predisposed to bark beetle attack and to study landing rates throughout the season.

In late April and early May, two groups of trees were chosen: 20 healthy *P. ponderosa* (mean dbh 34.7 $\pm$ 1.9 cm) showing no *V. wagenerii* stain in a 5% linear sample (6.4-mm arch punch sample every 12.7 cm) at the root collar, and 35 severely diseased *P. ponderosa* with 50% or more of the samples showing *V. wagenerii* stain in the sapwood. Trees surrounding the study trees were also sampled to ensure the absence of severely diseased trees which, if attacked by beetles, would influence landing rates on the study trees. Twenty

[6]For a discussion of the difficulty in dealing with ties and zeros, see Lehmann (1975) pp. 18–23, 60, 129–132. Information about the computer program may be obtained by contacting K. Q. Lindahl.

of the diseased trees (D) (mean dbh 34.6 ± 1.5 cm, mean percentage *V. wageneri* stain 74.9 ± 2.9) were screened (DS) to the base of the live crown by the ladder method as described in experiment 2 (Figure 1). The healthy (H) and remaining diseased trees (D) (mean dbh 44.2 ± 2.6 cm, mean percentage *V. wageneri* stain 62.6 ± 3.2) were left unscreened (HU and DU), and all were pruned of dead branches to 11 m with pole pruners. The latter group served as a check on the proportion of diseased trees coming under insect attack.

All trees were provided with six Stikem-coated hardware cloth traps, each 30.5 × 61 cm, two each at 1 m, midbole, and base of live crown; middle and upper traps were raised and lowered as described in experiment 2. Total trapping surface was 1.12 m^2/tree. All insects longer than 1 mm, except Psocoptera, were picked from the traps after each of 12 trapping periods which varied from 1 to 2 weeks in duration. Collections 2–5 were from half of the traps on each tree, whereas collection 6 was a cumulative count for periods 2–5 inclusive from the other half of the traps. Collections 1 and 7–12 were from all traps, for a total of 13 collections. In September, screened diseased trees with fading foliage or insect attack in the crown were cut, and the status of the insect infestation was determined.

The landing rate data for each insect species under investigation were statistically analyzed as follows: the observations were first sorted into 3 groups: namely, groups HU, DS, and DU, containing data from healthy unscreened, diseased screened, and diseased unscreened trees, respectively, except those subject to direct insect attack or pheromone interference from nearby attacked trees. Evidence of this was provided by boring dust on the traps or under the screen, presence of fresh pitch tubes on the tree or galleries in the bark as revealed by tree dissection at the end of the experiment; data from these trees for the affected trapping periods were placed into a fourth group A (attacked). All observations were then standardized to a trap-tree-day basis by dividing the numbers of insects caught by the number of days between observations. The resulting landing rates for groups HU, DS, and DU were then compared by the Kruskal-Wallis test (Lehmann, 1975). When this test was significant, indicating differences among the groups, multiple comparisons were made to determine which pairs of groups differed from one another (Miller, 1966). Groups HU and A were compared by the Wilcoxon rank-sum test (Lehmann, 1975). Where data were available the sexes were analyzed separately. All tests were judged significant if the significance probability was less than $\alpha = 0.05$.

RESULTS

Experiment 1

Very few Scolytidae were caught on any of the test materials except on traps containing manually infested bolts (Tables 1–3). These beetles were

TABLE 1. CATCH OF SCOLYTIDAE ON STICKY TRAPS CONTAINING PONDEROSA PINE BOLTS THAT WERE UNTREATED, ANAEROBICALLY TREATED, OR INFESTED WITH *D. brevicomis* FEMALES, EXPERIMENT 1, TREATMENTS 1 AND 2, BASS LAKE AND BLODGETT FOREST, CALIFORNIA, AUGUST–SEPTEMBER 1971

Treatment no.	Total trapping time	No. and species of Scolytidae trapped at: 5 bolts untreated	2 bolts infested with 25 ♀ *D. brevicomis* each[a]	5 bolts treated anaerobically[b]
1	42 hr	1 ♀ *D. valens*	13 ♂ 11 ♀ *D. brevicomis*	0
2	13 days	1 ♀ *I. paraconfusus*	14 ♂ 9 ♀ *D. brevicomis* 1 ♂ *G. retusus*	0

[a] 18 of 25 and 19 of 25 females in treatment 1, and 21 of 25 and 18 of 25 females in treatment 2, producing frass.
[b] Anaerobic treatment 12 and 24 hr in treatments 1 and 2, respectively.

responding to pheromones produced by female *D. brevicomis* (Table 1) as has been demonstrated by Bedard et al. (1969), and by male *I. paraconfusus* (Table 3), as shown by Wood and Vité (1961). Failure to catch *D. brevicomis* on female-infested bolts in treatment 5 (Table 3) remains unexplained, but Bedard and Wood (unpublished) found that such bolts were attractive in spring and early summer, but not in the fall, the season during which this test was conducted. Thus in treatment 5 no conclusion with regard to attraction of *D. brevicomis* can be drawn, since the beetles' presence was not verified. The few scolytids caught on untreated or treated host materials probably arrived by chance, since as many were trapped on the control traps that contained no host materials.

The gas chromatography results indicate that the large-scale anaerobic treatment of bolts, bark, and wood disks was ineffective in producing the high (0.4%) ethanol concentration obtained in earlier studies (Moeck, 1970b). The reasons for this are not known. The extract from *P. ponderosa* bark frozen 4 hr after cutting contained 0.011% ethanol and after 20 hr of anaerobic treatment in the drum it contained 0.026%, whereas the sample from the desiccator in the laboratory contained 0.40%. Similarly, treatment of the *P. ponderosa* disks increased the ethanol concentration only slightly from 0.003% to 0.006%. However, the absence of a response to the 10% ethanol-in-water mixture indicates that ethanol produced by anaerobiosis would not be attractive by itself even at 0.4%, the maximum concentration expected under natural conditions (Moeck, 1970b). However, other products produced under these conditions may have been attractive.

Although no proof was obtained that ambrosia beetles and bark beetles other than *D. brevicomis* and *I. paraconfusus* were flying during the

TABLE 2. CATCH OF SCOLYTIDAE ON STICKY TRAPS ON EMPTY CAGES AND ON CAGES CONTAINING UNTREATED OR ANAEROBICALLY TREATED PONDEROSA PINE OR SUGAR PINE BARK, EXPERIMENT 1, TREATMENTS 3 AND 4, BLODGETT FOREST, CALIFORNIA, SEPTEMBER 1971

Treatment no.	Total trapping time (days)	No. and species of Scolytidae trapped at		
		Empty cage	Untreated bark[a]	Anaerobically treated bark[b]
3	13	1 ♂ *I. paraconfusus* 1 *Xyleborus scopulorum* 1 *Hylastes gracilis*	1 ♀ *D. brevicomis* 1 *Pseudopityophthorus* sp.	2 *Scolytus* sp. 1 *Pseudopityophthorus* sp.
4	9	1 ♀ *D. brevicomis*	0	0

[a] *P. lambertiana* bark in treatment 3, *P. ponderosa* bark in treatment 4.
[b] Anaerobic treatment of *P. lambertiana* bark for 21 hr in treatment 3, and of *P. ponderosa* bark for 20 hr in treatment 4.

TABLE 3. CATCH OF SCOLYTIDAE ON STICKY TRAPS CONTAINING WATER, 10% ETHANOL IN WATER, *D. brevicomis* AND *I. paraconfusus* INFESTED PONDEROSA PINE BOLTS AND UNTREATED AND ANAEROBICALLY TREATED PONDEROSA PINE STEM DISKS, EXPERIMENT 1, TREATMENT 5, MCCLOUD, CALIFORNIA, OCTOBER 1971[a]

Treatment	No. and species of Scolytidae trapped
2 empty cages	0
6 water traps	1 ♀ *G. retusus*, 1 ♀ *G. sulcatus*
6 ethanol (10%) traps	0
2 *P. ponderosa* bolts infested with 30 ♀ *D. brevicomis* each[b]	1 *Hylurgops subcostulatus*
2 *P. ponderosa* bolts infested with 20 ♂ *I. paraconfusus* each[c]	16 ♂ 18 ♀ *I. paraconfusus*
2 boxes with untreated *P. ponderosa* disks	1 ♀ *I. paraconfusus*
2 boxes with *P. ponderosa* disks anaerobically treated for 20 hr	1 ♀ *G. retusus* 1 *Hylurgops reticulatus*

[a] Total trapping time 5 days.
[b] 28 of 30 and 28 of 30 females producing frass.
[c] 16 of 20 and 18 of 20 males producing frass.

experimental period, their absence is believed to be highly unlikely at the elevation and time of year of these experiments. Further, these experiments demonstrate that very little, if any, attraction is produced in host material subjected to the most extreme stress, i.e., killing of the tree. The duration of experiment 1, treatment 2 (13 days) was believed to be adequate for natural aging processes to take place and render the bolts suitable for attack, yet no bark beetles were trapped. Host suitability was indicated by the boring activity of *D. brevicomis* females in the monitor bolts and the presence of an active pair of *D. ponderosae* in an 8-cm gallery on Aug. 28 in the top of the tree cut on Aug. 25. Treatments 3–5 were an attempt to increase the surface area for evaporation of host volatiles, including terpenes, yet no significant catches were recorded for periods up to 13 days. Thus the question of whether anaerobic processes that produce higher alcohol levels in wood and/or phloem results in attraction of bark beetles remains unanswered by these experiments. If primary attraction occurs, it must be at a very low level and must be produced under conditions other than those imposed in experiment 1, i.e., longer periods of aging or anaerobic treatment.

Experiment 2

Tree screening did not appear to materially influence the landing rate of Scolytidae (Table 4, columns 1 and 2), confirming previous findings by Wood

TABLE 4. CATCH OF SCOLYTIDAE AND PLATYPODIDAE ON UNSCREENED AND SCREENED PONDEROSA PINES THAT WERE HEALTHY OR TREATED WITH CACODYLIC ACID, FRILLING, OR LOWER STEM FREEZING WITH DRY ICE, EXPERIMENT 2, MCCLOUD, CALIFORNIA, JULY–OCTOBER 1972

Species	Tree treatment (5 trees each) and no. of beetles trapped						
	Unscreened control[a]	Screened control[a]	Unscreened frill only	Unscreened frill with cacodylic acid	Screened frill with cacodylic acid	Unscreened frozen	Screened frozen
Dendroctonus brevicomis							
♂	4	2	3	8	2	13	2
♀	6	3	4	13	2	25	3
Dendroctonus ponderosae							
♂	2	4	2	16	9	13	4
♀	1	8	2	19	6	17	2
Gnathotrichus retusus	10	2	2	379	38[b]	28	1
Hylastes gracilis	2	1	1	1	5	3	1
Hylastes tenuis	0	0	0	1	0	0	0
Hylurgops reticulatus	1	1	0	3	4	9	0

Hylurgops subcostulatus	1	0	0	2	0	1	0
Ips latidens	18	26	8	171	63[b]	21	16[b]
Phloeosinus spp.	1	0	1	0	0	2	0
Pityophthorus scalptor	1	6	5	21	10	4	6
Pityophthorus serratus	1	4	3	0	1	8	7
Pseudohylesinus sp.	0	0	0	0	1	1	1
Pseudopityophthorus sp.	0	0	1	1	0	0	0
Scolytus spp.	3	6	0	1	0	1	1
Xyleborus saxeseni	1	0	0	0	0	0	0
Xyleborus scopulorum	5	0	2	2	0	0	0
Platypus wilsoni							
♂	5	3	2	5	2	0	1
♀	1	3	3	3	1	1	1
Total all species	63	69	39	646	144	147	46
No. of trap-tree-days	629	580	394	394	394	355	355
Mean No. per trap-tree-day	0.10	0.12	0.10	1.64	0.37	0.41	0.13

[a]Includes control trees and test trees prior to treatment.
[b]Beetles penetrated screen (see text).

TABLE 5. COMPARISON OF MEAN LANDING RATES OF FOUR SPECIES OF SCOLYTIDAE ON UNSCREENED AND SCREENED HEALTHY PONDEROSA PINES AND UNSCREENED AND SCREENED PONDEROSA PINES TREATED WITH CACODYLIC ACID, FRILLING, OR LOWER STEM FREEZING WITH DRY ICE, EXPERIMENT 2, MCCLOUD, CALIFORNIA, JULY–OCTOBER 1972

Comparison	*Dendroctonus brevicomis*	*Dendroctonus ponderosae*	*Gnathotrichus retusus*	*Ips latidens*
Unscreened untreated trees[a]	0.0138[b]	0.0034	0.0155	0.0207
Screened untreated trees[a]	0.0086	0.0207	0.0034	0.0448
Significance probability[c]	0.3072	0.0626	0.0312	0.6042
Unscreened untreated trees[d]	0.0076	0.0025	0.0152	0.0076
Unscreened cacodylic acid treated trees	0.0533	0.0888	0.9619	0.4340
Significance probability[e]	0.0059	0.0002	0.0002	<0.0001
Screened untreated trees[d]	0.0076	0.0228	0.0051	0.0102
Screened cacodylic acid treated trees	0.0102	0.0381	0.0964	0.1599
Significance probability[e]	0.3438	0.1084	0.0005[g]	0.0001[g]
Unscreened untreated trees[d]	0.0076	0.0025	0.0152	0.0076

Unscreened frill treated trees	0.0178	0.0102	0.0051	0.0203
Significance probability[e]	0.1797	0.1875	0.9063	0.2031
Unscreened untreated trees[f]	0.0085	0.0028	0.0169	0
Unscreened frozen trees	0.1070	0.0845	0.0789	0.0592
Significance probability[e]	0.3594[h]	0.0005	0.3750[h]	0.0078
Screened untreated trees[f]	0.0085	0.0254	0.0056	0.0113
Screened frozen trees	0.0141	0.0169	0.0028	0.0451
Significance probability[e]	0.3125	0.6367	0.8750	0.1875

[a]Includes control trees and test trees prior to treatment (580 trap-tree-days).
[b]Mean beetle landing rate per trap-tree-day = $\frac{\text{No. of beetles trapped}}{\text{No. of trees per treatment} \times \text{No. of days of trapping}}$
[c]Wilcoxon signed rank test, 2-sided (Lehmann, 1975).
[d]394 trap-tree-days.
[e]Wilcoxon signed rank test, 1-sided (H_1: treatment raises landing rate).
[f]355 trap-tree-days.
[g]Beetles penetrated screen (see text).
[h]See text for explanation of apparent lack of significance.

(1976). Of the species tested statistically (Table 5), only *G. retusus* had a significantly higher landing rate on unscreened trees compared to screened trees, but the numbers of beetles trapped were small (9 and 2, respectively, in 580 trap-tree-days), and these beetles probably arrived by chance.

Cacodylic Acid Treatment. The cacodylic acid treatment was effective in stressing the trees, resulting in bark and ambrosia beetle attack on treated trees; all trees died during the study period. There was some variation in the time taken for the foliage to turn yellow, the earliest symptoms of poisoning being observed about 2 weeks after treatment. This coincided with initial scolytid and siricid attacks on unscreened poisoned trees. There was a significant increase in landing rate on the unscreened cacodylic acid-treated trees as compared to the unscreened controls for *D. brevicomis, D. ponderosae, G. retusus,* and *I. latidens* (LeConte) (Table 5), all attributable to attractants produced by these species' boring activity on these trees. The numbers of beetles caught, however, were much lower than would be expected on normally mass-attacked trees. A possible explanation was obtained in 1973, when tree dissection revealed a low attack density, and very short and abnormal bark beetle galleries. Ambrosia beetles either died in their galleries or abandoned them. This apparently early lethal condition of the phloem and sapwood was probably due to the large dose of cacodylic acid used in the treatment, preventing the development of a normal scolytid mass attack, which depends on the health and activity of the first-attacking beetles. Lister et al. (1976) obtained lower attack densities of spruce beetles (*D. rufipennis*) on trap trees treated with full-strength cacodylic acid compared to controls or diluted cacodylic acid treatments, but they attributed this to repellency of the cacodylic acid.

There was no significant increase in landing rate of *D. brevicomis* and *D. ponderosae* on the screened cacodylic acid-treated trees over the screened controls (Table 5), indicating no increase in attraction, at least at a level where it could be detected by this experimental design. One treated screened tree received 5 *D. ponderosae* attacks above the screen, which may have produced attractant pheromones, resulting in slightly higher catches on this tree. There was a significant increase in landing rate of *G. retusus* and *I. latidens*, but tree dissection showed that both species had penetrated the screen, thus invalidating the experiment as a test for primary attraction for these species. The higher proportion of male *G. retusus* (1.5:1) caught on the screened poisoned trees, when compared to the unscreened (0.9:1), may, however, be an indication of primary attraction since the males initiate the galleries and presumably would be more responsive to host odors. Ethanol traps caught far more male than female *G. sulcatus* (males–females = 4.9:1; Moeck, 1971), whereas Byrne et al. (1974) found that the male-produced pheromone attracted far fewer males than females (0.38:1). Without contrary evidence, it

is reasonable to assume that *G. retusus* would behave in a similar manner.

Frilling Treatment. Landing rates on frilled trees were not significantly higher than landing rates on untreated trees (Table 5). Frilling alone did not predispose the trees to bark beetle attack during the study period. Three years later, 4 of the 5 trees still appeared healthy and were free of insect attack, while one tree succumbed to insect attack in the third year. Vité (1961) also found that girdling the bark, including the phloem and cambium of ponderosa pines, and cutting deep into the sapwood on the entire circumference of the trunk did not elicit bark beetle attacks for one or more seasons.

Freezing Treatment. Tree-base freezing with dry ice had the desired effect of imposing a severe moisture stress on the tree above the point of freezing and in effect simulating naturally induced drought conditions (Figure 2). Stress condition was reached 1 day after the application of dry ice, as indicated by dawn pressure-bomb readings on needle fascicles (average stress -382.6 ± 5.1 psi, controls -169.6 ± 2.7 psi) and was maintained as long as the lower stem remained frozen. Frozen trees also showed no regular diurnal fluctuations of moisture stress as did nearby untreated trees (Figure 3). However, moisture

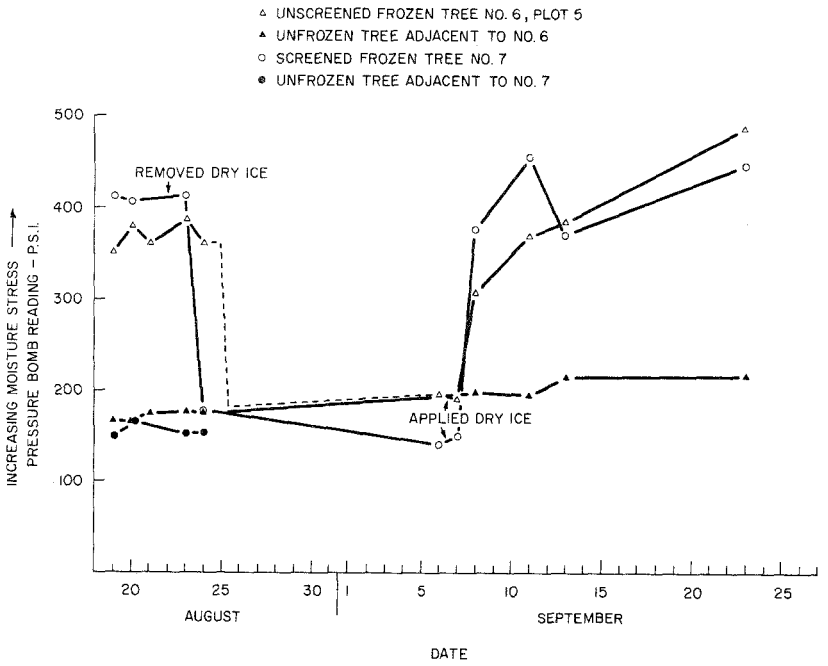

FIG. 2. Dawn pressure bomb readings on needles of ponderosa pines with lower stems frozen by dry ice and untreated controls, experiment 2, McCloud, California, 1972. Dry ice first applied August 16.

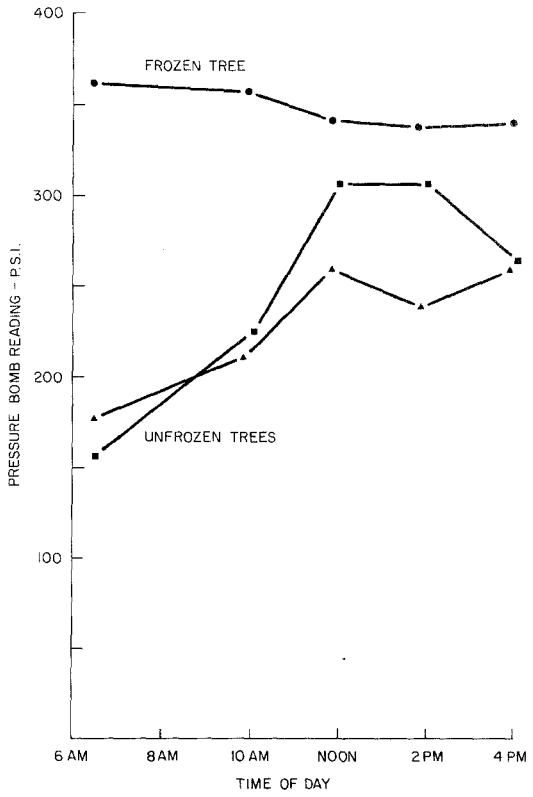

FIG. 3. Pressure bomb readings at different times of day on needles of ponderosa pines frozen by dry ice and untreated controls, experiment 2, McCloud, California, August 21, 1972.

stress increased with time (Figure 2), indicating that the trees continued to lose water in the absence of replenishment from the soil. Trees which were allowed to thaw out regained their turgor.

Freezing of the lower stem irreversibly damaged the trees. The phloem and cambium died in the region of freezing, effectively girdling the trees. The unscreened frozen trees were subsequently heavily attacked, mostly in the year following the experiment, by *D. valens* LeConte, *D. ponderosae, D. brevicomis, Hylurgops subcostulatus* Mannerheim, *I. latidens, G. retusus, Pityophthorus serratus* Swaine, and undetermined Buprestidae, Cerambycidae, and Siricidae. The screened treated trees received mostly *D. valens* attacks at the base and *Pityophthorus* spp., Cerambycidae, and Buprestidae attacks in the crown. The girdling could not have been solely responsible for these results since the mechanically girdled unscreened trees received no insect attacks whatsoever. The wood and crowns of the frozen trees appeared

abnormally dry, even in the screened trees which received no major bark beetle attacks, indicating that the water-conducting capacity of the sapwood was somehow permanently impaired by temporary freezing. On the other hand, mechanically girdled trees continue to conduct water (Vité, 1961). This method appears to be very useful for the experimental predisposition of standing trees to attack by various bark and woodboring insects, where tree survival is of no concern. Whitney (personal communication), in studies of host reaction to bark beetles and their symbiotic fungi, has devised a method of tree stem cooling without causing permanent injury, yet inducing moisture stress in the tree.

Owing to an irregular supply of dry ice and the activities of a bear which destroyed several dry ice packs on trees, the 10 trees were not continuously frozen during the study period. As a result, two unscreened trees, after thawing, pitched out several *D. ponderosae* from galleries initiated during the period when the trees were frozen. Vité (1961) has demonstrated a direct relationship between oleoresin exudation pressure (OEP) and moisture stress in *P. ponderosa*, i.e., as moisture stress decreases, OEP rises. Wood (1962) also found that induced *I. paraconfusus* mass attack was delayed on trees which were cut but retained some OEP because of branch removal, when compared to cut trees with branches intact. When *D. ponderosae* and *D. brevicomis* attacked unscreened trees while they were frozen, only dry frass and no pitch tubes were produced.

There was a significant increase in the landing rate of *D. ponderosae* and *I. latidens* on unscreened frozen trees compared to unscreened controls, and no statistically significant increase of *D. brevicomis* and *G. retusus* (Table 5). The lack of significance for the latter two species is probably due to the low number of matched observations not resulting in zeros or ties, and to the insensitivity of the Wilcoxon test to large numbers. Mean landing rates on unscreened frozen trees rose dramatically for both *D. brevicomis* and *G. retusus*, and the numbers of *D. brevicomis* caught on unscreened frozen trees were the highest of any treatment (Table 4). Furthermore, there was direct visual evidence of attack by both species. Thus tree-base freezing with dry ice subjected trees to severe moisture stress and predisposed them to bark and ambrosia beetle attack. However, landing rates of the above four species did not increase significantly on the screened frozen trees when compared to screened controls, again providing no evidence of primary attraction.

Experiment 3

Degree of sapwood staining caused by the root pathogen, *V. wagenerii*, as determined by use of the arch punch sampling method (Goheen, 1976), is an excellent criterion for selecting trees with a high probability of being attacked by insects in the current season. None of 20 healthy trees were killed, while 15

of the 35 diseased trees (43%) were dead by September. Twelve of the remaining 20 diseased trees were killed in the following 2 years, while 2 of the 20 controls were killed; one of these controls had some *V. wageneri* stain (5%) at the base at time of death. Thus, over the 3-year period, 77% of the severely root-diseased trees were killed. In his 4-year study, Goheen (1976) found that 79% of severely diseased trees, and only 2% of controls, were killed.

In 1973, 12 of the 35 diseased trees were mass attacked and killed by *D. brevicomis* and/or *D. ponderosae*. Thus those species are not the only tree killers in the study area. *Melanophila californica* Van Dyke and *M. gentilis* LeConte (Coleoptera: Buprestidae) were observed attacking most of the diseased trees (27 of 35), and in at least 10 cases preceded bark beetle attack.

Dendroctonus brevicomis. Of the healthy trees, one sustained an unsuccessful basal mass attack, probably as the result of the attraction of beetles to an attack on some nearby logs cut from a diseased tree. After the beetles were pitched out, the landing rates were comparable to those on healthy unattacked trees. A light attack on another tree was also overflow from a nearby attacked nonexperimental tree.

Of the diseased screened trees, one was attacked just prior to screening, and the greatly elevated landing rate on this tree indicated that tree screening did not interfere either with the liberation of attractant pheromones from the tree or with the landing behavior of arriving beetles. Thus the validity of the experimental method was confirmed. One other tree was lightly attacked just prior to screening and later, with three others, was mass attacked in the crown by *D. brevicomis*.

Of the unscreened diseased trees, four received heavy *D. brevicomis* attacks and were killed, whereas two were lightly attacked. Of the latter, one died early in 1973, while the other was again attacked in 1974 and killed (Goheen, personal communication).

Landing rate of male *D. brevicomis* was significantly higher on healthy trees (HU) than on both screened (DS) and unscreened (DU) diseased trees, but did not differ between the latter two (Table 6), indicating possible weak pheromone sources on or near several healthy trees. Males are more responsive to pheromones than females (Silverstein et al., 1968), and incomplete pheromone mixtures, which are probably present early in the attack phase, produce skewed sex ratios in favor of the males (Wood, 1972). However, the landing rate of females was not significantly different between healthy and screened or unscreened diseased trees prior to attack.

Dendroctonus ponderosae. A few attacks on two healthy trees probably resulted from attraction of beetles to nearby trees under attack. Three screened trees were attacked in low numbers prior to screening, while four others received light crown attacks. One unscreened diseased tree was lightly attacked, whereas two others were mass attacked and killed, one of these in combination with *D. brevicomis*.

TABLE 6. SUMMARY OF CATCHES AND MEAN LANDING RATES OF MAJOR SPECIES OF SCOLYTIDAE ON UNSCREENED HEALTHY AND UNSCREENED AND SCREENED ROOT-DISEASED PONDEROSA PINES, WITH RESULTS OF STATISTICAL ANALYSIS, EXPERIMENT 3, BLODGETT FOREST, CALIFORNIA, MAY–SEPTEMBER 1973

Species	Summary of trap catches and mean landing rates																*P* values	
	Group HU (healthy unscreened)				Group DS (diseased screened)				Group DU (diseased unscreened)				Group A (attacked)					
	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD	Kruskal-Wallis Test groups HU, DS, DU	Rank-sum test groups HU, A
D. brevicomis ♂	233	2064	133	0.064	199	1731	62	0.036	112	954	42	0.044	77	689	2156	3.129	0.011[a]	<0.001
♀	233	2064	108	0.052	199	1731	88	0.051	112	954	52	0.055	77	689	1768	2.566	0.725	<0.001
D. ponderosae ♂	234	2021	16	0.008	208	1811	17	0.009	138	1138	24	0.021	41	468	114	0.244	0.088	<0.001
♀	234	2021	11	0.005	208	1811	21	0.012	138	1138	17	0.015	41	468	141	0.301	0.061	<0.001
D. valens ♂	218	1944	1	0.001					23	194	0	0	380	3300	30	0.009	No test	No test
♀	218	1944	6	0.003					23	194	0	0	380	3300	16	0.005		
I. paraconfusus ♂	239	2110	4	0.002	217	1897	8	0.004	121	1028	2	0.002	44	403	289	0.716	0.669	<0.001
♀	239	2110	3	0.001	217	1897	6	0.003	121	1028	6	0.006	44	403	312	0.773	0.211	<0.001
I. latidens ♂ + ♀	239	2110	104	0.049	217	1897	88	0.046	121	1028	83	0.075	44	403	98	0.341	0.393	<0.001
G. retusus ♂	244	2158	140	0.065	154	1287	133	0.088	114	930	113	0.122	109	1063	2041	1.920	0.068	<0.001
♀	244	2158	129	0.060	154	1287	125	0.097	114	930	78	0.084	109	1063	1893	1.781	0.782	<0.001
H. subcostulatus ♂ + ♀	244	2158	29	0.013	236	2091	50	0.024	135	1154	55	0.048	6	35	209	5.971	0.005[b]	<0.001

[a] HU ≠ DS, HU ≠ DU: groups differing significantly according to multiple comparisons following significant Kruskal-Wallis test.
[b] HU ≠ DU: groups differing significantly according to multiple comparisons following significant Kruskal-Wallis test.

D. ponderosae was trapped at less than one tenth the *D. brevicomis* rate, and neither males nor females showed significantly different landing rates between healthy and screened or unscreened diseased trees prior to attack, thus giving no evidence of primary attraction.

Dendroctonus valens. There is a high correlation between *D. valens* attack and severe root disease symptoms at the root collar. Only 2 of 20 healthy trees exhibited *D. valens* attacks (one gallery each), whereas 33 of 35 diseased trees exhibited a few to many attacks (over 40 in one case). Since most attacks occurred prior to screening as well as at ground level below the screen where no protection to the tree was provided, no meaningful test of primary attraction exists for this species. Many galleries were started in the arch punch sampling holes and since the holes were present in equal numbers on the healthy trees and diseased trees, resinosis, which was prevalent on the healthy trees, does not in itself explain host selection by this species, as suggested by data of Vité and Gara (1962) and Furniss and Schmitz (1971).

Ips paraconfusus. Three diseased screened trees were mass attacked above the top of the screen by *I. paraconfusus*. Landing rates on most of the remaining trees were very low, with traps on 34 trees catching no beetles during the entire experimental period. Landing rates did not differ significantly for either sex between healthy and unscreened and screened diseased trees.

Ips latidens. Attacks by *I. latidens* were not confirmed on any of the experimental trees, although several diseased trees showed higher than normal landing rates. Because *I. latidens* was observed to penetrate the screen in experiment 2, this relatively high landing rate was presumably due to pheromone production. However, landing rates did not differ significantly on healthy, unscreened and screened diseased trees.

Gnathotrichus retusus. No attacks or high landing rates of *G. retusus* were noted on any of the healthy trees, whereas 19 of the 35 diseased trees were attacked. This ambrosia beetle was also able to penetrate the screen used in this experiment, as was the case in experiment 2. Attacks by this species occurred prior to, concurrent with, subsequent to, and in the absence of attacks by *D. brevicomis* or *D. ponderosae*, indicating a lack of dependence on tree predisposition by primary bark beetle invaders such as *Dendroctonus* spp. and *Ips* spp. Landing rates did not differ sigificantly for either sex between healthy and unscreened and screened diseased trees prior to attack.

Hylurgops subcostulatus. Only one tree received a mass attack of *H. subcostulatus*, and this occurred in early May, concurrently with *G. retusus* attacks. The relatively large number of beetles caught on this tree indicates the presence of a pheromone system for this species. Landing rates did not differ significantly between healthy and diseased screened trees, but were significantly higher on unscreened diseased trees when compared to controls,

indicating the possible presence of a few undetected attacks and pheromone production on the unscreened diseased trees.

Other Species. Many other Scolytidae and Platypodidae were caught on the experimental trees, some in considerable numbers (Table 7). Some species are too small to be excluded by the screen and hence no conclusions about primary attraction can be drawn from the numbers caught. Other species that were trapped have not been collected from *P. ponderosa* (Bright and Stark, 1973). Since they were landing on the experimental trees, a tree stem silhouette may be an adequate landing stimulus for these species. The absence of apparent boring activity, however, indicates that other stimuli are involved in host recognition.

Hylastes macer LeConte, which was found to attack the roots of ponderosa pine and has been strongly implicated as a vector of *V. wageneri* (Goheen, 1976; Goheen and Cobb, 1978), was caught in moderate numbers, mainly in late May. Traps on one dying diseased unscreened tree caught 15% of the beetles, but attacks by this species on this tree were not confirmed.

Of the remaining species which attack ponderosa pine, *Trypodendron lineatum* was caught in too low numbers to permit drawing valid conclusions. The biology of the other species [*Hylastes gracilis* LeConte, *H. longicollis* Swaine, *H. nigrinus, Hylurgops porosus* (LeConte), and *H. reticulatus* Wood] in pine is not known. Hence it cannot be established whether a valid test condition existed in this experiment.

DISCUSSION

In none of the field experiments described here could primary attraction, i.e., guided insect response to host-produced volatiles, be adequately demonstrated for *D. brevicomis, D. ponderosae*, or *I. paraconfusus*, despite the verified presence of beetles in the area and presentation of host material or trees known to be suitable for infestation. The results do indicate, however, that beetles landed apparently indiscriminately on healthy and stressed hosts at a rate of about one beetle per tree per day for *D. brevicomis* and one beetle per tree per 5 days for *D. ponderosae*, based on the extrapolation of the mean landing rates to the surface area of a tree of average height (24 m) and diameter (34.7 cm). Wood (1976) demonstrated that these species also land on nonhosts. Thus, even in the absence of primary attractants, there was ample opportunity for the beetles to determine the host species and its physiological condition after landing. Theoretically, only one beetle boring in a suitable tree can initiate a mass attack on that tree, owing to the production of pheromones (Wood, 1972). The average landing rate of *D. brevicomis* on the attacked trees was over 70 beetles per tree per day, with peak rates calculated to be in excess of 800 beetles per tree per day, while Stephen and Dahlsten

TABLE 7. SUMMARY OF CATCHES AND MEAN LANDING RATES OF INCIDENTAL SPECIES OF SCOLYTIDAE AND PLATYPODIDAE ON STICKY TRAPS ON UNSCREENED AND SCREENED ROOT-DISEASED PONDEROSA PINES, EXPERIMENT 3, BLODGETT FOREST, CALIFORNIA, MAY–SEPTEMBER 1973

Species	Summary of trap catches and mean landing rates															
	Group HU (healthy unscreened)				Group DS (diseased screened)				Group DU (diseased unscreened)				Group A (attacked)			
	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD	No. of observ.	Trap tree days (TTD)	No. of insects	Mean landing rate/TTD
Dryocoetes autographus	244	2158	2	0.001	236	2091	0	0	141	1189	1	0.001				
Gnathotrichus sulcatus[a]	244	2158	11	0.005	236	2091	8	0.004	135	1154	5	0.004	6	35	14	0.400
Hylastes gracilis	244	2158	2	0.001	236	2091	4	0.002	141	1189	0	0				
Hylastes longicollis	244	2158	291	0.135	230	2055	258	0.126	129	1119	71	0.063	18	106	218	2.057

Hylastes macer	244	2158	54	0.025	236	2091	86	0.041	135	1154	71	0.062	6	35	37	1.057
Hylastes nigrinus	244	2158	5	0.002	236	2091	10	0.005	141	1189	8	0.007				
Hylastes tenuis[a]	244	2158	201	0.093	231	2064	269	0.130	129	1116	77	0.069	17	100	306	3.060
Hylurgops porosus	244	2158	45	0.021	236	2091	98	0.047	135	1154	38	0.033	6	35	34	0.971
Hylurgops reticulatus	244	2158	8	0.004	236	2091	19	0.009	129	1119	7	0.006	12	70	50	0.714
Monarthrum scutellare[b]	244	2158	2	0.001	236	2091	1	0.001	141	1189	1	0.001				
Phloeosinus spp.[b]	244	2158	16	0.007	236	2091	4	0.002	141	1189	15	0.013				
Pityokteines ornatus[a]	244	2158	10	0.005	225	1978	18	0.009	124	1071	2	0.002	28	231	80	0.346
Pityophthorus confertus[a]	244	2158	7	0.003	236	2091	13	0.006	129	1119	5	0.004	12	70	27	0.386
Pseudohylesinus spp.[b]	244	2158	10	0.005	236	2091	5	0.002	141	1189	3	0.003				
Pseudopityophthorus pubipennis[b]	244	2158	3	0.001	236	2091	3	0.001	141	1189	0	0				
Scolytus spp.[b]	244	2158	42	0.019	236	2091	45	0.022	141	1189	40	0.034				
Trypodendron lineatum	244	2158	2	0.001	236	2091	4	0.002	141	1189	3	0.003				
Xyleborus saxeseni[a]	244	2158	16	0.007	232	2047	41	0.020	141	1189	10	0.008	4	44	11	0.250
Xyleborus scopulorum[a]	244	2158	112	0.052	185	1525	99	0.065	123	1049	71	0.068	69	706	457	0.647
Platypus wilsoni[b]	244	2158	29	0.013	236	2091	14	0.007	141	1189	9	0.008				

[a]Species too small to be held back by screen.
[b]*P. ponderosa* is not a known host of these species.

(1976a) recorded even higher landing rates. With such a powerful attraction source, it is not surprising that some beetles will land and bore into nearby healthy or resistant host trees only to be pitched out, as was observed in experiment 3. If beetles landing on unsuitable trees (healthy ponderosa pines) are not under the influence of pheromones, they presumably do not initiate galleries. If galleries were initiated, they would soon be marked by resin exudation, pitch tubes, and/or boring dust and, most importantly, by an increased landing rate of this bark beetle species and some associated insects, including predators and parasites (Stephen and Dahlsten, 1976b). These conditions could be readily detected but were not observed on the experimental trees.

CONCLUSION

The evidence thus far gathered for *D. brevicomis, D. pondersae*, and *I. paraconfusus* indicates that these beetles land on trees of various species and physiological states. However, sustained feeding is initiated selectively. Therefore, selection of suitable breeding material must occur while the beetles are in contact with the tree. Further observations of unrestrained beetles in contact with trees of known species and condition are required to elucidate this phase of their host selection behavior (Wood, 1972). Steps could then be taken to determine whether or not the observed behavior patterns are a result of chemoreception and, if so, to undertake identification of the chemical stimuli.

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SCENT TRAILING BY VIRGIN FEMALES OF *Pseudococcus calceolariae*[1,2]

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Abstract—Virgin females of the citrophilous mealybug *Pseudococcus calceolariae* (Mask.) deposit scent marks as trails on the substrate on which they rest or move. These substances elicit attraction and sexual behavior by conspecific males. The same responses were obtained when males were bioassayed on extracts from filter paper disks on which females had rested. The significance of scent trailing in mealybugs is discussed.

Key Words—Scent marking, scent trailing, mealybugs, sex pheromone(s), citrus, *Pseudococcus calceolariae*, Homoptera, Pseudococcidae.

INTRODUCTION

Scent trailing has never been observed in Homoptera. Scent marking of filter paper and of abdominal silky fluffs by virgin females was observed in the red pine scale *Matsucoccus resinosae* Bean et God. (Doane, 1966). In this case, however, there was no evidence that virgin females left scent trails while moving.

During our studies on short-range sex behavior of the citrophilous mealybug *Pseudococcus calceolariae* (Mask.) we noticed that males attempted copulation on the places where virgin females had been located. This behavior was examined more closely during the present research.

METHODS AND MATERIALS

A strain of *Pseudococcus calceolariae* was reared in glass jars (18 × 17 ID cm) on potato sprouts under controlled environmental conditions

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(26 ± 1°C and 60 ± 5% relative humidity). The jars were kept closed with a nylon screen (mesh opening 250 μm). New potatoes were synchronously colonized by crawlers by placing them on the screen of the jars which contained the mixed population (Rotundo, 1978). Virgin females were isolated by mechanically removing young male stages (cocoons).

A first group of bioassays was carried out in closed Petri dishes (ID 100 mm) containing two small citrus leaves. On one of them, a virgin female had rested for 1 min in a registered position. The other leaf, on which no females had rested, was left as a check. At least 3 unmated males were placed on each leaf. Males which exhibited courtship behavior within 5 min plus copulatory attempts were registered as positive responses. Each bioassay was replicated at least 5 times. All tests were carried out under fluorescent light (700 lux) at 26 ± 1°C temperature and 63 ± 5% relative humidity.

A second group of bioassays was carried out in empty Petri dishes in which females were left free to move. The displacements of each female were traced on the external glass surface by means of a soft pen. Males which were

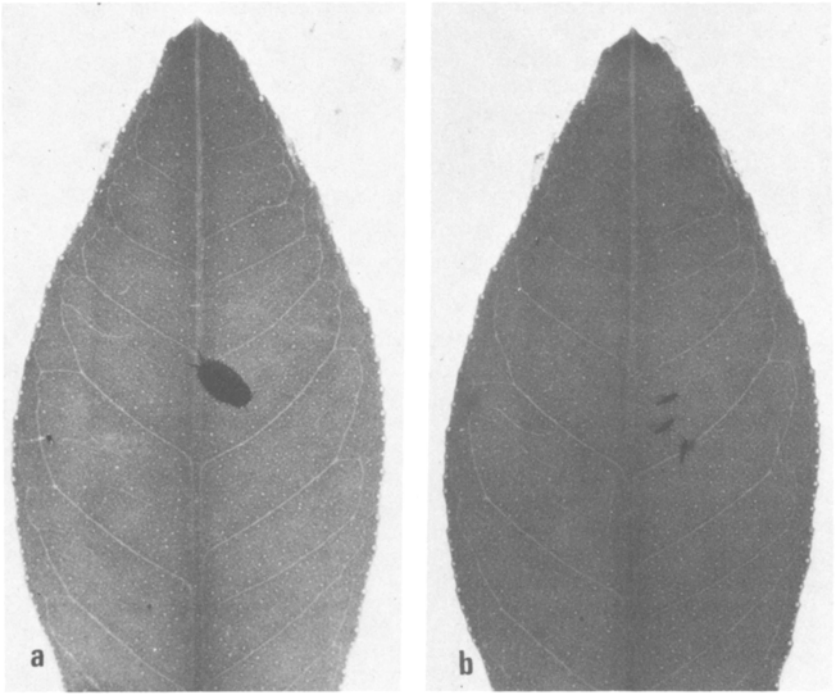

FIG. 1. Scent marking in *Pseudococcus calceolariae:* (a) virgin female while marking on a citrus leaf; (b) conspecific males exhibiting courtship behavior on the place where the female was located.

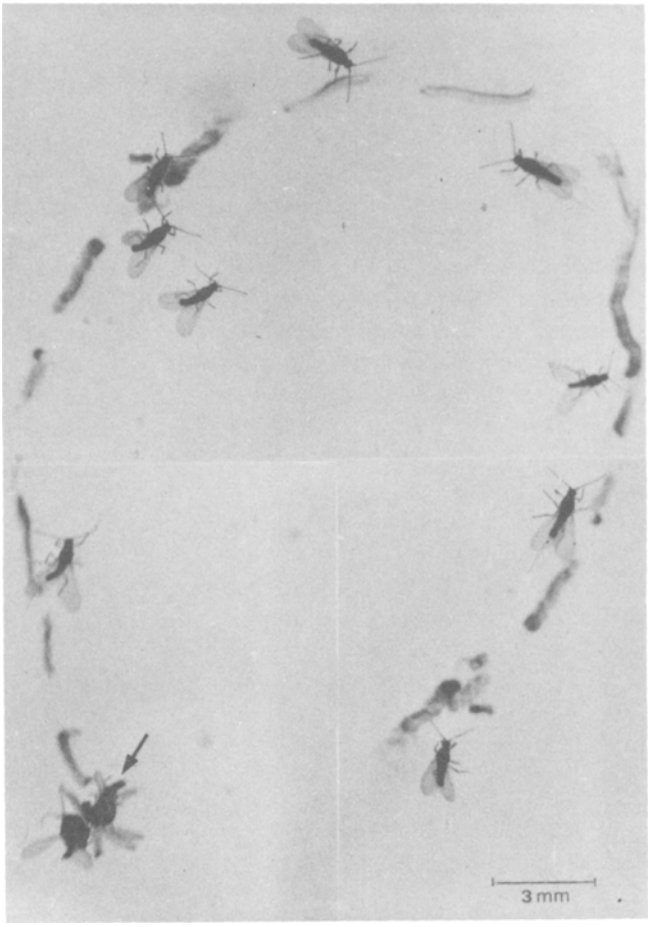

FIG. 2. Scent trailing in *Pseudococcus calceolariae*. Males in movement along the trail produced by a virgin female on glass; (arrow) copulatory attempts between males on the place from which the female had started.

able to follow the trails while exhibiting copulatory behavior were registered as positive responses. Replications and environmental conditions were as stated above.

A third group of bioassays allowed females to rest on filter paper disks (2 cm in diameter) for 3 hr under small glass capsules. After removal of the females, the disks were placed in 2 ml ethyl ether for 1 hr at room temperature. The extract was concentrated to 0.1 ml by means of nitrogen jets. The concentrate was bioassayed in microdosages of 30 μl according to a previous

technique (Rotundo and Tremblay, 1974). Male behavior was followed in all cases under low stereoscopic magnification (16×).

RESULTS AND DISCUSSION

Observations confirmed that the leaf and glass surfaces on which virgin females had rested or moved became very attractive to conspecific males (Figures 1 and 2). On contact with the marked surfaces males exhibited typical sexual behavior consisting in rapid sweeping abdominal movements and copulatory attempts. Similar results were obtained from concentrated extracts. Of special interest is the capability of males to follow the female trail along several centimeters. From the vigorous copulatory attempts between searching males even on glass (Figure 2, arrow), it may be inferred that a rather high concentration of scent is deposited by females. This sequence has not been observed by us in the related species *Pseudococcus obscurus* (Essig) (obscure mealybug) nor in *Planococcus citri* (Risso) (citrus mealybug).

Females of *Pseudococcus calceolariae* were never observed to extrude body structures except for the ventral adhesive circle (circulus), which is typical for pseudococcids. However, this structure seems not to be involved in the scent trailing by females. As a matter of fact, the circulus is extruded on the substrate only on occasion of upside-down resting phases. Instead, males behave as though they were in the presence of a continuous trail. Moreover, females of the two other mealybug species (the obscure and citrus mealybugs), in spite of having a regular circulus, do not deposit scent trails.

The sexual behavior of males suggests that the females utilize the sex pheromone(s) for scent trailing. We cannot exclude the possibility, however, that some other substance(s) may be involved.

It seems clear that scent deposition ensures the continuity of the pheromone plume among wax masses, crevices, and fruits.

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2-FURALDEHYDE FROM BALDCYPRESS
A Chemical Rationale for the Demise of the Georgia Silkworm Industry

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Abstract—2-Furaldehyde was growth inhibitory and toxic to larvae of *Bombyx mori* at concentrations as low as 1 ppm. Baldcypress, *Taxodium distichum*, heartwood released this volatile at concentrations in excess of these levels. The adverse effect of this chemical on silkworms may partially account for the demise of the Georgia silkworm industry (circa 1765), after the construction of a baldcypress rearing facility.

Key Words—Baldcypress, *Taxodium distichum*, 2-furaldehyde, *Bombyx mori*, Lepidoptera, Bombycidae, growth inhibition, toxicity, Georgia silkworm industry.

INTRODUCTION

The early Georgia colonists established a silkworm industry in Ebenezer village orphanage near Savannah in 1736. Silk production began in 1741 and increased to 1000 pounds a year from 1750 to 1760. For some unexplained reason production declined to only 500 pounds in 1763, and there was no further reference to an active industry after 1765 (Krispyn, 1978). Krispyn discovered a clue to the demise of the industry in the orphanage diary. Just prior to 1763 the diary mentions plans for building a new rearing house because the orphanage attic was too small. This new facility was to be constructed from baldcypress wood because cypress shingle cutting was a rapidly expanding local industry. Krispyn suggested that perhaps the

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baldcypress was deleterious to the silkworms. She tested this possibility by placing baldcypress heartwood, *Taxodium distichum* (L.) Rich., in a silkworm culture and found marked growth inhibition, reduced survivorship, and developmental abnormalities in comparison to controls (Krispyn, 1978). Since silkworms feed only on mulberry, *Morus* sp. (Ishikawa et al., 1969), baldcypress wood was not consumed in her experiments, and it was therefore likely that volatile heartwood components were responsible for these adverse effects.

We have determined the concentration and release rate of 2-furaldehyde, one volatile component of baldcypress heartwood, and investigated the effect of this compound on the growth, development, and survivorship of *Bombyx mori* L. (Lepidoptera, Bombycidae) as a possible explanation for the observed adverse effects of baldcypress and the decline of the Georgia silkworm industry.

METHODS AND MATERIALS

Extraction of Plant Material. Core and sap samples were taken from 6 adjacent baldcypress trees on the Athens campus in October 1978, using a 4.5-mm ID increment borer. Heartwood was removed from each core, weighed, measured, and placed in airtight glass tubes filled with N_2 (23 ml minus wood vol). Headspace analyses were carried out on these samples using heartwood of known mass and surface area (approx. 12 g, 120 mm × 4.5 mm diam). The 5-ml headspace aliquots were analyzed by gas chromatography (GC) after 24 hr at STP. Analyses were repeated at 24-hr intervals, flushing the tubes with N_2 after each sampling, to determine the temporal decay in release of 2-furaldehyde (dr/dt).

Ethanolic extracts of heartwood were prepared from pulverized fresh wood (1–3 g) extracted twice with ethanol (1 ml) at 0° C for 24 hr, centrifuged, and the supernatent analyzed by GC. Hydrolyzed wood extracts were prepared by reacting pulverized fresh wood (1–3 g) with ethanol–10% H_2SO_4 1:1 (1 ml) for 1 hr, neutralizing with Na_2CO_3, and analyzing the centrifuged supernatant by GC. Sap samples were centrifuged and analyzed by GC. Triplicate samples of each extract of each tree were analyzed.

GC and GC-Mass Spectroscopy. GC was carried out on a Perkin Elmer Sigma 3 chromatograph fitted with dual-compensated flame-ionization detectors. Detectors were optimized at 195 kPa H_2 and 140 kPa air with a carrier flow of 30 ml/min of N_2. Three GC stainless-steel 1.83 m × 2.0 mm ID columns were used: 10% SP 1200 1% H_3PO_4 on 80/100 Chromasorb WAW, programed from 50° C to 180° C at 10° C/min, detectors and injectors at 230° C; 10% SP 2100 3% OV-1 on 80/100 Supelcoport, programed with the same conditions; and 60/80 Tenax GC, programed at 100–300° C at 10° C/min, detectors and injectors at 350° C.

Detector responses were linear over the range used. The GC was connected to a Varian CDS III integrator. Quantification and retention times were determined from triplicate injections of 1 μl (ethanolic and hydrolyzed samples) and 5 ml (headspace, Precision Sampling Corp., Pressure-Lok gas syringe) using internal and external standards of 2-furaldehyde.

GC-mass spectroscopy was carried out on a Varian MAT 112S with a Varian MAT SS200 data system. The GC column was 1.83 m × 4.0 mm ID, 3% OV 17 on 100/120 Gas Chrom Q at 60°C. The mass spectrum of 2-furaldehyde in baldcypress was compared to that of an authentic sample.

Maintenance of Silkworm Culture. Bombyx mori L. (multivoltine variety) larvae were maintained in open-topped trays at 28°C, 16-hr light cycle, and fed twice daily with fresh, washed leaves of *Morus alba* L., obtained on campus. Adults were allowed to mate in small Petri dishes, and gravid females oviposited on filter paper. Viable eggs turned from a light yellow color to dark brown within 4 days. Egg diapause was prevented by treating light brown eggs (24–48 hr) with 6 N HCl at 45° ± 1° for 5 min, rinsing thoroughly in distilled water, and air drying (Nayar and Fraenkel, 1963). 81–92% hatching then occurred within 10–12 days.

Exposure of Silkworms to 2-Furaldehyde. Fifty 1st instar larvae were placed in each of matched pairs of cylindrical glass chambers (113 mm × 170 mm diam) lined with moist filter paper and sealed with a heavy glass lid made airtight with inert silicon grease. Fresh, weighed, washed leaves of *Morus alba* L. were added twice daily and the old leaves removed and weighed. Calibrations of leaf fresh weight (FW) loss in the absence of larvae were also made. Consumption of leaves was determined from leaf dry weights (DW). The apparatus was kept at 28°C, 80% relative humidity, on a 16-hr light cycle.

Each chamber had an outlet (12 mm diam) and inlet (24 mm diam) at the base, and the outlet was connected to a fume hood. Each inlet was connected to an adjacent volatilizing chamber (106 mm × 24 mm diam) containing a calibrated scale and an open-ended microcapillary (0.5 or 1.0 μl). This chamber was made lightproof to reduce photodecomposition of 2-furaldehyde (Dora-Gonzalez et al., 1972) but with an accessible flap so that the microcapillary volume could be recorded with the aid of a microscope. One of each pair of volatilizing chambers contained an empty microcapillary (control), while the other contained 2-furaldehyde. Each volatilizing chamber was supplied from a common, filtered, humidified air supply via a stream splitter and calibrated variable flow meters (Matheson models 7621, 4.72–74.2 ml/min; 7622, 47.2–943.9 ml/min). The rate of flow of 2-furaldehyde from the volatilizing chamber was varied by changing the carrier air flow and microcapillary volume. Larvae were periodically removed for weighing and the chambers cleaned. Equilibrium concentrations of 2-furaldehyde were regained within 1 hr of resealing the system.

Concentrations in the chamber (ppm) were calculated based on calibrations of 2-furaldehyde release in the absence of larvae and leaves, assuming no adsorption. Measurement of 2-furaldehyde release (μg/hr) in the presence of leaves and larvae was considered to be a more accurate determination of exposure since it was independent of adsorption in the exposure chamber. A regression analysis between the concentration in ppm and the release rate in μg/hr for the carrier air flow rates used in the experiments showed that:

$$y(\text{ppm}) = 20.61 \cdot x(\mu\text{g/hr}) - 44.5(r = 0.95), \text{ when } x > 2.16\ \mu\text{g/hr}$$

Calculation of Persistence of Inhibitory Concentrations of 2-Furaldehyde. Estimation of the time period, t, that 2-furaldehyde would remain at growth-inhibitory and/or toxic concentrations in the air of a hypothetical baldcypress rearing house, $3 \times 3 \times 5$ m $\times$ 0.0508 m thick, was calculated from:

$$t = \frac{\ln(I) - \ln(C_i)}{-k/2.3} \text{ days}$$

where I = the minimum inhibitory concentration (ppm) to silkworm larvae, determined experimentally; $-k/2.3$ = the slope of the first-order kinetic (log-linear) of dr/dt. r_i, the initial release rate of 2-furaldehyde from wood (g per cm^2 wood surface area per g 2-furaldehyde in wood per day) and dr/dt were determined experimentally by headspace analysis; C_i= the initial concentration of 2-furaldehyde (ppm) that would occur in the air of the rearing house and is calculated from:

$$C_i = \frac{r_i \cdot a \cdot b \cdot 10^6}{m \cdot d} \text{ ppm}$$

where a = the internal surface area of the rearing house (cm^2), a is 49.28% of the total wood surface area; b = the amount (g) of 2-furaldehyde in the wood available for release and is calculated from the concentration of 2-furaldehyde found by GC analysis (ppm) multiplied by 49.28% of the total wood mass (where ρ wood = 1.122 g/cm^3), i.e., of the total amount of 2-furaldehyde present, only that proportion equivalent to the internal surface area would be released to the interior; m = the mass (g) of the internal volume of air per unit time in the rearing house. m is calculated from:

$$m = V_2 \cdot \rho \text{ air}$$

Where V_2 = the internal volume of the rearing house (liters) and ρ_{air} = 1.29 g/liters at STP; d = the dilution factor dependent on the flow rate of air through the system, f_2 (liter/day) and is calculated by extrapolation from the flow rates, f_1 (liter/day), used in the exposure experiments, i.e.,

$$f_2 = \frac{f_1 \cdot V_2 \text{ liters}}{V_1}$$

where V_1 = the internal volume of the experimental chamber (liters), Hence:

$$d = \frac{f_2}{V_2}$$

d is then the number of changes of air per day.

Using these calculations a family of curves was derived for the duration of inhibitory and toxic effects to silkworm larvae with different initial concentrations of 2-furaldehyde in wood, with different values of d. The following assumptions were made: (1) The rearing house remains at STP with a constant flow rate, f_2 liters/day, of air through the system. (2) The initial release rate, r_i ($g/cm^2/g/day$), is directly proportional to the initial concentration in wood, b (g), and the surface area, a (cm^2), available for release. This relationship was shown to be true for headspace analyses of baldcypress. (3) 2-Furaldehyde is not readsorbed into the wood and the decomposition rate of 2-furaldehyde is negligible in comparison to the concentration per unit time in the rearing house.

RESULTS AND DISCUSSION

Baldcypress heartwood contained a number of volatile components. One major component comprising greater than 10% of the total peak area was identified by GC retention time and GC-MS as the volatile furan, 2-furaldehyde, by comparison with an authentic standard. The other components have yet to be identified. Concentrations of 2-furaldehyde in tree samples are shown in Table 1. The levels of 2-furaldehyde varied considerably between trees, but were consistent ($\pm 10\%$) within trees.

The presence of 2-furaldehyde in heartwood is not surprising since it is a fairly ubiquitous natural product in plants (McCullough, 1972). 2-Furaldehyde is reported to occur in Old World cypress, *Cupressus sempervirens* L., *Pinus, Betula, Citrus, Helianthus, Zea*; in representatives of the Anonaceae, Compositae, Graminae, Labiatae, Lauraceae, Myrtaceae, and Umbelliferae; and in the leaves, stems, bark, and fruit pericarps of a wide variety of plants (Guenther, 1965; Stecher, 1968; McCullough, 1972; Cerning and Guilbot, 1973; Dinsmore and Nagy, 1974; Filatova and Korol'kov, 1977; Flodin and Anderson, 1977). In fact, the starting material for commercial and laboratory extraction of 2-furaladehyde is corn cobs (Adams and Voorhees, 1967). 2-Furaldehyde is formed by decomposition of aldo- and keto-pentose polymeric "pentosans" (Lehninger, 1970; Barker, 1971), sugars that are of

TABLE 1. Concentrations of 2-Furaldehyde in Baldcypress Heartwood (*Taxodium distichum* L.) Determined by GC Analyses.[a]

Tree sample	Concentration (ppm ± 10%)		Ratio (hydrolyzed/ethanol)	r_i	
	Ethanol extracts	Hydrolyzed extracts		$\times 10^{-7}$ g/cm^2/g/day	dr/dt (±10%)
1	0.4	5	12.5	ND	ND
2	125	399	3.2	1.489	−0.0264
3	27	81	3.0	1.533	−0.0282
4	12	35	2.9	1.421	−0.0278
5	11	29	2.6	ND	ND
6	6	23	3.8	ND	ND

1-6 (pooled) Sap. 53 ppm.

[a]ND = not determined. For explanations of r_i and dr/dt see text.

almost universal distribution in plants. The occurrence of between 2 and 4 times as much 2-furaldehyde in hydrolyzed extracts from 5 out of 6 trees (Table 1), almost certainly reflects this decomposition process.

2-Furaldehyde is notable for its toxicity to bacteria, fungi, protozoa, insects, and mammals (Stecher, 1968; Starzyk et al., 1969; Pashev et al., 1970; Saubenova et al., 1973; Soboleva et al., 1973; and see Jones et al., 1980). The widespread occurrence of 2-furaldehyde in plants, its broad-spectrum toxicity, and its presence as a major volatile constituent of baldcypress, prompted us to investigate the effects of this compound on silkworm larvae. We wished to demonstrate whether or not inhibition by 2-furaldehyde was a realistic chemical rationale for the adverse effects of baldcypress on silkworm larvae (Krispyn, 1978).

Silkworm larvae were exposed to volatilized 2-furaldehyde at a number of different concentrations (2.21–4.45 μg/hr ≏ 1–47 ppm); these concentrations fell within the range of 2-furaldehyde concentrations found in headspace from baldcypress. Larval growth rates were markedly reduced in comparison to air-exposed controls at all concentrations (Figure 1a). Control larvae exhibited a linear rate of change of growth rate (gr) with time, $d\ln(gr)/dt$. At 2.21 and 2.52 μg/hr 2-furaldehyde there was an initial lag or slight decline in growth rates but a linear $d\ln(gr)/dt$ of the same slope as controls was achieved after 8 days. At higher concentrations (3.89–4.45 μg/hr) a marked decline in $d\ln(gr)/dt$ occurred, the slope of $d\ln(gr)/dt$ was reduced in comparison to controls, and linearity of $d\ln(gr)/dt$ was maintained only from 8 to 32 days.

The effect of 2-furaldehyde was reversible. Larvae exposed to 4.07 μg/hr for 10 days, and then transferred to a control environment showed a rapid

recovery, with the slope of $d\ \ln(gr)/dt$ greater than, but not significantly different from controls (t test, $P = 0.2$); although the growth curve was temporally displaced by 3–5 days (Figure 1b). This pattern was in marked contrast to continuous 2-furaldehyde exposure at this concentration (see Figure 1a). The initial decline in growth rates exhibited by larvae exposed to 4.45 μg/hr 2-furaldehyde in the first instar (see Figure 1a), was also characteristic of larvae exposed to the same concentration after a 10-day control air exposure, although the growth curve was temporally displaced by 10 days (Figure 1b).

The growth inhibition resulted in an extension of the duration of the life cycle by 15–64% ± 5% over concentrations from 2.21–4.20 μg/hr 2-furaldehyde, respectively. The greatest proportional extension (with controls set to unity) occurred in the 2nd instar, but all stages were significantly extended in comparison to controls, at all concentrations (t test, $P < 0.1$) (Figure 2). The dramatic proportional extension shown by 2nd instar larvae may have been characteristic of the exposure to 2-furaldehyde of 1st instar larvae, since

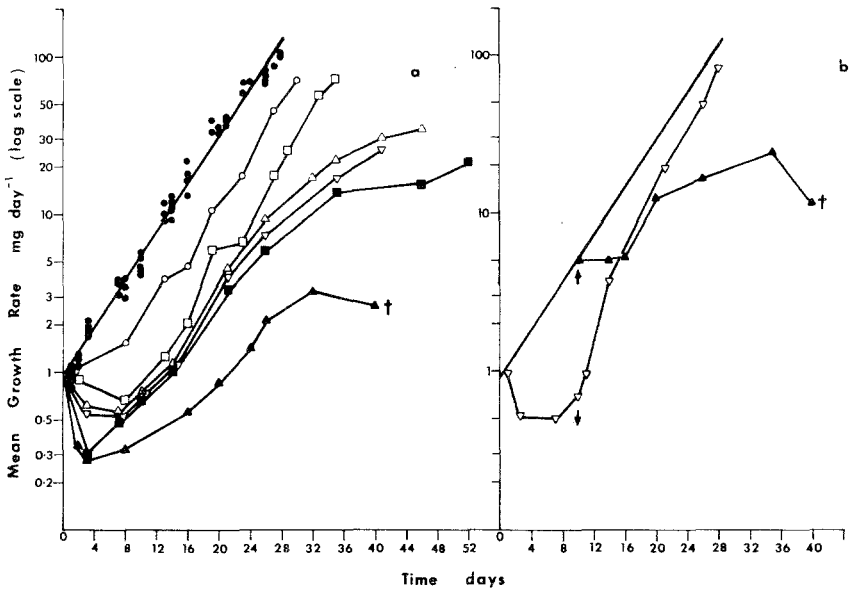

FIG. 1. Effect of 2-furaldehyde exposure on the growth rate of *Bombyx mori* larvae. Each data point is the mean growth rate of 50 larvae (1 SE, not shown, $\leqslant$ 15% for all samples). (a) ● : Controls, regression for growth rate $= e^{(0.173t-0.0238)}$, $r = 0.967$; ○ = 2.21 μg/hr; □ = 2.52 μg/hr; △ = 3.89 μg/hr; ▽ = 4.07 μg/hr; ■ = 4.20 μg/hr; ▲ = 4.45 μg/hr; 100% mortality by 40 days (†). (b) Control regression as in a; ▲ = 4.45 μg/hr, exposed after 10 days control environment (↑), 100% mortality by 40 days (†); ▽ = 4.07 μg/hr, exposed for 10 days and then removed to control environment (↓).

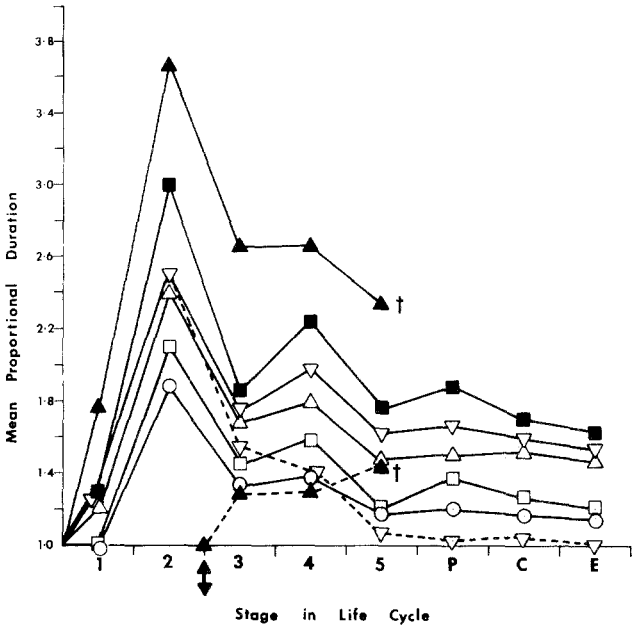

FIG. 2. Effect of 2-furaldehyde exposure on the duration of *Bombyx mori* life cycle. Each data point is the mean duration of 50 larvae. Control values have been set to unity (mean cumulative durations for controls ± 1 SE were **1**: 2 ± 0.5; **2**: 4.5 ± 1.0; **3**: 11 ± 3; **4**: 17 ± 4; **5**: 24.5 ± 5.5; **P**: 30 ± 2; **C**: 44.5 ± 5; **E**: 53 ± 7; $N = 300$). **1–5**: larval instars; **P**: prepupal period; **C**: pupal period; **E**: period over which adults emerged. ○: 2.21 μg/hr; □ = 2.52 μg/hr; △ = 3.89 μg/hr; ▽: 4.07 μg/hr; ▽: 4.07 μg/hr, exposed for 10 days only (↓); ■: 4.20 μg/hr; ▲: 4.45 μg/hr; ▲: 4.45 μg/hr 2-furaldehyde, exposed after 10-day control period (↑).

exposure of late 2nd instar larvae (i.e., after a 10-day control air exposure) to the same concentration of 2-furaldehyde (4.45 μg/hr) did not result in the same proportional extension (see Figure 2). The recovery of larvae transferred to a control environment after 10 days of exposure to 4.07 μg/hr 2-furaldehyde can also be seen in the subsequent reduced proportional extension of each stage in the life cycle (see Figure 2, cf. Figure 1b).

We consider that this extension of larval instar duration is related to a critical weight or size requirement for molting, such as is the case for some other insects (Wigglesworth, 1934; Van der Kloot, 1961; Steel and Harmsen, 1971; Nijhout and Williams, 1974; Nijhout, 1975; Blakeley and Goodner, 1978). This may explain why the durations of nonfeeding stages (prepupal, pupal, and the period over which adults emerged) were not proportionally extended as much as the larval instars.

Growth inhibition was not the result of reduced food consumption. Consumption rates (mg FW leaves consumed per day, determined from DW) of controls and 2-furaldehyde-exposed larvae (2.21–3.89 μg/hr) of the same weight, were not significantly different (t test, $P > 0.05$). At higher concentrations (4.07–4.45 μg/hr) larvae became sluggish after 24–28 days, and consumption was reduced. However, we consider this to have been the result rather than the cause of growth inhibition; since lower concentrations, and higher concentrations up to 24 days, inhibited growth but not feeding, and no evidence of the repellency or deterrency of leaves was observed. Since growth was inhibited in the absence of reduced consumption, utilization efficiency therefore decreased. This implied that 2-furaldehyde was indirectly or directly adversely affecting the nutrition of the insect. This may be of considerable significance in relation to the inhibition of silkworm enteric microorganisms by 2-furaldehyde (Jones et al., 1980 this volume).

2-Furaldehyde was also toxic to silkworm larvae at concentrations that fell within the range of concentrations determined in baldcypress headspace (Figure 3). The LD_{50} was determined as 3.8 μg/hr ($\simeq$34 ppm) for 1st instar to adult emergence, and 4.2 μg/hr ($\simeq$42 ppm) for 1st instar to pupation; 10% mortality (minimum significant lethal doses) occurred at 2.4 ($\simeq$5 ppm) and

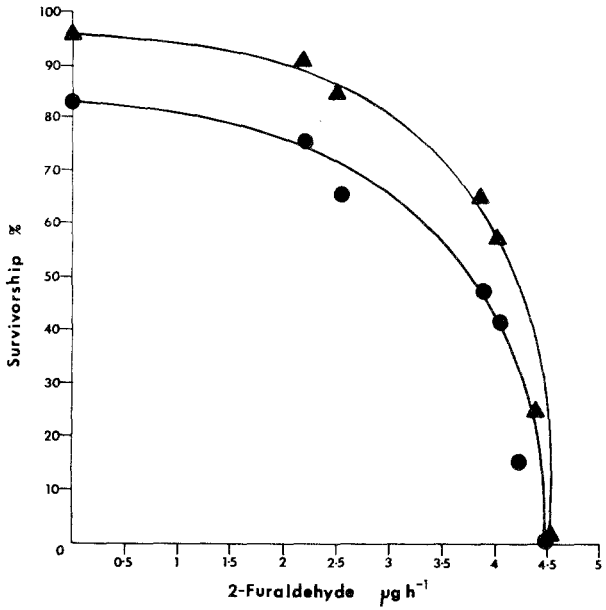

FIG. 3. Effect of 2-furaldehyde exposure on the survivorship of *Bombyx mori*. Each data point is the survivorship of 50 larvae exposed to each 2-furaldehyde concentration and 300 control larvae. The curves are fitted by eye. ●: survivorship to adult emergence; ▲: survivorship to pupation.

2.6 μg/hr ($\triangleq$ 9 ppm), respectively. These values demonstrated that sublethal doses were growth inhibitory. At concentrations below 2.7 μg/hr ($\triangleq$11 ppm; with survivorship >80% compared to controls) there were no noticeable adverse effects on mating behavior, oviposition (mean egg lay ± 1 SE = 341 ± 93, N = 17, t test, $P > 0.1$), egg fecundity (mean egg fecundity ± 1 SE = 86.3 ± 5%, N = 17, t test, $P > 0.2$), or adult size (mean prothoracic width ± 1 SE = 6.82 mm ± 0.58 mm ♂, N = 14; 7.14 mm ± 0.45 mm ♀, N = 17; mean length, head to distended abdominal tip ± 1 SE = 19.38 mm ± 1.48 mm ♂, N = 14; 19.82 mm ± 1.58 mm ♀, N = 17, t tests, $P > 0.2$). Provided that larvae reached a weight or size sufficient for successful pupation, emergence and adult behavior were normal; however, these processes took considerably longer than those of the controls. No developmental abnormalities were observed at any concentration, so the adverse developmental effects observed by Krispyn (1978) were probably the result of baldcypress volatiles other than 2-furaldehyde. Since a number of other, as yet unidentified, components were found in headspace and extracts, it is conceivable that mixtures of these compounds could have acted synergistically in Krispyn's experiment, but this must remain speculative until other single compounds and multiple mixes of components are tested.

Our experiments demonstrated that 2-furaldehyde was growth inhibitory and toxic to silkworm larvae at concentrations that are released naturally from baldcypress wood. However, to assess realistically the role of 2-furaldehyde as a chemical rationale for the demise of the Georgia silkworm industry, it is necessary to demonstrate that the release of growth inhibitory and toxic concentrations of 2-furaldehyde would persist in the air of a rearing house for a sufficient period of time to cause a decline in the silk production.

Headspace analyses, determinations of the initial release rate of 2-furaldehyde from heartwood (r_i), and measurements of dr/dt were used to calculate the persistence of growth inhibitory and toxic levels of 2-furaldehyde (see Methods and Materials). The data for calculation of persistence and calculated values are shown in Figure 4. The time period varies with the initial concentration of 2-furaldehyde in wood, b (g) and the flow rate of air through the rearing house (f_2 liters/day). Flow rates equivalent to the lowest rates used in our exposure experiments ($\equiv$ to 5.49 air changes per day) gave values from 15 to 200 days, depending on wood concentrations. However, at some of the higher flow rates (550 air changes per day), persistence would still be 40 days at concentrations of 400 ppm. Although we cannot say how well ventilated the rearing house would have been, it is unlikely that it was extremely well ventilated; it may in fact have lacked vents or windows (i.e., a box with a door, with only a few air changes per day, say 2–3). In this case, the use of baldcypress wood with concentrations in excess of 300 ppm could have adversely affected 4–5 generations of silkworms (generation time$\triangleq$53 days

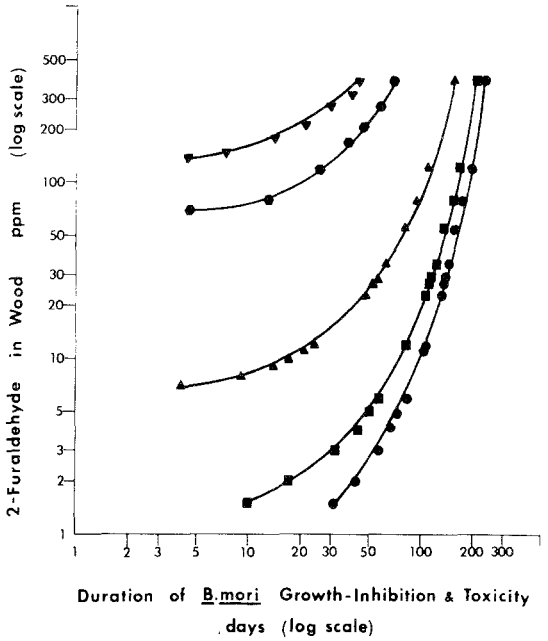

FIG. 4. Family of decay curves calculated for the persistence (days) of 2-furaldehyde at toxic and/or growth-inhibitory levels in a hypothetical baldcypress rearing house, $3 \times 3 \times 5$ m $\times$ 0.0508 m thick, with varying concentrations of 2-furaldehyde initially in the wood (ppm) and varying dilution factors (d). For explanation of formulas shown below, see Methods and Materials. ●: d, number of changes of air/day, = 2.74; ■: d = 5.49; ▲: d = 27.46; ⬢: d = 274.6; ▼: d = 549. Assumed I = 1 ppm; f_1 = 6.78, 13.59, 67.96, 679.6, or 1358.8 liters/day; V_1 = 2.475 liters; $V_2 = 4.305 \times 10^4$ liters; $m = 5.553 \times 10^4$ g; $a = 7.578 \times 10^5$ cm^2; b = (2-furaldehyde, ppm/10^6) 2.1906×10^6 g; $r_i = 1.481 \times 10^{-7}$ g/cm^2/g/day; $-k/2.3 = -0.0274$.

for multivoltine species). This could have considerably reduced the yield of silk since larval survivorship would be decreased and the generation time extended by at least 70% at these concentrations. In addition, the stunted larvae may well have been more susceptible to disease. If a univoltine species was used, silk production would have been drastically curtailed.

Although 2-furaldehyde concentrations in the rearing house could have reached levels that were toxic to many of the larvae (i.e., well above the LD_{50}), it is not necessary to propose that baldcypress decimated the entire silkworm population. If the silk yield was reduced considerably over a 6-month period, this may have been economically disastrous to the industry, even though some silkworms may have survived. Our study therefore suggests that volatile baldcypress components, one of which is 2-furaldehyde, may well have been responsible for the demise of the colonial Georgia silkworm industry. This

conclusion leads us to wonder to what extent plant chemicals have unbeknowingly affected the economy of man in previous times.

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BALDCYPRESS ALLELOCHEMICS AND THE INHIBITION OF SILKWORM ENTERIC MICROORGANISMS
Some Ecological Considerations

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Abstract—Exposure of *Bombyx mori* larvae to 2-furaldehyde, a major volatile component of baldcypress heartwood, *Taxodium distichum*, resulted in in vivo inhibition of enteric microorganisms at concentrations (1–47 ppm) that were released naturally from heartwood. The 7 bacterial and 2 fungal enteric isolates were also inhibited in vitro at the same concentrations. It is suggested that inhibition of leaf surface microorganisms or in vivo inhibition of silkworm enteric microflora, as a result of indirect or direct action of 2-furaldehyde, exacerbates the growth-inhibitory effects of this compound on larvae by reducing the microbial nutritional contribution. The ecological significance of insect enteric microbial inhibition by plant allelochemics is discussed.

Key Words—Baldcypress, *Taxodium distichum*, 2-furaldehyde, 2-furoic acid, *Bombyx mori*, Lepidoptera, Bombycidae, enteric microbial inhibition, *Bacillus*, *Micrococcus*, *Arthrobacter*, *Mucor*, *Curvularia*.

INTRODUCTION

In a recent study we demonstrated that 2-furaldehyde was growth inhibitory and toxic to silkworm larvae, *Bombyx mori* L. (Lepidoptera, Bombycidae), when present in air at concentrations as low as 1 ppm. Baldcypress heartwood, *Taxodium distichum* (L.) Rich, contained and released volatile 2-furaldehyde at inhibitory concentrations (Jones et al., 1980). We suggested that the growth

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inhibition and toxicity of 2-furaldehyde may partially explain why baldcypress reduced the survivorship and inhibited the growth of silkworm larvae (Krispyn, 1978). In addition, we suggested that the adverse effects of 2-furaldehyde may partially account for the demise of the colonial Georgia silkworm industry (circa 1765) after the construction of a new baldcypress rearing house.

Our studies on larval growth in the presence of 2-furaldehyde demonstrated that growth inhibition occurred despite the fact that consumption of mulberry leaves, *Morus alba* L., was not significantly reduced in comparison to air-exposed controls. It therefore appeared to us that 2-furaldehyde reduced the utilization efficiency of silkworm larvae (Jones et al., 1980). The adverse effects of 2-furaldehyde, in terms of growth inhibition and toxicity, do not explain the mode of action of this compound.

We wanted to know whether or not larval exposure to 2-furaldehyde was adversely affecting the enteric microorganisms, since many 2-substituted furans, including 2-furaldehyde, have potent antimicrobial activity (Stecher, 1968; Starzyk et al., 1969; Pashev et al., 1970; Saubenova et al., 1973; Soboleva et al., 1973; Flodin and Andersson, 1977; Stehlik et al., 1978). It has been suggested that ingestion of leaf-surface microorganisms from mulberry contribute to the nutrition of the silkworm by breaking down plant material in the gut (Portier, 1911). We hypothesized that if 2-furaldehyde inhibited these microorganisms on the leaf surface, or inhibited them in the gut of the insect by direct action or indirectly by altering the physiological state of the gut, then the loss of the microbial nutritional contribution could be a contributory factor in the reduction of utilization efficiency we obtained in larvae on exposure to 2-furaldehyde (Jones et al., 1980). Therefore we carried out a series of experiments on in vivo and in vitro growth of enteric microorganisms of *Bombyx mori*, in the presence of 2-furaldehyde and its oxidation product, 2-furoic acid. The latter compound would be expected to occur as an oxidation product both in the insect and on the leaf surface as a result of penetration of the aldehyde and ingestion of leaves exposed to 2-furaldehyde.

METHODS AND MATERIALS

Methods on the culture of silkworms and exposure of larvae to volatilized 2-furaldehyde are described in Jones et al. (1980). Control larvae and larvae exposed to 2-furaldehyde, using the methods described previously, were used for in vivo studies and for isolation of enteric microorganisms for in vitro studies.

Aseptic procedures were used throughout. All nutrient agar (NA) plates (100-mm × 15-mm-diam Petri dishes) were poured with 20 ml of autoclaved

2.3% Difco Bacto Nutrient agar in distilled water and incubated inverted at 28 or 37° C in the dark or light for 24 or 48 hr.

In Vivo Determinations of Viable Counts. Control (air-exposed) and 2-furaldehyde-exposed larvae were weighed, killed (CO_2 and chloroform), and shaken gently with surface sterilizing agent (saturated $HgCl_2$, 10% Tween 80 as a surfactant) for 10 min (Ritter, personal communication). Larvae were washed twice in 70% ethanol for 5 min and individually transferred to sterile Petri dishes, to permit the ethanol to evaporate from the larval surface. The surface sterility technique was checked by wiping larvae over the surface of NA plates. No colony growth was observed.

Each larva was then separately homogenized in sterile ground-glass homogenizers with 1 ml sterile distilled water. Serial dilutions (10^{-1}–10^{-6}) of the homogenate were prepared in 100-ml sterile water blanks in medicine flats. A known volume (0.1 ml) of each dilution was applied to the center of each of 6 replicate NA plates, surface-spread with a sterile glass spreader, and incubated. Replicates of dilutions containing between 30 and 300 colonies were counted at 24 and 48 hr, and the viable count calculated from colony count and dilution factor (Cruickshank, 1965).

To determine whether or not there was a significant number of nonenteric microorganisms, 5th instar control larvae of known weight were surface sterilized, dissected under sterile conditions, and the whole fore-, mid-, and hind-gut homogenized, serially diluted, and plated out as before. There was no significant difference in the viable counts of gut homogenate and whole homogenate control larvae of the same weight (mean viable count $\pm$ 1 SD, for 1000 ± 50 mg larvae $= 7.2 \pm 3.1 \times 10^5$ for whole homogenate, $N = 32$; $6.9 \pm 4.2 \times 10^5$ for dissected gut homogenate, $N = 10$). This demonstrated that nonenteric microorganisms did not constitute a significant proportion of the total larval microflora that grew under these conditions.

Morphological characteristics of microbial isolates from fresh gut squash and smears were determined microscopically. Pure subcultures of each organism were prepared on NA slants. Each organism was then subcultured five times. Bacterial identification and standard bacteriological tests (Cruickshank, 1965) were carried out by a commercial agency.

In Vitro Determination of Microbial Inhibition by 2-Furaldehyde. Filter paper disks (6 mm diam, 0.75 mm thick, 9.5 ± 0.1 mg) were sterilized in waterproof containers and impregnated with 2-furaldehyde (11.6, 1.16, 0.116, 0.0116 mg in methanol) or 2-furoic acid (5, 1, 0.1, 0.01 mg in water). Inhibition standards were Mycostatin (Grand Island Biological Co.) fungal inhibitor-impregnated disks (10, 100, and 1000 units/disk in phosphate-buffered saline), and pre-prepared disks of the same size of penicillin G (10 units/disk) and chloramphenicol (30 μg/disk) (Becton-Dickinson Co.). Controls were disks

impregnated with 10 μl of methanol, water, or phosphate-buffered saline.

Dilutions of each organism, at a previously determined concentration that would give confluent growth at 24 or 48 hr, were prepared from 100-ml sterile water blanks; innocula (0.1 ml) were surface-spread on NA plates. A single disk treated with a given concentration of 2-furaldehyde, 2-furoic acid, inhibitor standards, or controls was placed in the center of each of five replicate plates for each treatment for each organism and incubated. Zones of inhibition of growth were measured at 24 and 48 hr and the inhibition expressed as a percentage of inhibitor standards. Dose–response curves for each organism were determined.

Test for Cellulolytic Activity. Cellulose powder (Whatman CF 11) was incorporated (5%) into NA or minimal medium (Difco, Davis) in 2.3% agar (Difco, Bacto-agar). Inocula of each organism (0.1 ml) were surface-spread on 10 replicate plates and incubated. Growth and zones of clearing were recorded.

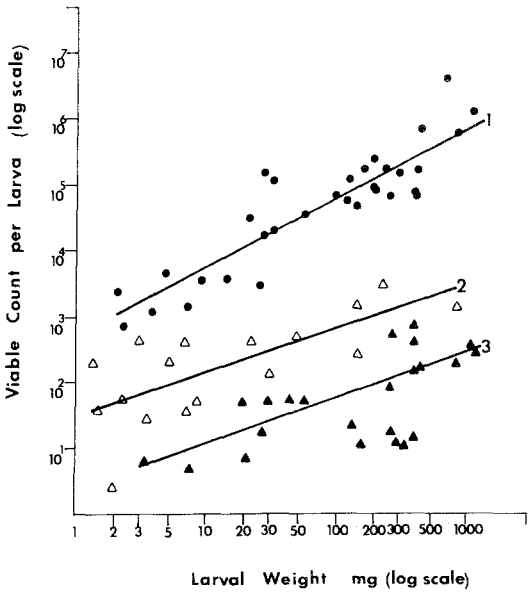

FIG. 1. Effect of 2-furaldehyde on the in vivo viable population of enteric microorganisms of *Bombyx mori* larvae. Linear regressions: 1, ● : controls, $N = 32$, $y = e^{(1.04 \ln x + 6.24)}$, $r = 0.89$; 2, Δ: 3.89 μg/hr (≙36ppm) 2-furaldehyde, $N = 17$, $y = e^{(0.66 \ln x + 3.44)}$, $r = 0.72$; 3, ▲: 4.45 μg/hr (≙47ppm) 2-furaldehyde, N = 23, $y = e^{0.64 \ln x + 0.77)}$, $r = 0.62$. Additional linear regressions for two other concentrations were used to derive Figure 2. These values were: 2.21 μg/hr (≙1ppm) 2-furaldehyde, $N = 19$, $y = e^{(0.84 \ln x + 6.03)}$, $r = 0.75$; 2.52 μg/hr (≙7ppm) 2-furaldehyde, $N = 21$, $y = e^{(0.73 \ln x + 5.14)}$, $r = 0.77$.

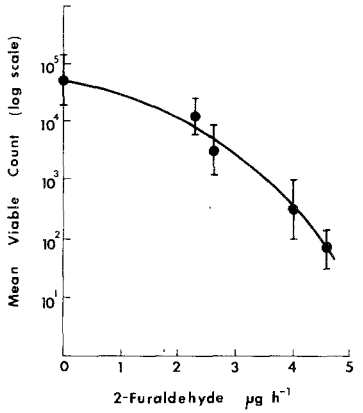

FIG. 2. Dose–response curve of inhibition of in vivo viable populations of enteric microorganisms of *Bombyx mori* larvae exposed to 2-furaldehyde (mean viable count for 100 mg larvae ± 1 SD). The curve is fitted by eye. Data were derived from linear regression values for 100 mg larvae (controls, $N = 32$; 2-furaldehyde exposed, $N = 17$–23; see legend of Figure 1).

RESULTS AND DISCUSSION

Larvae exposed to 2-furaldehyde at 2.21–4.45 μg/hr (1–47 ppm, for calculations of concentrations see Jones et al., 1980) contained significantly lower numbers of viable enteric microorganisms than controls of the same weight (Figure 1 shows controls and 2 of the 4 concentrations tested). At the highest concentrations (4.45 μg/hr, ≏47 ppm), the viable count was reduced 3.9×10^3-fold, and young larvae were effectively axenic. Values for 100 mg fresh-weight larvae obtained from regression analyses were used to derive a dose–response curve (Figure 2). A 50% inhibition of enteric microorganisms in vivo, occurred at 3.90 μg/hr (≏36 ppm), a value similar to the larval LD_{50} determined previously (Jones et al., 1980), 10% inhibition occurred at 1.25 μg/hr (<1 ppm). In vivo microbial inhibition occurred over the range of concentrations that were toxic and growth inhibitory to silkworm larvae (2.21–4.45 μg/hr) (Jones et al., 1980) and at concentrations within or below the levels found for baldcypress headspace samples (1.48×10^{-7} g/cm^2 wood area/g 2-furaldehyde in wood/day, the initial release rate from wood) (see Jones et al., 1980).

Although our experiments demonstrated that larval exposure to 2-furaldehyde resulted in in vivo inhibition of enteric microorganisms, they did not indicate whether or not inhibition was a direct consequence of 2-furaldehyde exposure. For example, inhibition could have been an indirect consequence of physiological changes in the insect, such as gut pH, resulting from 2-furaldehyde exposure, or leaf microorganisms could have been

inhibited before ingestion. Therefore we investigated the in vitro inhibitory activity of 2-furaldehyde and its oxidation product, 2-furoic acid, on nine species of microorganisms (see later and Table 2) consistently isolated from the gut of control larvae. All nine species were inhibited to varying degrees by both the aldehyde and the acid (Table 1), with three species inhibited more by the acid than by the aldehyde. All but one species (*Micrococcus* sp., M_2) were inhibited by at least 15% at the highest concentrations of 2-furaldehyde that were used in the in vivo experiments (47 ppm), with the majority being inhibited by between 30 and 100%. The in vitro microbial inhibition would indicate that leaf-surface microorganisms could have been inhibited but does not preclude in vivo enteric microbial inhibition.

Although microbial inhibition occurred at 2-furaldehyde concentrations used in larval growth experiments and at levels released by wood, this did not demonstrate that microbial inhibition contributed to larval inhibition. In order to test that this is the case, axenic larvae (i.e., aseptically reared) should have a lower utilization efficiency on mulberry leaves than septic larvae (i.e., larvae with a normal complement of enteric microorganisms). We did not attempt to rear axenic larvae on aseptic mulberry, and therefore we do not have direct evidence that this is the case. However, we do have substantive evidence that microbial inhibition and larval growth inhibition are intimately related.

TABLE 1. CONCENTRATIONS OF 2-FURALDEHYDE AND 2-FUROIC ACID (PPM) RESULTING IN 10% (I_{10}) OR 50% (I_{50}) INHIBITION OF GROWTH OF ENTERIC MICROBIAL ISOLATES FROM *Bombyx mori* IN VITRO[a]

Microorganism[b]	2-Furaldehyde			2-Furoic acid		
	I_{10} ppm	I_{50} ppm	% inhibition at 47 ppm	I_{10} ppm	I_{50} ppm	% inhibition at 47 ppm
Arthrobacter (A)	0.4	8.0	75	1.2	34.6	58
Bacillus B_1	12.3	308	15	10.2	86.9	34
Bacillus B_2	3.3	97.5	39	14.6	146	28
Micrococcus M_1 (1)	4.9	275	33	7.7	123	28
Micrococcus M_2 (1)	184	>490	0	15.4	164	29
Micrococcus M_3 (1)	2.4	36.6	57	13.8	138	28
Rhodopseudomonas (R) (1,2)	14.5	116	30	14.6	123	29
Mucor racemosus (Mr) (3,4)	3.6	4.4	100	56	245	10
Curvularia (C) (1,3,5)	13.8	54.8	50	2.6	17.3	64

[a]Calculated from zones of inhibition compared to penicillin, chloramphenicol, and Mycostatin inhibitors. Percent inhibition calculated to occur in the highest in vivo concentrations (47 ppm) used in 2-furaldehyde exposure experiments (Jones et al., 1980) are also shown.

[b](1) 48 hr (remainder incubated for 24 hr); (2) growth in light only; (3) not inhibited by penicillin; (4) not inhibited by chloramphenicol; (5) slight inhibition by chloramphenicol.

The maximum proportional increase in the duration of the larval instars (a function of growth rate, see Figure 2 in Jones et al., 1980) took place in the 2nd instar; this instar was effectively axenic (see larvae of 3–5 mg weight at 4.45 μg/hr, Figure 1). A study of the morphological (Table 2) and biochemical (Table 3) characteristics of the nine isolated microorganisms reveals that both fungal species and one *Bacillus* species (B_1) possessed some degree of cellulolytic activity; five species of bacteria possessed some degree of proteolytic activity (A, B_1, B_2, M_1, M_3). It is conceivable that these microorganisms may render carbohydrate and amino acids more available for assimilation by the larvae. This may be of more importance in the digestion of older leaves that have a reduced free carbohydrate and protein availability. The variety of biochemical transformations exhibited by the isolates (e.g., utilization of seven sources of carbohydrate, catalase activity, nitrate reduction, etc., see Table 3) may also be important in the production of micronutrients, although this must remain speculative at present.

The viable enteric microorganisms we isolated probably enter the gut during feeding, as previously shown with some fungi and bacteria (Portier, 1911; Khokhlacheva and Azimdzhavov, 1977). This may explain why the majority were obligate aerobes (with the exception of a consistently isolated facultative, aerobic, photosynthetic *Rhodopseudomonas*, which was obviously an airborne leaf-surface organism. This organism could not be of nutritional significance, since it would not grow in the dark). The gut of the silkworm is obviously sufficiently aerobic for the growth of these organisms, especially since a number of aerobic bacteria and fungi have been isolated in other studies (Iizuka and Takizawa, 1969; Nakasuji and Kodama, 1969; Iizuka et al., 1970; Kodama and Nakasuji, 1971). Whether or not anaerobes were present and involved in nutrition was not investigated, since procedures were designed for aerobes.

Substantive evidence for the nutritional role of enteric microorganisms in the silkworm comes primarily from studies on axenic culture. Our in vivo data for control silkworms suggest that these nonpathogenic microorganisms are resistant to the natural antibiotics (e.g., protocatechuic acid, *p*-hydroxybenzoic acid, and caffeic acid) present in the gut of mulberry-reared larvae (Iizuka et al., 1974; Kinoshita and Inoue, 1977; Koike et al., 1979). These antibiotic factors are absent from the guts of silkworms reared on artificial diets (Iizuka et al., 1974; Kinoshita and Inoue, 1977; Koike et al., 1979), and the growth and survivorship of these larvae can then be drastically reduced by a proliferation of pathogens (Kodama and Nakasuji, 1969; Nakasuji and Kodama, 1969; Iizuka et al., 1970, 1974). Although the additon of antibiotics to artificial diets reduces mortality due to pathogens (Kodama and Nakasuji, 1969; Nakasuji and Kodama, 1969; Iizuka et al., 1970, 1974), in the absence of pathogens, growth and survivorship is reduced in axenic culture, compared to septic culture, on artificial diets of a nutritional quality comparable to

TABLE 2. MORPHOLOGICAL AND TAXONOMIC CHARACTERISTICS OF ENTERIC MICROBIAL ISOLATES FROM *Bombyx mori*

Microorganism	Gram stain	Shape	Size (μm)	Aggregates[a]	Spores	Pigmentation
Bacteria						
Arthrobacter sp. (A)	+	Young, rod; old, ovate/spheroid	0.8 × 1.5–2.4	Si, Pr	None observed	Yellow nondiffusable
Bacillus sp. 1 (B_1)	+	Rod	1.2 × 2.3	Si, Pr, Lgch	Endospores elliptical/central, no distension of sporangial wall 1–1.2 × 1.2–2 μm	Clear
Bacillus sp. 2 (B_2)	+	Rod	1.2 × 2.5–5	Si, Pr	Endospores elliptical, not attached to vegetative cell 1.2–1.5 × 1.5–2 μm	Clear
Micrococcus sp. 1 (M_1)	+	Coccus	0.8–1.0 diam	Si, Pr, Cl, 4	None observed	White
Micrococcus sp. 2 (M_2)	+	Coccus	0.6–0.8 diam	Si, Pr, Cl, 4	None observed	Faint white
Micrococcus sp. 3 (M_3)	+	Coccus	0.7–0.9 diam	Si, Pr, Cl, 4	None observed	White
Rhodopseudomonas sp. (R)[b]	−	Rod	0.7 × 1.5–2.5	Pr	None observed	Yellow
Fungi						
Mucor racemosus Fresenius (Mucorales, Mucoraceae) (Mr)						
Curvularia sp. (Deuteromycetes, Mycelia Sterilia) (C)						

[a]Si: single; Pr: pairs; Lgch: long chains; Cl: clusters; 4: tetrads.
[b]Growth in light only.

TABLE 3. BIOCHEMICAL CHARACTERISTICS OF ENTERIC MICROBIAL ISOLATES FROM *Bombyx mori*[a]

Microorganism	Growth in trypticase soy broth + 5, 10, 15% NaCl	Range of growth (°C)	Anaerobic growth	Aerobic growth	Catalase	Nitrate reduction	Utilization of							Hydrolysis of		Indole	Methyl red	Voges Proskauer	Simons citrate
							Glucose	Mannitol	Maltose	Sucrose	Lactose	Cellobiose	Starch	Litmus milk	Gelatin				
Bacteria																			
A	ND	20–37	−	+	+	−	a	−	−	a	−	−	+	+	+	−	−	−	+
B_1	ND	20–37	−	+	+	−	a	−	a	a	−	a	+	+	+	−	+	+	+
B_2	ND	20–37	−	+	+	−	a	a	a	a	a	−	−	+	−	−	+	−	−
M_1	+	20–37	−	+	+	−	a	a	a	a	a	−	−	−	+	−	+	−	(+)
M_2	+	20–37	−	+	+	+	a	(a)	a	a	a	−	−	−	−	−	+	−	−
M_3	+	20–37	−	+	+	−	a	a	a	a	a	−	−	−	+	−	+	−	−
R^1	(+)	ND	(+)	+	+	−	−	−	−	−	−	−	ND	ND	−	ND	ND	ND	−
Fungi																			
Mr C	Both produce clear zones in minimal agar + 5% cellulose—cellulolytic																		

[a]Substrate utilization and hydrolysis tests were carried out aerobically. Indole, methyl red, Voges Proskauer and Simons citrate refer to standard IMViC bacterial tests (Cruickshank, 1965). For abbreviations of genera and species of microorganisms see Table 2. ND: not determined; +: positive; (+): weakly positive; −; negative; a: acid but no gas; [1]: only in the presence of light.

mulberry (Ito and Tanaka, 1962; Matsubara et al., 1967). However, when the nutritional quality of the diet is improved, there is no difference in growth and survivorship between axenic and septic cultures (Matsuda and Matsuura 1967; Ito, 1969). This would indicate that some nutritional benefit to the silkworm is derived from its nonpathogenic microorganisms in conditions of low nutrient availability.

Our study suggests that larval growth inhibition by 2-furaldehyde may be exacerbated either by in vivo direct inhibition of enteric microorganisms or indirectly as a result of physiological changes in the gut of the insect. Alternatively, inhibition of leaf-surface microorganisms prior to ingestion could take place. This phenomenon does not appear to be unique. For example, gossypol, caryophyllene, gallic acid, and tannins from cotton suppressed the growth of gut bacteria of the boll weevil (Hedin et al., 1978). Terpenes from *Abies balsamae* inhibited several entomopathogenic species of bacteria used as biological control agents of pests of *Abies* (Smirnoff, 1972). Nicotine has been shown to inhibit symbiotic yeasts in *Lassioderma serricorne*, the nicotine having little or no effect on the insect if vitamin supplements were added to the diet (Milne, 1961). In fact, it may be that many plant allelochemics of broad-spectrum toxicity or antimicrobial action inhibit endosymbionts (enteric and in mycetosomes). This phenomenon would appear to be eminently worthy of further investigation.

If this phenomenon is widespread, then the inhibition of endosymbiotic microorganisms by plant allelochemics could be of considerable ecological significance in regard to insect feeding behavior and insect resistance to plant allelochemics. For example, the host-plant specificity of an insect with an obligate requirement for endosymbionts (e.g., lower termite dependence on protozoa) may be partially determined by the resistance or susceptibility of the endosymbionts to specific allelochemics. In this case, the deterrent or repellent qualities of nonhost plants may, in some insects, be the consequence of selection for avoidance of "antiendosymbiotic" allelochemics, rather than selection for avoidance of "antiinsect" allelochemics (Jones, in preparation). Therefore, an important component of the host-plant specificity of some insects may be the result of subtle selection mechanisms involving endosymbiotic microorganisms. This example of the diversity of biological activity of plant allelochemics may add an extra dimension to our understanding of the evolutionary role of these compounds in plant resistance and insect adaptation.

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LABORATORY EXPERIMENTS TO DETERMINE IF CRAYFISH CAN COMMUNICATE CHEMICALLY IN A FLOW-THROUGH SYSTEM

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Abstract—The importance of chemical cues for transmitting information concerning sexual identity, agonistic state, and stress-related condition in the crayfish *Procambarus clarkii* (Girard) was examined in a flow-through system. Experiments tested the effects of "conditioned water" from stimulus tanks on the behavior of solitary male or female crayfish. Twenty males and 20 females were subjected to a random sequence of five treatments: unconditioned water (control), conditioned water flowing through tanks containing a solitary male or female, and conditioned water from tanks holding either two males or two females. Durations of the following behaviors were recorded: chelae up, chela(e) in baffle hole, chela waving, climbing, digging, grooming, gross body movement, and meral spread. Results indicated that crayfish chemically detected another animal within 0.25 m without additional visual or tactile stimuli; however, crayfish apparently did not "communicate" information on sexual identity, agonistic state, or stress condition, nor does this detection necessarily imply discrimination between stimuli from crayfish and other taxa (e.g., fish). Our conclusions are contrasted with the two previous reports on chemical communication in crayfish in which experimental animals were tested in static systems. We suggest that a temporal separation of molting and copulation and a long reproductive receptivity period for females (which would allow abundant intersexual encounters) could account for a lack of selective pressure to evolve long-distance sex pheromones.

Key Words—Agonistic behavior, chemical communication, crayfish, pheromone, *Procambarus clarkii.*

INTRODUCTION

Biological communication as defined by Wilson (1970) is ". . . action on the part of one organism (or cell) that alters the probability pattern of behavior in

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another organism (or cell) in an adaptive fashion" (see also Burghardt, 1970). The critical portion of this definition is the requirement for a real or perceived advantage to the signaler (or its kin) to encode the message. In other words, information transfer (e.g., mere chemical detection of another organism) does not necessarily imply communication.

Pheromones are a form of chemical communication functioning within species or, in certain instances, between closely related species (Karlson and Lüscher, 1959). In Crustacea, experimental evidence for a sex pheromone is limited to only two of the eight major subclasses, the Malacostraca and the Copepoda (for a review, see Dunham, 1978). The majority of such studies have dealt with marine species with much less attention directed toward freshwater crustaceans (e.g., Decapoda: Kamiguchi, 1972; Ameyaw-Akumfi and Hazlett, 1975; Little, 1975; Thorp and Ammerman, 1978; Amphipoda: Dahl, et al. 1970a,b). Little (1975) showed that third-stage larvae of the freshwater crayfishes *Cambarus virilis* (now *Orconectes*), *Orconectes sanborni*, and *Procambarus clarkii* discriminate chemically between brooding and nonbrooding females. Ameyaw-Akumfi and Hazlett (1975) and Ameyaw-Akumfi (1976) suggested that sex recognition by males of the crayfish *Procambarus clarkii* was mediated by a sex pheromone. Males responded agonistically to male chemical stimuli and submissively to female stimuli. Thorp and Ammerman (1978), however, found no chemical sex recognition in *Procambarus a. acutus* and suggested that the ecological importance of pheromones for adult crayfish was species specific and perhaps niche specific.

Dunham (1978) reviewed experiments on sex pheromones in Crustacea and concluded that "The lack of appropriate control procedures casts doubt upon most of the available evidence for a sex pheromone in Crustacea." He cited a lack of (1) "blind" observation techniques, (2) novel stimuli, and (3) randomized sequences of treatments. Some previous studies, in our opinion, also failed to eliminate vibrations as a means of communication nor did the design of the apparatus take into account a pheromone's dispersal through its active space (area of threshold concentration for behavioral response; Bossert and Wilson, 1963; Wilson, 1970).

To maximize active space and minimize "background noise" from chemical signals overlapping spatially and temporally, highly motile crayfish may have evolved pheromones with short fade-out times (both lotic and lentic species) and/or they could release them into currents (lotic species) (see related discussion in Wilson, 1970). Unfortunately, few pheromone experiments have either examined behavioral responses of aquatic organisms when the temporal separation between stimulus release and reception are reduced (thus obviating response to frequently unrealistic accumulations of wastes in laboratory experiments) or have employed flow-through conditions (fewer wastes and also rapid dispersal of pheromones).

In this study we evaluated whether information concerning sexual identity, agonistic state, or stress-related condition is transmitted chemically in the crayfish *Procambarus* (*Scapulicambarus*) *clarkii* (Girard, 1852). Crayfish were tested in flow-through systems in which conditioned water passed immediately from stimulus tanks (or head tanks, after Thorp and Ammerman, 1978) to observation tanks. Behaviors which were used as experimental indicators of chemical detection and/or communication were applicable to laboratory situations and were not necessarily completely representative of normal crayfish behavior in natural environments, although we have observed similar behaviors in the field and many have frequently been described in laboratory experiments on crayfish behavior (e.g., Bovbjerg, 1970).

METHODS AND MATERIALS

Reproductively active male (form I; wet weight 13–44 g) and female (wet weight 12–30 g) *P. clarkii* were purchased from a commercial supplier in Louisiana and tested in the summer of 1978. Crayfish were acclimated for several weeks under a 12-hr L:D photoperiod at 22 ± 2°C in individual aquaria and were fed commercially pelleted fish food 3 times a week.

Five experiments were designed to test the effects of "conditioned water" (possibly containing pheromones) from head tanks (stimulus tanks) on the behavior of solitary male or female crayfish in observation tanks. A random group of 20 males and 20 females were subjected individually to a random sequence of five treatments: unconditioned water (control), water flowing through tanks containing a solitary male or female, and water from tanks holding either two males or two females. The series of experiments was formulated to reveal (1) any sexual differences in production and reception of chemical cues, and (2) any differential production of chemical cues from solitary (relatively undisturbed) and communally housed (stressed) crayfish.

Each experiment consisted of a 2- to 3-day isolation period, a 90-min period for acclimation to observation and head tanks, and a 20-min observation period during the light phase of the photoperiod. The acclimation period allowed crayfish to adjust to their new surroundings and, in most cases, to complete "exploration" of the tanks (thus increasing the likelihood of detecting treatment-related behavior). For experiments with two head tank animals, the second crayfish was not introduced until the beginning of the observation period to promote maximum stress (from agonistic contact) at this point. The two head tank crayfish were separated by a transparent flow-through baffle (Figure 1) to prevent physical contact and the loss of body fluids from potential wounds. Head tank animals were observed to respond agonistically to the other stimulus crayfish during at least the initial portions

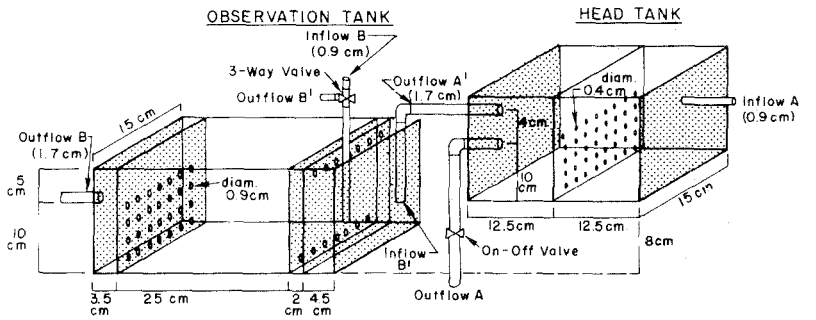

FIG. 1. Flow-through test chambers. During acclimation, water flowed through inflows A and B, and out through outflows A and B. During subsequent observation periods outflow A and inflow B were closed which caused water to pass out of the head tank through outflow A′ and into the observation tank through inflow B′. When two male or two female stimulus crayfish were in the head tank, they were separated by a transparent partition. Tanks were constructed of ¼-in. Plexiglas and contained 1 cm of fine gravel on the bottom.

of the "observation period" despite their inability to achieve direct physical contact. Observation and head tanks were designed to maintain constant flow rates (approximately 1.5 cm/sec) near the bottom of the tanks (as indicated by dye tests of dispersion rates). Stable flow was achieved with valves which kept pressure constant in the entire system during both the acclimation period, when tanks were independently supplied with well water (20°C), and the observation period, when tanks were connected in series. Observation and stimulus tanks were placed close enough together so that maximum separation of animals was less than 0.5 m but were not touching in order to prevent intertank vibrations. The 0.5-m distance was selected somewhat arbitrarily on the basis of behavioral observations of an approximate, maximum detection (chemical or tactile) distance for some blind cave crayfish (James H. Thorp and H. H. Hobbs III, personal observation) which were used in other pilot experiments. After each replicate the tanks were flushed for 5–10 min, and any remaining detritus or fecal matter was removed with a small mesh dipnet. All animals were tested between 1000 and 1800 hr and only intermolt crayfish were used. Because of a shortage of suitable crayfish, some observation animals also served as stimulus crayfish. Crayfish were returned to their aquaria for at least 2 days prior to a new experiment. Behavioral data for crayfish which died within 3 days of their trial were discarded.

Behaviors of crayfish in observation tanks were recorded with a Sanyo videotape system and were analyzed for duration and/or frequency of individual behaviors. The 20-min observation periods were subdivided for analysis into 80 intervals of 15 sec each. During each 15-sec interval the

occurrence of any of eight defined crayfish behaviors were noted (Table 1). In addition, observation tanks were subdivided for analysis (no physical barriers) into three equal-sized zones (zone 1 was upstream); and, the position of the crayfish was noted at the end of each 15-sec interval.

Data were analyzed for statistical significance ($P < 0.05$) with the general linear model procedure (GLM; analysis of variance portion) of the statistical analysis system (SAS). GLM procedures were run comparing treatment effects on duration of the eight behaviors listed in Table 1 as well as on the probability of a crayfish being in zone 1 (upstream third) and on the mean of the quantity "average duration per trial" for the following behaviors: chela(e) up, chela wave, and meral spread. Tendencies toward "handedness" (i.e., right or left chela waving) were also analyzed. Treatments were designated with the letters A through J as described in Figure 2 and Table 2. Grouped letters (e.g., AD or ACDF) indicate that these treatments were statistically compared with the GLM procedure.

TABLE 1. BEHAVIORAL RESPONSES RECORDED IN EACH EXPERIMENT

Chelae up	Both chelae raised off the substratum.
Chela(e) in baffle hole	Insertion of one or both chelae in hole of the inflow or outflow baffles.
Chela waving	Moving the right or left chela back and forth horizontally in front of the body with propodus of chela held approximately perpendicular to substratum; crayfish not simultaneously grooming that appendage.
Climbing	Cephalothorax pointed at angle above the horizontal with at least 4 walking legs off the substratum.
Digging	Digging in the gravel on the bottom of the tank using walking legs and chelae.
Grooming	Rubbing, picking, or scratching at parts of the body with one or more walking legs.
Gross body movement	Movement of the entire body around the tank (climbing is a subset of this behavior).
Meral spread	Both chelae raised above level of coxae of walking legs and spread laterally from normal walking position. Cephalothorax may be raised but crayfish not climbing (meral spread is a subset of chelae up).

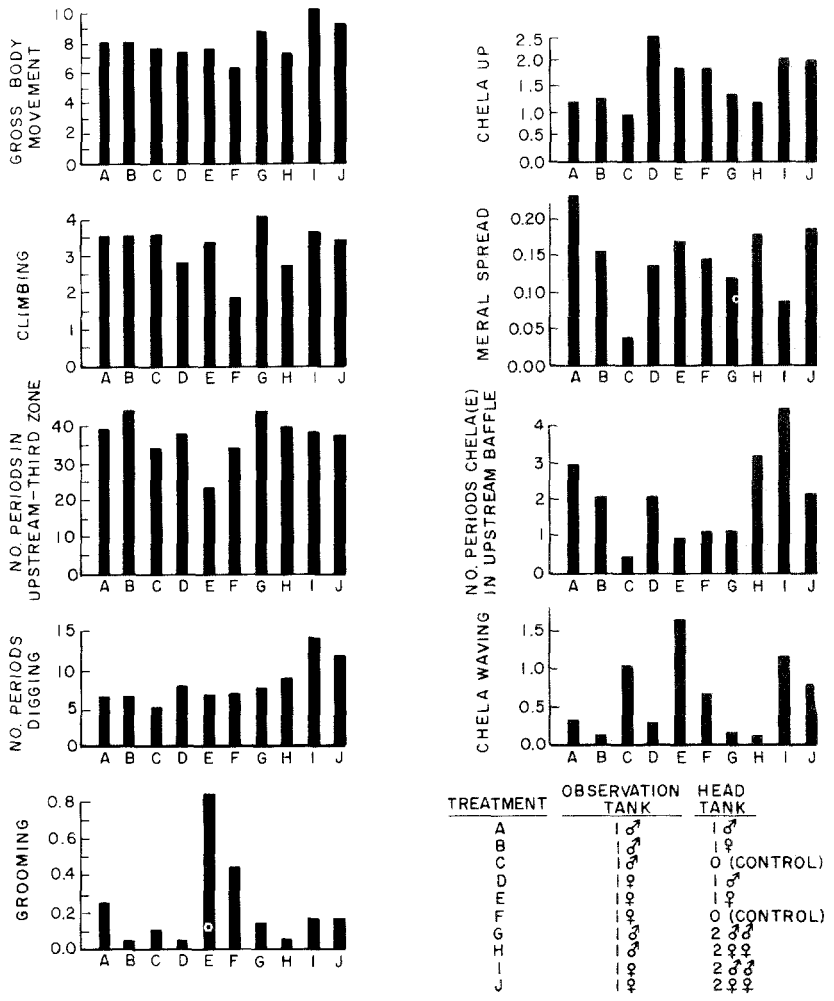

FIG. 2. Effect of conditioned water from stimulus animals in head tanks on specified behaviors of male or female crayfish in observation tanks. Letters on abscissa indicate treatment groups as described below. Behaviors are described in Table 1. Values on ordinates expressed as mean duration in minutes for each behavior per 20-min experiment or as mean number of 15-sec periods out of 80 that behavior occurred. Results of statistical comparisons of various treatments are in Table 2.

RESULTS

Chemical Detection of Conspecifics. Observation tank crayfish appeared able to detect chemically animals in head tanks. Despite the large probability of type I errors (from over 300 GLM procedures), the frequency of significant

TABLE 2. RESULTS OF GENERAL LINEAR MODEL PROCEDURES FOR COMPARING VARIOUS TREATMENT EFFECTS ON DURATION OF SPECIFIED CRAYFISH BEHAVIORS[a]

TRT	Observation tank	Head tank	TRT	Observation tank	Head tank
A	1 ♂	1 ♂	F	1 ♀	0 (control)
B	1 ♂	1 ♀	G	1 ♂	2 ♂♂
C	1 ♂	0 (control)	H	1 ♂	2 ♀♀
D	1 ♀	1 ♂	I	1 ♀	2 ♂♂
E	1 ♀	1 ♀	J	1 ♀	2 ♀♀

Comparisons[b]	Significant behaviors[c]	Rank order	Total *DF*	*F* ratio	Prob. > *F*
ABC, ACG, EFJ, AG, BH, DI, HJ, AB, GH, and IJ	All nonsignificant ($P > 0.05$)				
DEF	Chela wave	(E>F>D)	62	3.90	0.026
CGH	Chela(e) in upstream baffle	(H>G>C)	60	3.73	0.030
FIJ	Gross body movement	(I>J>F)	62	4.72	0.013
	Climbing	(I>J>F)	62	3.51	0.036
	Chela(e) in upstream baffle	(I>J>F)	62	3.22	0.047
BCH	Chela(e) in upstream baffle	(H>B>C)	59	3.42	0.040
DFI	Gross body movement	(I>D>F)	61	4.01	0.023
	Grooming	(F>I>D)	61	3.34	0.042
	Chela(e) in upstream baffle	(I>D>F)	61	3.38	0.041
ACDF	Grooming	(F>A>C>D)	81	2.78	0.046
	Chelae up	(D>F>A>C)	81	3.08	0.032
BCEF	Chela(e) in upstream baffle	(B>F>E>C)	83	3.10	0.031
CFGI	Digging	(I>G>F>C)	83	3.20	0.270
	Chelae in upstream baffle	(I>G>F>C)	83	5.78	0.001
CFHJ	Grooming	(F>J>C>H)	82	3.27	0.025
	Chelae up	(J>F>H>C)	82	2.69	0.051
	Chela(e) in upstream baffle	(H>J>F>C)	82	2.58	0.059
EJ	Time in upstream zone	(J>E)	40	5.77	0.021
AD	Chelae up	(D>A)	38	3.79	0.059
BE	Chela wave	(E>B)	40	8.13	0.007
	Time in upstream zone	(B>E)	40	9.77	0.003
GI	Chela(e) in upstream baffle	(I>G)	40	5.03	0.031
CF	Chelae up	(F>C)	42	4.35	0.043
	Chela(e) in upstream baffle	(F>C)	42	4.23	0.046
	Average chelae up per trial	(F>C)	39	4.94	0.032
DE	Chela wave	(E>D)	39	6.07	0.018
	Time in upstream zone	(D>E)	39	4.87	0.034
A–J	Grooming		203	1.97	0.044
	Chelae up		203	2.04	0.036
	Chela(e) in upstream baffle		203	1.97	0.044

[a] Those tested were chelae up, chela(e) in upstream baffle, chela waving, climbing, digging, grooming, gross body movement, meral spread, presence in upstream zone, and mean of the quantity "average duration per trial" for chelae up, chela wave and meral spread.
[b] Grouped letters indicate that means of these experiments were compared statistically.
[c] Behaviors described in Table 1. Probability values listed only for significant behaviors.

comparisons (Table 2) for the behavior "chela(e) in upstream baffle" supports our conclusion about chemical detection. This behavior suggests that observation crayfish may have been orienting towards the chemical cues and were attempting to approach the source. Only one behavior (chela wave) out of 28 possible was significant for the statistical comparisons ABC and DEF (see treatment descriptions in Figure 2 and Table 2), which suggests that neither males nor females react outwardly to a single male or female in head tanks. When either two males or two females were in the head tank, however, observation males and females tried to reach through holes in the upstream baffle (comparisons CGH and FIJ), moved (FIJ), and climbed (FIJ) significantly more than in controls. In general, observation crayfish were more responsive when two head tank crayfish were present than when a single animal was in the head tank.

Although chemical detection of the head tank animals was shown, these experiments do not imply that crayfish can discriminate between conspecifics and either congeneric crayfish or, in fact, noncrustaceans. Likewise, significant demonstration of "detection" does not necessarily imply "communication" as defined in the introduction.

Communication: Sexual Recognition. Sexual recognition would be demonstrated by either quantitative or qualitative differences in behavioral response to the chemical presence of males or females. In most comparisons with significant differences, females were more active than males to water conditioned by either one or two males (comparisons AD, GI, ACDF, CFGI), or one or two females (BE, HJ, BCEF, CFHJ) (Figure 2, Table 2). This partially resulted because females in control treatments (C and F) showed significantly more "chelae up" and "chela(e) in upstream baffle" than did males. Despite this result, however, for almost all behaviors either no significant differences among treatments occurred or, when differences were significant (ACDF, CFGI, BCEF, CFHJ), treatment values were greater than controls.

Most importantly, males did not recognize differences between water conditioned by head tank males or females (comparisons ABC, AB, GH) nor, in general, did females distinguish chemically between males or females (DEF, DE, IJ). Although females waved their chelae more often (DE) to solitary females than to solitary males, there were no differences in response to water conditioned by either two males or two females (IJ).

Communication: Agonistic Behavior. Although each of the behaviors graphed in Figure 2 could represent agonistic responses (fight, flight, or displacement activities), only meral spread is consistently associated with agonistic behavior (personal observation). Meral spread behavior, however, was infrequently shown (Figure 2) and never significantly affected by experimental treatment (Table 2).

Communication: Stress. Differential response to one versus two head

tank crayfish could indicate (1) a special "stress pheromone" was released as a result of agonistic encounters between head tank animals, (2) another pheromone, not specifically coding for stress, was released intermittently, e.g., when two crayfish interacted, or (3) the response threshold for a pheromone in the flowing system required output from at least two crayfish. Although responses were generally greater to two animals than one in head tanks, comparisons of single versus multiple crayfish treatments (AG, BH, DI, EJ) were rarely significant.

DISCUSSION

The crayfish *Procambarus clarkii* seems able to detect chemically the presence of another animal at a distance of at least 0.25 m without additional visual or tactile stimuli. The information received produced significant, but slight, quantitative differences from controls in releaser behavior (Wilson and Bossert, 1963) and in chemokinetic and chemotaxic reactions (Dunham, 1978) but no significant change in qualitative behavior. Increasing the number of head tank animals tended to enhance the response of the observation crayfish. Despite evidence for chemical detection, however, the results do not suggest that crayfish "communicated" information on sexual identity, agonistic state, or stress condition, nor does this detection necessarily imply discrimination between stimuli from crayfish and other taxa (e.g., fish).

On the basis of experiments with larval crayfish (Little, 1975), it appears that crayfish possess at least one pheromone; however, the existence of sex pheromones or other pheromones acting between adults remains controversial at best. Ameyaw-Akumfi and Hazlett (1975) and Ameyaw-Akumfi (1976) concluded that *Procambarus clarkii* produced sex pheromones, but Thorp and Ammerman (1978) reported no significant sex recognition in *P. a. acutus*, although they demonstrated significant chemical detection. An obvious question at this point is "Why are the findings of these studies on interadult sex pheromones in conflict?"

All studies were conducted under artificial conditions in the laboratory; however, in our opinion, the apparatus and methods employed in the present study produced physical and chemical conditions (and thus behaviors) more representative of those occurring in the natural environment. Flow-through systems (1) provide better directionality for chemical cues than do static systems, (2) allow dispersal of chemical cues at rates more comparable to natural lotic conditions (a factor particularly important for pheromones with short fade-out times), and (3) prevent possible pheromones and toxic waste products from accumulating at levels unnaturally high for either lotic or most lentic environments. In previous studies with static systems, however, metabolic waste products and possible pheromones were allowed to build up

for 1 day (Thorp and Ammerman, 1978) or 1–2 days (Ameyaw-Akumfi and Hazlett, 1975) before addition to the observation chamber. Pheromones with 1- to 2-day fade-out times could create confusing "signal noise" in aquatic systems, and therefore selective forces should prevent their evolution in motile species. Separating the responses caused by either pheromones or toxic wastes would be difficult in studies employing static systems and long acclimations.

Methodological problems and differences in experimental design make it difficult to compare results of studies on interadult pheromones in crayfish. For example, acclimation and test temperatures affect agonistic behavior (Thorp, 1978), and acclimation time in test tanks can alter dramatically the expressions of many behaviors (personal observation). Unfortunately, information on acclimation temperature, acclimation period in test tanks, prior "housing" conditions, and sequences of experimental treatments (including randomization procedures) was not reported in Ameyaw-Akumfi and Hazlett (1975) nor in Ameyaw-Akumfi (1976). Additional problems may be differences in definition of behaviors (e.g., chelae up versus meral spread) and in interpretation of what constitutes agonistic and submissive behaviors. Methodological differences could perhaps explain why Ameyaw-Akumfi (1976) reported for *P. clarkii* high frequencies of agonistic behavior with low variability (e.g., average minutes chelae up per 30-min experiment = 27.6 ± 0.976 SD), whereas we found reduced agonistic response with high variability. However, Thorp and Ammerman (1978) and unpublished pilot studies by Thorp have shown similar low responses for other crayfish species in both static and flow-through conditions (e.g., with *Cambarus latimanus*, *Procambarus troglodytes*, and with the blind, troglobitic crayfish, *Orconectes i. inermis*).

If we are correct in stating that adult crayfish do not communicate with each other over long distances with pheromones, then two questions arise. First, are there interadult sex pheromones with small active spaces? Second, what selective pressures were missing which, had they been present, would have promoted evolution of "long-distance" pheromones?

Behavioral displays by crayfish suggest that visual and tactile stimuli are important for intra- and interspecific communication, but it is not clear which means (i.e., chemical, tactile, visual, and/or behavioral) are utilized for sex recognition. At least as early as 1909, investigators were questioning the ability of crayfish to discriminate visually or chemically between sexes. Pearse (1909) reported amplexus between two males and between a live male of one species and a dead female of another (although the latter behavior was rare). Mason (1970) observed intrasexual copulation of crayfish in the laboratory and reported finding spermatophores on the exterior of a mature male in the field. Copulation between *Procambarus* (*Ormannicus*) *hirsutus* and *P.* (*O.*) *acutus* has been noted in our laboratory (personal observation). Occurrences of successful and even unsuccessful attempts at interspecific and intrasexual

amplexus argue against "facile" acceptance of sex pheromones in crayfish. Sex recognition and successful intersexual copulation appear to involve lowered levels of agonistic behavior by receptive females (Chidester, 1912; Mason, 1970) with the possible partial contribution of sex pheromones which are operative only over short distances.

Natural selection should promote development of sex pheromones for species in which mate location is difficult or haphazard, or in which females are receptive for only relatively short periods. For most epigean crayfish species, however, locating suitable mates would probably never limit a species because females are reproductively active over a period sufficiently long (several months) to allow encounters with a large number of form I males (see Penn, 1943, for life history information on *Procambarus clarkii*). In contrast to mating in crayfish, copulation between lobsters is assumed usually to be a molt-related phenomenon restricted to a 24-hr period following the female's molt (Templeman, 1934, 1936, in Dunham, 1978) although copulation has been observed at other times (Dunham, 1978). Evidence for sex pheromones has been reported for lobsters and other crustaceans, although these results are not definitive (Dunham, 1978). If mating attempts are more likely to be successful during intermolt periods (e.g., because of reduced agonistic behavior and/or heightened receptivity in the female), then it would be an advantage for that species to have evolved a molt-related, sex pheromone.

In summary, the results of this study suggest that chemical communication between adult crayfish does not occur or is not efficient at distances greater than the effective range for visual communication. A temporal separation of molting and copulation and a female receptivity period which is sufficiently long to allow abundant intersexual encounters could account for a lack of selective pressure to evolve long-distance sex pheromones. Finally, there is no definitive evidence in crayfish for interadult pheromones which act over short distances or which are related to nonsexual responses (e.g., alarm, stress, and/or agonistic behaviors).

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SOLDIER-SPECIFIC CHEMICALS OF THE TERMITE *Curvitermes strictinasus* MATHEWS (ISOPTERA, NASUTITERMITINAE)

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Abstract—The defense secretion of the soldier termites of *Curvitermes strictinasus* Mathews has been analyzed. Seven components have been isolated and identified, including limonene, terpinolene, *p*-cymen-8-ol, tridecan-2-one, tridecen-2-one *cis,trans*-farnesal, and *trans,trans*-farnesal.

Key Words—Isoptera, Nasutitermitinae, isolation, identification, *Curvitermes strictinasus* (Mathews), defense secretion.

INTRODUCTION

Defense in some termite species is achieved through toxic and/or repellent substances produced by the soldier caste. Many of the highly specialized soldiers in the subfamily Nasutitermitinae (Termitidae) are characterized by the reduction of the mandibles and the development of a nasus with a gland opening at the end. The mode of defense in such species is by forcible ejection of an irritating and sticky fluid (Ernst, 1957; Eisner, 1976). Chemical studies of these secretions have shown the components to be a variety of terpenoid compounds. Many common monoterpenes such as α-pinene, β-pinene, and limonene have been found together with mixtures of oxygenated diterpenes (Prestwich, 1979). *Curvitermes strictinasus* is a member of the *Paracornitermes subulitermes* branch of Nasutitermitinae. It is a neotropical species found in the cerrado vegetation (wooded savanna) of Brazil. The termite inhabits large, earthen, epigeic mounds built by other species, and lines its galleries with a characteristic, shiny, smooth, soil-colored material of fecal

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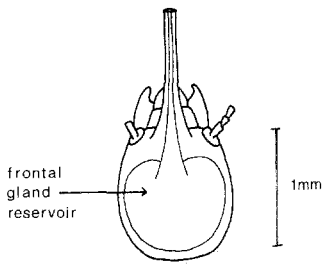


FIG. 1. Dorsal view of the head capsule of a *Curvitermes strictinasus* soldier showing the long frontal tube.

origin. It is thought to feed on organic residues in the soil and within the host termite mound (Mathews, 1977; H.R. Coles, personal observations).

The soldier caste of *C. strictinasus* has a well-developed nasus and small but functional mandibles. The frontal gland occupies almost all of the head capsule. Using measurements of dissected glands, the approximate volume was calculated as 370 μl. (Figure 1). In common with other termites the main invertebrate predators of *C. strictinasus* appear to be ants, while anteaters and armadillos constitute the main vertebrate predators. When the nest is breached, the soldiers immediately rush to the disturbance and, with forward lunging movements of the whole body, daub large amounts of a clear liquid from the nasus onto the substratum. This liquid has a characteristic smell of lemons. The soldiers remain near the breached galleries until the workers begin to reconstruct them, commonly 5–10 min after the disturbance. This paper reports a chemical study of the components of the defense secretion of the soldiers of *Curvitermes strictinasus.*

METHODS AND MATERIALS

Collection Procedures. Workers and soldiers of *Curvitermes strictinasus* were collected from opened mounds in the Distrito Federal, Brazil. Live specimens were dropped into vials of methylene chloride, which were then sealed and stored under refrigeration until dispatch by air to Southampton. This procedure was adopted because substantial loss of material would have taken place if collection had involved decapitation since the secretion appeared to be fairly volatile. An extract was prepared by crushing the insect bodies under methylene chloride, followed by filtration of the crude extract through a cotton wool plug. In this way separate extracts were obtained from workers and soldiers which enabled the soldier-specific components to be identified.

Instrumental Methods. Nuclear magnetic resonance (NMR) spectra were obtained on 50- to 100-μg samples in deuteriochloroform solution

containing 1% TMS using a Varian XL-100, Fourier transform spectrometer.

Mass spectra (MS) were obtained using a Kratos MS-30 spectrometer with a DS-505 data system, interfaced with a Pye Unicam 204 chromatograph via a Ryhage molecular separator. The chromatograph was equipped with 3 m × 0.2 cm ID glass columns packed with 5% OV-101 on 100–120 mesh CLQ (A) and 5% diethylene glycol succinate (DEGS) on 100–120 mesh Diatomite CLQ.

Gas–liquid chromatography (GLC) was performed using either a Varian 3700 gas chromatograph or a Pye Unicam 104 gas chromatograph both equipped with flame ionization detectors and 3 m × 0.2 cm glass columns packed as (A), (B) and 5% FFAP on 100–120 mesh Diatomite C (C). The glass columns used for preparative gas chromatography were 1.5 m × 0.4 cm ID and were packed with 10% DEGS on 60–80 Diatomite C (D), 10% OV-101 on 60–80 Diatomite C (E), and 10% FFAP on 60–80 Diatomite C (F).

RESULTS

The GLC trace of the dichloromethane extract was obtained from approximately 1000 crushed soldiers (Figure 2). Each soldier contains about

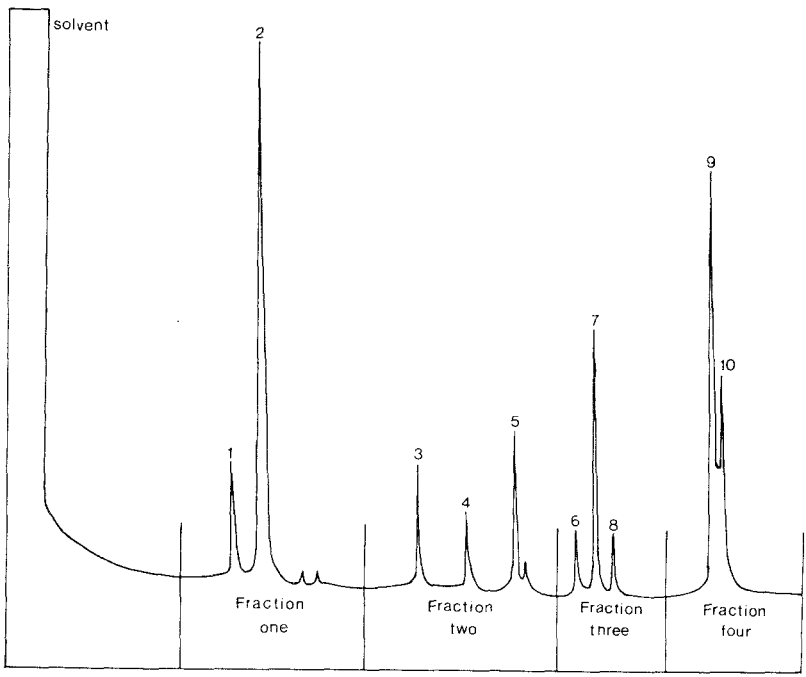


FIG. 2. Chromatogram of an extract of the soldiers of *Curvitermes strictinasus* (Pye Unicam 104, FFAP 70–200°C at 5°C/min).

320 μg of volatile material. Individual collections of soldiers showed minor variations ($\pm$5%) in the amount of each peak. None of the peaks were present in a similar extract of the worker termites.

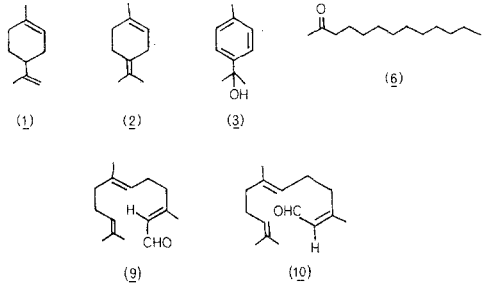
The extract was passed through column B (70–250° C at 5° C/min) and fractions were collected as indicated (Figure 2), using the all-glass micro-preparative splitter of Baker et al. (1976). These fractions were then rechromatographed and individual components collected in a pure state in deuteriochloroform ready for Fourier transform NMR.

Fraction one was passed through column A (50–100° C at 5° C/min) and components (1) and (2) were collected. Components (3), (4), and (5) in fraction two were separated on column A (70–170° C at 5° C/min). On passage through column C (100–170° C at 5° C/min) fraction three yielded the separate components (6), (7), and (8). Fraction four was also passed through column C (165° C isothermal), and components (9) and (10) were collected.

The mass spectra of components (1) and (2) suggested that they were monoterpene hydrocarbons (M^+, m/e 136). The NMR spectrum δ($CDCl_3$, 100 MHz) of (1) showed diagnostic peaks at 1.66 (CH_3—=), 1.76 (CH_3—=), 4.71 (CH_2=) and 5.42(H—=). Thus (1) was identified as limonene (1) (Scheme 1) which was confirmed by comparison of mass spectrum with that reported by Ryhage and Von Sydow (1973).

Component (2) was identified as terpinolene (2) by its mass spectrum which was identical to that reported by Thomas and Willhalm (1964) and its NMR spectrum δ($CDCl_3$,100 MHz) which showed peaks at 1.6–1.72 (3 × CH_3—=) and 5.31 (vinyl proton).

The structure of (3) was identified as *p*-cymen-8-ol (3) by its NMR and mass spectra, δ($CDCl_3$,100 MHz), 1.59 (6H), 2.36 (3H), 7.12–7.25(4H) and *m/e*, M^+ 150 (2%), 132 (100%), 117 (72%), and 91 (29%). Confirmatory evidence was provided by comparison with an authentic sample. Component (6) was found to be tridecan-2-one (6) by its NMR and mass spectra, δ($CDCl_3$, 100 MHz), 0.88 (3H, CH_3), 1.2–1.3 [18H, $(CH_2)_9$], 2.14 (3H, CH_3—CO), and 2.21 (2H, CH_2—CO) and *m/e* M^+ 198 (6%), 183 (2%), 140 (5%), and



(1) (2) (3) (6) (9) (10)

SCHEME 1.

38 (100%). The NMR and mass spectra of (7) indicated that it was a closely related monounsaturated ketone, δ ($CDCl_3$, 100 MHz), 0.99 (3H, t, CH_3), 1.25–1.35(CH_2envelope), 2.16(CH_3—C=O), 2.23 (CH_2—C=O), 5.36 (CH=CH, m) and *m/e*, M^+ 196 (12%), 138 (6.8%), and 58 (17%). From these data it was clear that the component was a tridecen-2-one; although the double bond could not be placed in the molecule with any certainty, it was evident it was neither conjugated nor terminal.

Components (9) and (10) were identified as farnesals by their mass spectra, *m/e* M^+ 220 (1%), 107 (17%), 84 (47%), 69 (98%), 55 (22%), 41 (100%), and *m/e*, M^+ 220(1%), 93 (92%), 84 (49%), 69 (100%), 55 (15%), 41 (67%), which compare favorably with those published by Von Sydow et al. (1970). The NMR spectrum of (9) δ($CDCl_3$, 100 MHz), 1.6(3H,s,CH_3—=), 1.62(3H,s,CH_3—=), 1.68(3H,s,CH_3—=), 2.95(3H, CH_3—=—CHO), 4.76, 5.06, and 5.88 (3 × 1H, m, H—=) and 10.0 (1H, CHO) provided confirmation that this compound was *trans,trans*-farnesal (9). The latter compound was identified as *cis,trans*-farnesal (10) which was confirmed by its NMR spectrum, δ($CDCl_3$, 100 MHz), 1.6 (2H, CH_3—=), 1.69 (3H, CH_3—=), 1.96 (3H, CH_3—=—CHO), 4.76, 5.06, and 5.85 (3 × 1H, H—=), and 9.9 (1H, CHO).

Due to the small amounts of available material, we have so far been unable to assign structures to components (4), (5), and (8) on the available data. The first of these, (4) appears to a dioxygenated monoterpene and (5) and (8) sesquiterpene hydrocarbons.

DISCUSSION

The defense secretion of *Curvitermes strictinasus* contains the monoterpene hydrocarbons limonene and terpinolene. Common monoterpenes have frequently been found in the secretions of the family Termitidae (Moore, 1968; Vrkoč et al., 1973; Prestwich, 1979a), but the role of these compounds is not fully understood. Preliminary tests carried out in Brazil (to be reported elsewhere) have shown that limonene, α-phellandrene, α-pinene, *cis*-β-ocimene, and α-thujene, all known components of termite defense secretions, are, to varying extents, toxic on topical application to several indigenous species of camponotine ants. Hrdý et al. (1977) have reported that several monoterpenes are toxic to the housefly *Musca domestica*. Monoterpenes have been shown to act as alarm pheromones for several species. The effect of limonene with a small amount of terpinolene and α-phellandrene on *Drepanotermes rubriceps* is to produce a short-lived snapping frenzy (Moore, 1968). Soldiers of *Nasutitermes rippertii* and *N. costalis* exhibit alarm behavior on exposure to certain monoterpenes (Hrdý et al., 1977; Vrkoč et al., 1978). The major component of the secretion of *N. rippertii* is α-pinene, which

evokes the greatest response from soldiers of this species. In *N. costalis* the strongest alarm effect is elicited by Δ^3-carene which is the major monoterpene constituent of the soldier secretion in this species. Limonene, *cis*-β-ocimene, and β-pinene act as feeding deterrents to the anteater (*Myrmecophaga*), a vertebrate termite predator (Coles, 1980).

Straight-chain saturated and unsaturated ketones similar to those identified from *C. strictinasus* have been found in a number of more advanced termites of the family Rhinotermitidae. Tridecan-2-one has been identified as 8% and 0.7% of the secretions of *Schedorhinotermes putorius* and *S. lamanianus*, respectively (Quennedey, 1973; Prestwich, 1975). Topical application of the secretion of *S. putorius* or exposure of ants to the vapor led to immobilization and death (Quennedey, 1973). The presence of the aliphatic side chain in these ketones has led to speculation that it may aid penetration through the ant cuticle (Prestwich, 1979).

p-Cymen-8-ol, *trans,trans*-farnesal, and *cis,trans*-farnesal in the defense secretion of the soldiers of *C. strictinasus* are the first aromatic alcohols and unsaturated sesquiterpene monoaldehydes to have been identified from insect defense secretions. The only related compounds which have been isolated from termite defense secretions are two dialdehydes, cavidial and ancistrodial, which have, respectively, been found in the secretion of the major and minor soldiers of *Ancistrotermes cavithorax* (Baker et al., 1978). Further investigations as to the properties of these compounds with respect to their role in defense are being carried out.

Vrkoč et al. (1977, 1978) and Preswich (1979a,b) found that a number of nasutes produce a complex mixture of monoterpene hydrocarbons and diterpenes. *C. strictinasus* is one of the first members of the Nasutitermitinae in which analysis of the defensive secretion did not yield this mixture of compounds; although monoterpene hydrocarbons and sesquiterpene aldehydes are present, no diterpenoid compounds were found. These results indicate that large variations can be found in the defensive secretions within families of termites. More studies on the role and biochemical origin of diterpenoids in other species would be interesting.

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CHEMICAL COMPOSITION OF THE FRONTAL GLAND SECRETION OF *Syntermes* SOLDIERS (ISOPTERA, TERMITIDAE)

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Abstract—The defensive secretions from the frontal glands of soldier termites of the genus *Syntermes* contain similar mixtures of mono- and sesquiterpene hydrocarbons. The major components in *S. dirus, S. molestus, S. brevimalatus, S. peruanus,* and a new species (*Syntermes* sp. n), is *cis*-β-ocimene. A substantial amount of aristolochene is found in *Syntermes* sp. n. and is present at lower levels in all the other species; *S. brevimalatus* contains only *cis*-β-ocimene and aristolochene. The four other species also contain minor amounts of epi-α-selinene and germacrene A. The latter compound has been identified on the basis of its rearrangement product β-elemene. The termite *S. grandis* differed markedly from the other *Syntermes* species in that no terpenoid components were found in the soldier extract. With the obvious exception of *S. grandis*, the same soldier-specific mono- and sesquiterpenes occurred in all species. The total amount of secretion per unit weight of soldiers varies with the species and is inversely proportional to the development of the mandibular apparatus. In *S. molestus* smaller gland size is compensated for by a greater number of soldiers foraging trails.

Key Words—Isoptera, Nasutitermitinae, *Syntermes* species, defensive secretion, isolation, identification, terpenoid compounds.

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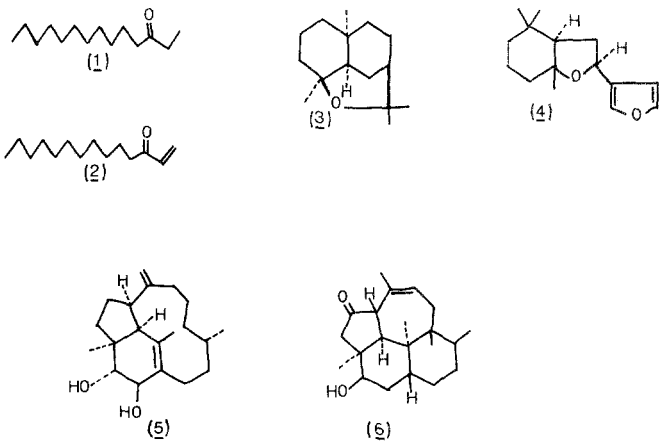
INTRODUCTION

In common with other social insects, termite societies display a marked degree of division of labor. In most termite species the task of defense is performed by the soldier caste which is morphologically specialized for this function. These specializations vary considerably but in general take the form of modification of the mandibles into powerful, cutting, snapping, or reaping blades (Deligne, 1965, 1971; Deligne and Quennedey, 1977) or the regression of the mandibles and production of toxic and/or repellent secretions from the enlarged, cephalic frontal gland (Noirot, 1969; Moore, 1968). The types of compounds that have been isolated from termite defense secretions range from simple alkanones and alkenones represented by (1) and (2) (Scheme 1) found in *Schedorhinotermes lamanianus* (Prestwich et al., 1975) to complex mixtures of terpenoids. The sesquiterpene 4,11-epoxy-*cis*-eudesmane (3) was found in *Amitermes evuncifer* (Wadhams et al., 1974) and ancistrofuran (4) was among the terpenes identified from *Ancistrotermes cavithorax* (Baker et al., 1978). A series of tricyclic diterpenes exemplified by (5), together with several simple monoterpene hydrocarbons, constitute the sticky secretion of *Trinervitermes gratiosus* and *T. bettonianus* (Prestwich et al., 1976). The soldiers of *Nasutitermes octopilis* produce the tetracyclic diterpene (6) and other closely related compounds (Prestwich et al., 1979a). A review of the chemistry of termite defensive secretions has recently been published (Prestwich, 1979b).

Some of the most highly advanced specializations for chemical defense can be found in soldiers of species within the subfamily Nasutitermitinae. The neotropical genus *Syntermes* Holmgren is generally regarded as the most



SCHEME 1.

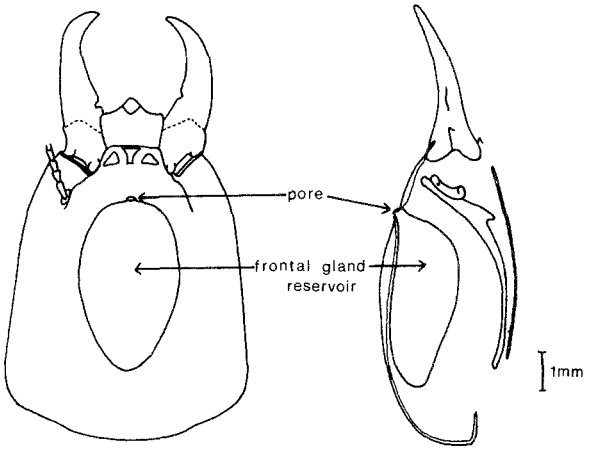


FIG. 1. Dorsal and sagittal sections of the head capsule of a *Syntermes* soldier (head width approximate).

primitive member of the subfamily (Emerson, 1952; Sands, 1957) and appears to be morphologically specialized for mandibular defense with large, heavily sclerotized mandibles. The soldiers also possess a frontal gland and, when disturbed, secrete a clear liquid from the poorly developed nasus (Figure 1). We report here the results of a chemical examination of these secretions in six *Syntermes* species and discuss their possible use in defense.

METHODS AND MATERIALS

Collection Procedures. Collections of termites were made from excavated epigeic mounds and subterranean nests and also from above-ground foraging parties. Four species, *Syntermes molestus* (Burmeister) 1839, *S. dirus* (Burmeister) 1839, *S. grandis* (Rambur) 1842, and *S. brevimalatus* Emerson, 1945, were collected from the cerrado vegetation around Brasilia, Distrito Federal, Brazil. *S. peruanus* Holmgren, 1911, was collected from primary tropical rain forest, Amasonas, and *Syntermes* sp n.,[3] from Vicosa, Minas Gerais, Brazil.

Soldiers and workers were collected separately and placed into vials of purified methylene chloride which were then sealed and stored under refrigeration prior to being dispatched by air. In Southampton an extract was prepared by crushing the whole insects under methylene chloride, followed by

[3] This was originally identified as *S. wheeleri* by the late R.L. Araujo (personal communication, 1977) but was subsequently found to be a new species.

filtration of the crude extract through cotton wool. "Milked" secretions were collected from the soldiers of *S. dirus* and *S. molestus* using 1-μl microcapillaries. These were also analyzed (see below) and results compared with those from extracts of whole bodies to determine which compounds were present in the defense secretion.

Instrumental Methods. Nuclear magnetic resonance (NMR) spectra were obtained on solutions in deuteriochloroform containing 1% TMS using either a Varian XL-100 spectrometer or a Bruker WH 360 NMR spectrometer. Microscale Fourier transform spectra were run with an internal deuterium lock. Samples were collected in deuteriochloroform at room temperature by micropreparative gas chromatography using the all-glass micropreparative splitter of Baker et al. (1976).

Mass spectra (MS) were obtained using an AEI-MS-12 spectrometer interfaced with a Pye Unicam 104 gas chromatograph via a Watson-Bieman glass frit separator. The gas chromatograph was equipped with a 2 m × 2 mm ID glass column packed with 5% OV-101 on 100–120 mesh CLQ(A).

Gas chromatography (GLC) was performed on a Pye Unicam 104 gas chromatograph or a Pye Unicam GCD chromatograph both of which were fitted with flame ionization detectors (FID). All glass columns were used fitted with 6 mm OD ground glass ends. Column A was used for analytical and a 1.5 m × 6 mm ID column packed with 10% OV-101 on 60–80 mesh CLQ was used for micropreparative work.

General Biology. Extensive ecological and behavioral studies (Coles, 1980) have been carried out over the last two years on several species of *Syntermes* in the cerrado vegetation zone of Brazil. *Syntermes* are large (head width 3–6 mm), grass-harvesting species which forage crepuscularly, or nocturnally, above ground and do not protect themselves by soil sheeting (Mathews 1977). The workers leave the ground through small exit holes and form trails to the food source where they cut pieces of grass and leaves and return with them to the nest. The columns of workers are flanked by soldiers aligned with heads raised, mandibles opened and pointing outwards from the column. The distances from the exit holes to the food source and the ratio of soldiers to workers in the foraging parties vary between the species (Table 2).

The nests built by the different species of *Syntermes* range from purely subterranean excavations to large epigeic mounds several meters in diameter and up to 0.80 m high. In common with other termites, their major invertebrate predators are ants and include species from the genera *Pachycondyla* Fr. Smith, *Odontomachus* Latreille, *Ectatomma* Fr. Smith, and *Camponotus* Mayr. Their major vertebrate predators are anteaters, particularly the giant anteater, *Myrmecophaga tridactyla*, Linnaeus, and a variety of armadillo species.

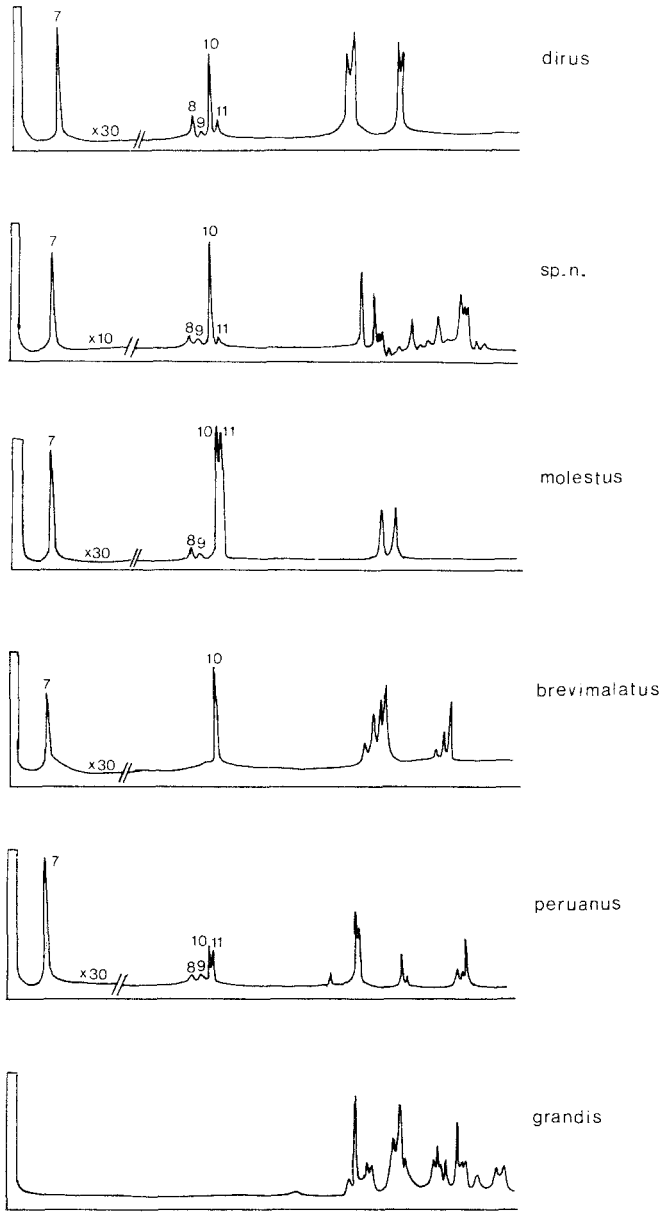


FIG. 2. Chromatograms of extracts of the soldier caste of six *Syntermes* species (Pye Unicam 104, 5% OV-101, 70–300°C at 5°/min). The identities of the peaks are indicated numerically as follows: 7, *cis*-β-ocimene; 8, germacrene A; 9, β-elemene; 10, aristolochene; 11 *epi*-α-selinene.

RESULTS

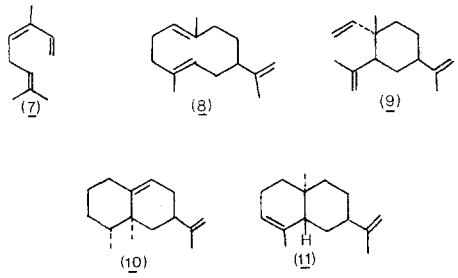
Chemical Analysis of Secretions. The GLC traces of the six species studied are shown in Figure 2. The compounds of major interest were those labelled 7–11 since these were absent from comparable traces of the worker termites. In addition "milked" secretions of *S. dirus* and *S. molestus* were shown to contain only 7–11.

The major component 7 was shown to be the monoterpene *cis*-β-ocimene (7) and was identified by its MS and NMR spectra. (Ryhage and Von Sydow, 1963; Ohloff et al., 1964). Coelution with an authentic sample of *cis*-β-ocimene confirmed the assignment (7) (Scheme 2).

Components 8–11 were identified as sesquiterpene hydrocarbons from their mass spectra. The major sesquiterpene 10 was found to be aristolochene (10), *m/e* 204 (5%), M^+, 105 (100%), 41 (87%), 189 (72%), 91 (69%), 93 (63%). 107 (59%), 161 (53%), 79 (48%) and δ ($CDCl_3$, 360 MHz), 0.80 (3H, d, J = 6 Hz), 0.93 (3H, s, angular CH_3), 1.70 (3H, s, $CH_3-=$), 4.65 (2H, bs, $CH_2=$), 5.25 (1H, m, vinyl proton) (Govindachari et al., 1970).

Compound 11 was identified as *epi*-α-selinene (11), *m/e* 204 (5%, M^+), 55 (100%), 57 (86%), 69 (54%), 91 (46%), 105 (44%), 107 (42%), 81 (37%), 79 (37%) and δ ($CDCl_3$, 100 MHz), 0.85 (3H, s, angular CH_3), 1.61 (3H, s, $CH_3-=$), 1.75 (3H, s, $CH_3-=$), 4.84–4.92 (2H, m, $CH_2=$), 5.28–5.40 (1H, m, vinyl proton) (Klein and Rojahn, 1970).

The situation with components 8 and 9 was more complicated since it was clear that 9 was a thermal rearrangement product of 8; a steady change of 8 to 9 occurred during gas chromatography. Component 9 was identified as β-elemene (9): δ ($CDCl_3$, 360 MHz), 0.95 (3H, s, angular CH_3), 1.67 (3H, s, $CH_3-=$), 1.72 (3H, s, $CH_3-=$), 4.59–4.93 (6H, m, 3 × $CH_2=$), 5.78–5.86 (1H, q, $J_{AX(obs)}$ = 10 Hz, $J_{BX(obs)}$ = 18 Hz) (Vig et al., 1968). It has been previously shown that germacrene A readily undergoes rearrangement to the β-elemene above 40° C. (Weinheimer et al., 1970; Bowers et al., 1977). Component 8 is considered to be the former, although it could not be isolated under our GLC conditions.



SCHEME 2.

TABLE 1. RELATIVE QUANTITIES OF SOLDIER—SPECIFIC COMPONENTS FOUND IN *Syntermes*[a]

	No of colonies examined.	*Cis*-β-ocimene		Germacrene A and β-elemene		Aristolochene		*epi*-α-selinene	
		$\bar{X}$	*SE*	$\bar{X}$	*SE*	$\bar{X}$	*SE*	$\bar{X}$	*SE*
S. dirus	7	93.3	2.2	5.19	1.26	5.56	1.89	0.59	0.23
S. molestus	3	89.05	2.03	0.2		5.9	2.33	0.67	0.29
Syntermes sp. n	1	90.1		0.6		8.8		0.5	
S. brevimalatus	4	96.95	0.37			3.03	0.38	Trace	
S. peruanus	3	91.8	3.91			4.07	1.89	4.1	1.95

[a]Figures expressed as mean percentages and standard error.

The constituents of the defensive secretion of each termite are summarized in Table 1. *Cis*-β-ocimene (7) is the major component in each species constituting at least 90% of the total secretions. Aristolochene (10) is the major sesquiterpene constituent and is the only other component in the secretion of *S. brevimalatus*. It is also the major sesquiterpene in the other species and constitutes about 7% of the total secretion in the case of *Syntermes* sp n. A truer reflection on the relative amount of germacrene A is probably best obtained by adding the estimated β-elemene to the value for germacrene A.

An examination of the GLC trace of an extract of *S. grandis* soldiers showed the complete absence of monoterpene and sesquiterpene hydrocarbons. The presence of a number of long-chain alkanes and alkenes was detected in the extract of *S. grandis*, some of which were not present in extracts of the worker termites of this species. These compounds were C_{19}–C_{23} saturated and monounsaturated hydrocarbons which were identified by their mass spectra.

DISCUSSION

The chemical examination of the secretions of six *Syntermes* species illustrates that the major component, in all but *S. grandis*, is *cis*-β-ocimene. Preliminary tests in Brazil show that *cis*-β-ocimene is toxic on topical application to *Camponotus spp* (LT_{50} = 37 min at 0.15 μl/ant) (Coles, 1980). Food selection tests using a giant anteater (*Myrmecophaga tridactlya*) in captivity showed that *cis*-β-ocimene was an olfactory feeding deterrent (Coles, 1980). It would appear, therefore, that even in this genus with obvious mandibular specialization, some chemical defense is apparent.

Prestwich (1979b) raises the possibilities of chemosystematics based on the different compositions of termite defense secretions for several species of *Nasutitermes*. He also reports (Prestwich, 1979c) that the African species *Macrotermes subhyalinus* (closed-mound type)[4] and the partially sympatric *Macrotermes* sp. near *subhyalinus* which are very difficult to distinguish morphologically, can be distinguished by the differences in gas chromatograms of their defensive secretions without examination of soldier or mound morphology. Similar chemical differences would be of great value in *Syntermes* systematics as there are several species that are difficult to distinguish on morphological characteristics alone. However, the results presented show considerable conservatism within the genus. With the exception of *S. grandis* there is little qualitative variation in the secretions, and the differences that do occur are mainly of a quantitative nature. Furthermore, quantitative intraspecific variations are of the same magnitude as quantitative interspecific variations (Table 1).

The similarity in composition of the defense secretions of five of the *Syntermes* species studied contrasts with the high interspecific variability, noted by Prestwich (1979c), in the secretions of the genus *Nasutitermites*. This is of interest if the positions of the two genera at opposite ends of the nasutitermitine phylogenetic line are considered. The primitive genus, *Syntermes*, shows a high degree of specialization for mandibular defense and has apparently limited chemical defense-producing secretions dominated by one compound. *Nasutitermes*, in contrast, has vestigial mandibles but components of the secretions differ markedly among species.

The marked difference between the secretions of *S. grandis* and the other species confirms a distinct behavioral difference noted in these species. Their thick-walled epigeic mounds are often breached by vertebrate predators. When this is simulated in the field, *S. dirus* and *S. molestus* soldiers congregate at the breach and secrete large droplets of secretion with a strong odor from the nasus. *S. grandis* soldiers, however, retreat into the mound and rarely return to the breach. As *cis*-β-ocimene has been shown to be a feeding deterrent to the vertebrate predator, *Myrmecophaga tridactyla* (Coles, 1980), it seems reasonable to suggest that the behavior of *S. dirus* and *S. molestus* is associated with the large quantities of this compound in their defense secretions. *S. grandis* which produces none of this deterrent, shows an alternative form of defense involving a retreat into the complex nest structure (Coles, 1980).

Differences in the defensive behavior of *S. dirus* and *S. molestus* include differences in the soldier–worker ratio in foraging parties (Table 2). *S.*

[4]Sands has proposed that the closed-mound type should be called *Macrotermes* sp. near *subhyalinus* and may be synonymous with *M. michaelseni*. The open-chimney type is believed to be *M. subhyalinus* (sensu stricto) (Prestwich, 1979c).

TABLE 2. SUMMARY OF BIOLOGICAL DATA FOR SOLDIERS OF THREE *Syntermes* SPECIES (FIGURES ARE EXPRESSED AS MEANS ($\bar{X}$) AND STANDARD ERRORS (SE)).

Variables	*Syntermes grandis*	*Syntermes dirus*	*Syntermes molestus*
Soldier to worker	1:12.1a	1:9.7a	1:1.6a
ratio (1:x) in	1.7	0.8	0.08
foraging parties	14	11	16
$\bar{X}^a$			
SE			
N			
Frontal gland	2.39a	2.27a	1.05b
volume per soldier	0.36	0.03	0.17
(μl)[b]	5	5	5
$\bar{X}^a$			
SE			
N			
Frontal gland volume	0.022a	0.037b	0.122c
to head volume	0.002	0.007	0.1
ratio per soldier	5	5	5
$\bar{X}^a$			
SE			
N			
Biomass of soldiers	1624.9a	1390.5a	1277.6a
in foraging parties	49.8	114.3	161.6
of 100 workers (mg)	14	11	16
$\bar{X}^a$			
SE			
N			

[a]Ranked using Hartley's multiple range test. For each variable, any two means not followed by the same letter are significantly different at $P < 0.05$

[b]Frontal glands were dissected using a stereomicroscope and graticule. The shape of the gland was approximated to a tube (Coles, 1980) and the volume calculated using the equation $\pi r^2 l$ where l = length of the gland and r = the mean of 3 radius measurements.

molestus has a relatively large number of small soldiers protecting foraging workers, while *S. dirus* has a relatively small number of large soldiers. Biometric studies show that the total soldier biomass involved in foraging parties is approximately the same in both species (Table 2). It has also been shown that although the frontal gland volume per soldier is smaller, in absolute terms, in *S. molestus* than in *S. dirus* (Table 2), the total gland volume present in foraging parties is much larger in the former than in the latter (Figure 3). If one makes the reasonable assumption that an increase in gland volume is related to an increase in secretion, then *S. molestus* has more defense secretion in foraging parties than *S. dirus*.

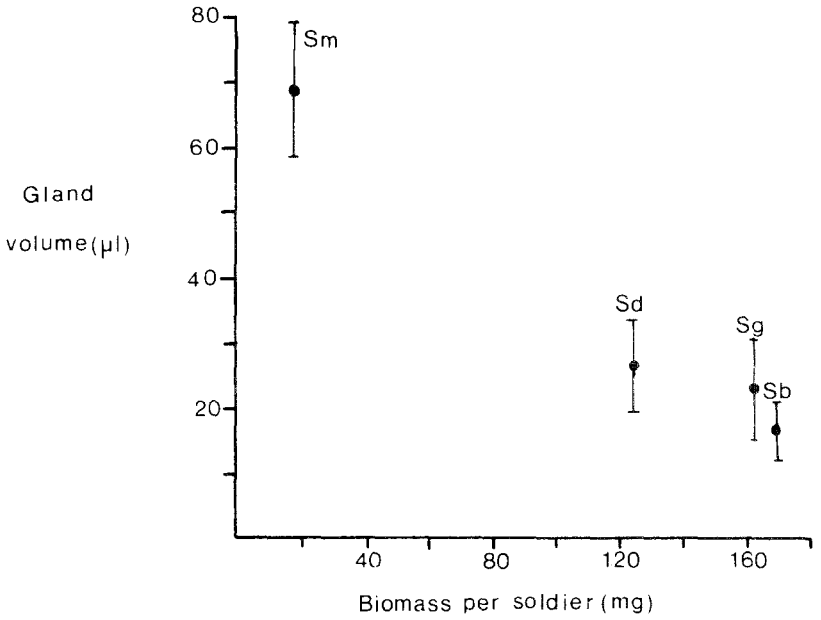


FIG. 3. Total frontal gland volumes (mean and *SD*) of soldiers protecting foraging parties of 100 workers.

The largest organs in termite soldier head capsules are the mandibular muscles and the frontal gland. If the frontal gland volume is expressed as a percentage of the head volume, *S. molestus* shows a much higher percentage gland volume than *S. dirus*. The corollary is that *S. dirus* shows a much higher mandibular muscle volume.

It would seem from these observations that in these two *Syntermes* species there is a difference in chemical and mandibular defense and that the size of the mandibular apparatus is balanced mutalis mutandis by the amount of chemical secretion.

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SOLDIER DEFENSE SECRETIONS OF *Trinervitermes bettonianus* (ISOPTERA, NASUTITERMITINAE): Chemical Variation in Allopatric Populations

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Abstract—The soldier defense secretions of major and minor soldiers of the East African nasute termite *Trinervitermes bettonianus* were analyzed chromatographically and spectrometrically, and the diterpene and monoterpene constituents were determined for three allopatric populations. Incipient colonies from one population raised on food from a second population produced their population-specific terpene pattern independent of food source. Secretion compositions are thus genetically rather than nutritionally determined, in agreement with biosynthetic studies in other nasutes. A minor volatile constitutent, previously proposed to be 3-ethyl-2-octanol was re-identified as (*R*)-2-decanol.

Key Words—Termite, *Trinervitermes bettonianus*, Isoptera, Nasutitermitinae, defense secretions, terpenes, diterpenes, allopatric populations, (*R*)-2-decanol, trinervitene.

INTRODUCTION

Nasute termite soldiers (Isoptera, Termitidae, Nasutitermitinae) eject an irritating, sticky defense secretion from a highly developed frontal gland. The secretions have been shown to possess a variety of monoterpene hydrocarbons as solvents (Prestwich, 1975) and a species-specific selection of novel tricyclic and tetracyclic diterpenoids as solutes (Prestwich, 1979a,b). Considerable progress has been made in the elucidation of the structures of the individual diterpenes (Prestwich, 1978, 1979; Prestwich et al., 1976, 1979, 1980; Vrkoč et al., 1978a,c), the overall composition, including intra- and interspecific

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variation of the secretions (Prestwich, 1977, 1978, 1979a,b,c), and the use of the secretions in defense (Eisner et al., 1976; Nutting et al., 1974). In this paper we present additional data on the secretion of the East African grass-feeding termite *Trinervitermes bettonianus* (Sjöstedt): (1) a nonisoprenoid alcohol previously alleged to be 3-ethyl-2-octanol (Gebreyesus, 1973; Prestwich, 1979a) is now shown to be (*R*)-2-decanol; (2) intraspecific variation among three allopatric populations of *T. bettonianus* is presented; and (3) the diterpenoid compositions from soldiers of laboratory-reared incipient colonies are shown to be independent of food source, but uniquely determined by the population from which the founding king and queen are selected.

METHODS AND MATERIALS

Collection Procedures. Intact *T. bettonianus* mounds were excavated in Ruiru, Machakos, and Narok, Kenya, and transported to the ICIPE laboratories in Nairobi for soldier collection. Major and minor soldiers were collected individually from the mounds and kept at 0° until the collection was completed. They were then decapitated, and the heads were transferred into a 25% ether–hexane solution and crushed. Prior experimentation had shown the secretion to be present only in the heads, so that decapitation was a convenient method of eliminating extraneous lipid-soluble materials. For analytical purposes, 100 major soldier and 200 minor soldier heads were crushed in separate 1.0-ml Microflex (Kontes) vials containing 0.4 ml each of solvent. These solutions were then analyzed by GLC and by coupled GLC–mass spectrometry (see below). For preparative purposes, the extracts of several thousand crushed heads (from the Ruiru colonies) were chromatographed on Florisil by gradient elution with ethyl acetate–benzene mixtures. Chromatographic details are discussed below.

To isolate a larger quantity of the C_{10} alcohol for structure determination, 4000 major soldiers from several nests were pooled, chilled, and macerated in distilled hexane. The crude extract was concentrated at 30°/20 mm and then evaporatively distilled at (75°/0.1 mm) using a Büchi kugelrohrofen. The volatile material, collected using a collector chilled to −78°, contained the monoterpene myrcene (2) and alcohol 4. The alcohol was purified by preparative GC (Varian Aerograph, 15% Carbowax, ¼ in. × 10 ft, 150°) to give 6 mg of pure 4.

Incipient Colonies. At the onset of the rainy season, male and female (de)alates attracted to light traps or excavated from preflight nests were allowed to form tandem pairs in plastic dishpans. Colonies were initiated using both Narok and Ruiru alates. Pairs were carefully transferred to sterilized reddish soil in Petri dishes, provided with cooled, boiled water and were held at 85% relative humidity, 26° C in the insectary at ICIPE. Egg-

laying occurred soon after colony establishment, the first larvae appeared within two weeks, the first workers after 6–8 weeks, and the first minor soldiers after 3–6 months. Dried grass collected from the Ruiru site was provided at weekly intervals to colonies in which workers had developed.

A total of four 6-month-old incipient colonies of the Ruiru population were sacrificed by freezing. The heads of the 7–12 minor soldiers present in each were crushed in hexane and the extracts were analyzed as described below. For the Narok incipient colonies, a total of five colonies were examined, four at 3 months and one at 6 months. The resulting GLC traces are shown in Figure 3.

Analytical Methods. GLC analyses (ICIPE) were performed on a Hewlett-Packard HP402 gas chromatograph equipped with a flame-ionization detector (FID) and glass columns. Figure 1 shows the GLC trace of the major and minor soldier secretions (Ruiru) using a 1 m × 3 mm ID column of 5% OV-1 on 80/100 Gas Chrom Q, temperature programmed from 80° to

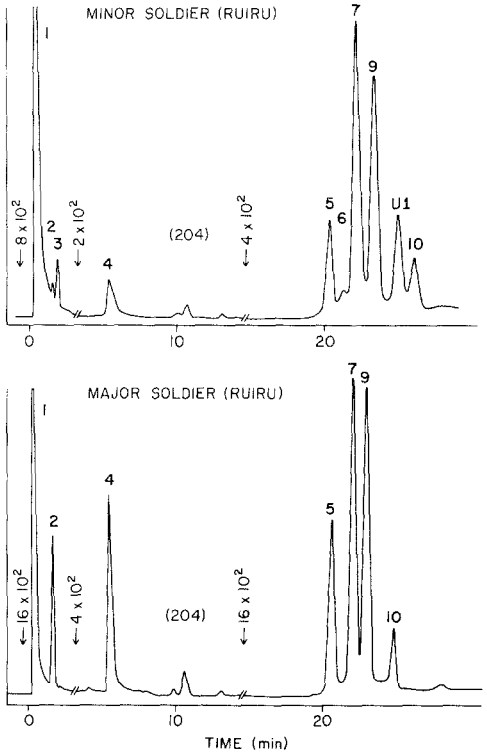


FIG. 1. Gas chromatograms of the major and minor soldier secretions from a Ruiru-type *T. bettonianus* mound (5% OV-1, 1 m × 3 mm ID, 80–240° at 5°/min).

240° at 5°/min after a 4-min delay. The identity of the monoterpene hydrocarbons was established by comparing retention times on a 1 m × 3 mm column of 5% Carbowax 20M on 80/100 Gas Chrom Q at 70° and mass spectra with those of authentic samples. GLC analyses at Stony Brook were performed on a Perkin-Elmer model 900 gas chromatograph equipped with FID and a glass column (2 m × 2 mm ID) packed with 3% OV-17 on 100/120 Gas Chrom Q or 3% FFAP on 100/120 Gas Chrom Q.

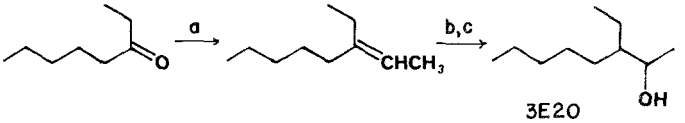
Gas chromatography–mass spectrometry (GC-MS) analyses were performed in Nairobi using a Finnigan 1015D quadrupole mass spectrometer (EI mode, 70 eV) equipped with a 2 m × 3 mm ID column of 5% OV-1 on 80/100 Gas Chrom Q, temperature programmed from 50° to 280° at 6°/min. Further GC-MS analyses were performed at Stony Brook using a Hewlett-Packard 5710A GC (3% OV-17) interfaced to an HP5980A mass spectrometer with an HP5933A data system.

The diterpenes were isolated by column chromatography of 630 mg of crude major soldier secretion (2000 crushed heads) by elution from 100–200 mesh Florisil with 10% to 75% ethyl acetate in benzene. (Note: We have substituted hexane for benzene in later work to avoid unnecessary exposure to a known carcinogen.) The order of elution (relative R_f on Merck Silica HF254 TLC with 15% ethyl acetate benzene) was 5 (0.47), U1 (minor soldiers only, 0.27), 7 (0.23), 10 (0.14), and 9 (0.07). Identities of 7, 8, and 9 were determined by chromatographic and spectral comparison (NMR, GC-MS, TLC) with authentic materials from *T. gratiosus*. Compounds 5 and 10 were identified spectrometically as described by Prestwich et al. (1976). Compound U1 from minor soldiers is a triacetoxytrinervitadiene of undetermined structure.

Nuclear magnetic resonance (NMR) spectra were determined using a Varian CFT-20 instrument operating at 20 MHz for carbon and 80 MHz for protons, using deuteriochloroform as the lock solvent. Chemical shifts are expressed in ppm downfield from δ(TMS) = 0.

Synthesis. The synthesis of 3-ethyl-2-octanol is illustrated in Scheme 1. Experimental details are given below.

A solution of dimsyl sodium in DMSO was prepared by warming 0.682 g (16.2 mmol) of sodium hydride (57% dispersion in oil) in 40 ml of dry DMSO (distilled from dimsylsodium-triphenylmethide) at 55° under N_2 for 1 hr



SCHEME 1. Synthesis of 3-ethyl-2-octanol. Reagents: (a) $(C_6H_5)_3P^+CH_2CH_3I^-$, $DMSO^-Na^+$, DMSO, 60°, 16 hr; (b) 9-BBN, THF, 3 hr, 25°; (c) H_2O_2, ^-OH, THF, 3 hr, 80°.

(Greenwald et al., 1963). To this solution was added 8.89 g (23 mmol) of freshly recrystallized ethyl triphenylphosphonium bromide, and the resulting orange ylid solution was stirred 1 hr at 25°. A solution of 0.822 g (8.01 mmol) of 3-octanone in DMSO was added and the mixture warmed at 65° for 16 hr. The mixture was cooled, poured into water, and the desired olefin was extracted with pentane. The pentane solution was washed (H_2O), dried ($MgSO_4$), filtered thru Florisil, and concentrated by distillation. The crude 3-methyl-2-octene showed a broad quartet for the vinyl proton (5.2 ppm) and broad singlet for the vinyl methyl (1.6 ppm).

To a solution of the crude olefin in 25 ml dry tetrahydrofuran (distilled from benzophenone sodium ketyl) was added via syringe 24 ml of a 0.5 M solution of 9-borabicyclononane (9-BBN, 12 mmol) in THF (Brown et al., 1976), and the reaction mixture was stirred 2 hr at 25° C. Hydrogen peroxide (5 ml of 30% solution) and 3 M sodium hydroxide (5 ml) was added and the mixture refluxed 1 hr. Aqueous workup and ether extraction afforded 1.01 g (79%) of 3-ethyl-2-octanol after short-path distillation (70°/0.5 mm).

GC-MS analysis (3% OV-17, 80–150° at 4°/min) showed a single slightly asymmetric peak (both diastereomers) with m/z 157 ($M^+ - 1$, 0.3), 143 ($M^+ - 15$, 5.1), 140 ($M^+ - 18$, 1.4), 125 ($M^+ - 18$–15, 1.7), 112 ($M^+ - 46$, 100), 45 (62.7). This material differed in both retention time (*rt* 5.51 min) and mass spectrum from the authentic termite alcohol 4 (*rt* 6.37 min), which had mass spectrum, m/z 157 (0.4), 143 (4.2), 140 (2.9), 125 (0.9), 112 (10.5), 45 (100). The termite alcohol 4 was identical in retention time (OV-17, FFAP) and mass spectral fragmentation to authentic 2-decanol (Aldrich).

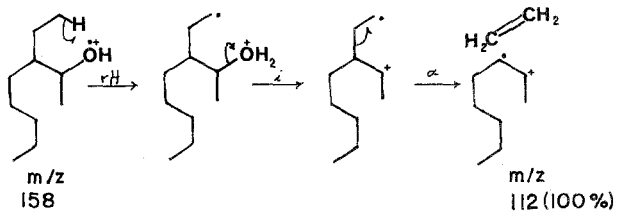
The (+)-α-methoxy-α-trifluoromethylphenylacetate esters of racemic commercial 2-decanol and termite 2-decanol were prepared by a modification of the procedure of Plummer et al. (1976), in which the new direct esterification technique of Neises and Steglich (1978) was employed. Thus, to a stirred, 0° solution of 35 mg (0.23 mmol) of 2-decanol in 2 ml of dry CH_2Cl_2 containing 4 mg (0.033 mmol) 4-dimethylaminopyridine (DMAP) and 63 mg (0.26 mmol) of (*R*)(+)-α-methoxy-α-trifluoromethylphenylacetic acid (Aldrich) was added 76 mg (0.37 mmol) of dicyclohexylcarbodiimide. The ice bath was removed and the mixture stirred 3 hr at 25° C under N_2. The cloudy solution (dicyclohexylurea precipitates) was diluted with hexane, extracted with 2 N HCl, and filtered through a short pad of Florisil. Removal of solvent gave 6.73 mg of the TLC- and GLC-homogeneous (+)-MTPA esters of (*R,S*)-2-decanol. A similar procedure was followed to obtain the (+)-MTPA ester of 4, which was indistinguishable (TLC, GLC) from the unresolved diastereomeric mixture prepared above. [^{1}H]-NMR spectra were obtained using Eu(fod)$_3$ to separate the already partially resolved methoxy signals in the two diastereomeric esters of the racemic alcohol (Plummer et al., 1976; Yamaguchi et al., 1976).

RESULTS AND DISCUSSION

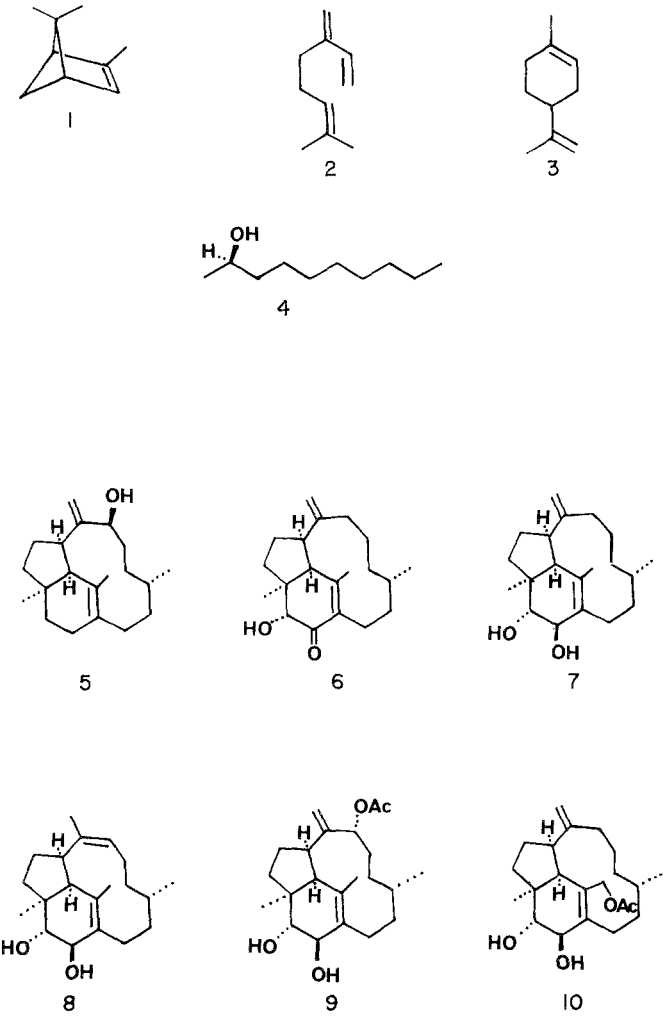
An unpublished synthesis (B. Chong and J. Meinwald) was cited (Prestwich, 1979a; Gebreyesus, 1973) in support of the structure of 3-ethyl-2-octanol. Sequential alkylation of ethyl acetoacetate with bromoethane and then with 1-tosyloxypentane gave a keto ester which was saponified, decarboxylated, and reduced to give a low yield of 3-ethyl-2-octanol claimed to be coincident on GLC with the *T. bettonianus* alcohol. Unfortunately, neither a sample nor the spectra of this compound could be found; a new synthesis was undertaken to determine the relative and absolute stereochemistry of the two asymmetric centers of 3-ethyl-2-octanol.

The synthesis of this alcohol is shown in Scheme 1. Wittig olefination of 3-octanone with ethylidenetriphenylphosphorane in DMSO (Greenwald et al., 1963) gave a mixture of (*E*)- and (*Z*)-3-methyl-2-octene which was not purified but used directly in the next step. Hydroboration of the olefin mixture with 9-BBN (Brown et al., 1976), followed by basic peroxide treatment, generated a mixture of diastereomeric 3-ethyl-2-octanols which were poorly resolved by GLC. However, neither diastereomer had the same retention time as the natural alcohol. The mass spectrum of the 3-ethyl-2-octanol showed a base peak at *m/z* 112, which suggested a hydrogen atom rearrangement followed by loss of water and ethylene as shown in Scheme 2 (McLafferty, 1973). The termite alcohol 4 showed a base peak at *m/z* 45, and exhibited only 20% relative intensity for *m/z* 112.

The identity of the termite alcohol 4 with 2-decanol was established by comparison (GLC, NMR, MS) with an authentic sample (Aldrich). However, we had insufficient natural material to determine an optical rotation for 4. Examination of the Eu(fod)$_3$-shifted NMR spectra of the (*R*)-(+)-MTPA esters of racemic 2-decanol showed two methoxyl signals moving at different rates as shift reagent was added. The empirical correlations of Yamaguchi et al. (1976) lead us to assign the (2*R*) configuration to the more rapidly shifting methoxyl signal. Comparison to the (*R*)-(+)-MTPA ester of 4 under analogous conditions leads to the assignment of (2*R*) as the absolute configuration of the natural alcohol. Within our limits of detection (ca. 2%),



SCHEME 2. Mass spectral fragmentation of 3-ethyl-2-octanol.



SCHEME 3. Chemical constituents of *Trinervitermes bettonianus* frontal gland secretions.

none of the (2*S*) enantiomer was present. This is the first report of (*R*)-2-decanol from an insect source, although the odd-carbon 2-alkanols (C_7–C_{17}) of undetermined absolute configuration have been found as alarm substances in the cephalic secretions of stingless bees (*Trigona*) (Luby et al., 1973).

The major components identified in the *T. bettonianus* soldier secretions are shown in Scheme 3. The monoterpenes serve as the solvents for the trinervitane diterpenes (Prestwich, 1979b) and may function as alarm substances, irritants, and toxicants (Eisner et al., 1976; Prestwich, 1979a;

Vrkoč et al., 1978b). The physicochemical nature of the gluey secretion is such that it effectively wets the lipophilic cuticle of an attacking ant and thus remains sticky for extended periods. The high concentration of diterpenes 80–90%) retards the evaporation of the volatile monoterpenes (Eisner et al., 1976; Prestwich, 1979a). 2-Decanol is intriguing as the only nonterpenoid constitutent of the secretion. It could be functioning as a cosolvent or as an alarm pheromone, but no further biological data are available on its role in the secretion. The secretion also contains traces of sesquiterpene hydrocarbons, on the basis of TLC mobility and strong *m/e* 204 parent peaks in the mass spectra.

The distribution of the trinervitane diterpenes in the major and minor soldiers of *T. bettonianus* is constant in a given population but different in geographically isolated populations. Figure 2 summarizes our analyses of 17 *T. bettonianus* mounds in three noncontiguous populations in Ruiru, Machakos, and Narok, Kenya. The populations appear to be isolated by

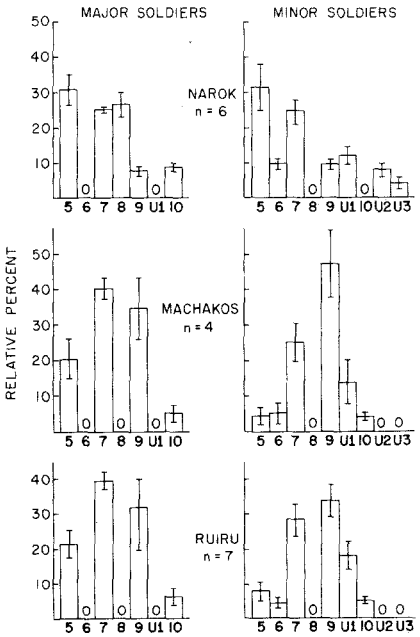


FIG. 2. Allopatric variation of trinervitene composition in soldier defense secretions in *Trinervitermes bettonianus*. Horizontal axes indicate order of GLC elution (3% OV-17 2 m × 3 mm, 200–250° at 5°/min). Structures corresponding to the numbers are given in Scheme 3. Compounds U1–U3 are uncharacterized di- and triacetoxytrinervitadienes. The vertical scales indicate mean relative percents of each component as determined from the integrated GLC traces. Sample sizes are shown for each location, and error bars indicate $\pm 1\sigma$.

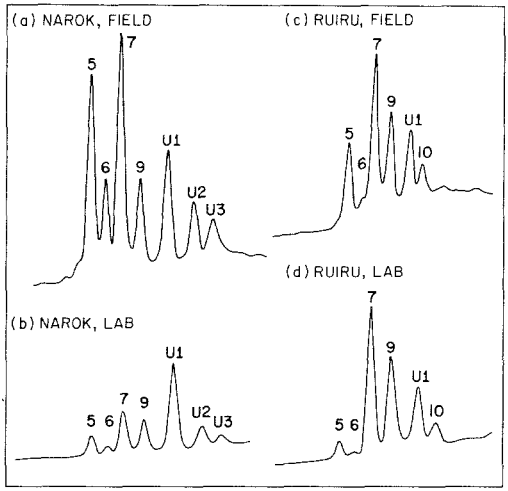


FIG. 3. Comparison of laboratory-raised and field-collected *T. bettonianus* minor soldier secretions by GLC: (a) field-collected, Narok-type; (b) incipient colony reared on Ruiru food from field-collected, Narok-type alates; (c) field-collected, Ruiru-type; (d) incipient colony reared on Ruiru food from field-collected, Ruiru-type alates.

geological features, i.e., a mountain range (Machakos Hills) between Ruiru and Machakos populations and the Eastern Rift Valley separating the Narok population from the other two. Genetic drift in these isolates has apparently resulted in minor alterations in the biosynthetic enzyme complex. We have already demonstrated this effect for three allopatric populations of *T. gratiosus* (Prestwich, 1978).

In addition to different relative ratios of components, several striking additions and omissions are evident in the *T. bettonianus* secretions. The presence of the double-bond isomer 8 in the Narok major soldier contrasts to the total absence of this compound in Ruiru and Machakos soldiers. In addition, Ruiru and Machakos minor soldiers completely lack two unidentified polyacetoxy trinervitenes (U2, U3) found in the Narok minor soldiers.

Evidence (Vrkoč, private communication) for the biosynthetic origin of the nasute diterpenes is provided by the incorporation of [2-^{14}C]-mevalonate into the diterpenes of *Nasutitermes rippertii* (cf. Prestwich et al., 1980). Further evidence for the genetic vs. environmental (e.g., diet) dependence of the secretion composition was obtained using incipient colonies from field collected alates of the Ruiru and Narok type *T. bettonianus*, both raised on dried grass collected in Ruiru. The results, illustrated in Figure 3, clearly demonstrate that even the first few minor soldiers produced by an incipient colony possess secretions reflecting their place of collection rather than the

origin of their food. This is best explained by differences among populations at the level of the biosynthetic enzymes which prepare this unusual assortment of diterpenes. Demonstration of the details of the biosynthetic sequences and the utility of chemical variation in addressing evolutionary trends in termites are subjects of ongoing research.

Acknowledgments—We thank the University Awards Committee/Joint Awards Council of State University of New York and the NSF (DEB-7823257) for partial financial support, scientists and staff at ICIPE (Nairobi, Kenya) for technical assistance during the early stages of this work, Mr. Stephen Spanton for NMR spectra, and Mr. P. Chang for mass spectra.

Note Added in Proof—Diterpene biosynthesis by nasute termite soldiers has now been conclusively established using an injection technique described in Prestwich, G.D., Jones, R.W., and Collins, M.S. 1981. Terpene biosynthesis by nasute termite soldiers (Isoptera: Nasutitermitinae). *Insect Biochem.* (in press).

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SYNTHESIS OF SEX PHEROMONE COMPONENTS OF THE FOREST TENT CATERPILLAR, *Malacosoma disstria* (HÜBNER) AND OF THE WESTERN TENT CATERPILLAR, *Malacosoma californicum* (PACKARD)

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Abstract—All four geometrical isomers of 5,7-dodecadien-1-ol have been stereoselectively synthesized by using Wittig condensation reactions. (5*Z*,7*E*)-5,7-Dodecadien-1-ol and its corresponding aldehyde are components of the sex pheromone of the forest tent caterpillar. (5*E*,7*Z*)-5,7-Dodecadienal is a component of the pheromone of the western tent caterpillar. These compounds have been successfully tested in the field.

Key Words—Synthesis, isomers, 5,7-dodecadienal, sex pheromone, *Malacosoma disstria*, *Malacosoma californicum*, Lepidoptera, Lasiocampidae.

INTRODUCTION

The forest tent caterpillar (*Malacosoma disstria* Hübner) and the western tent caterpillar (*M. californicum* Packard) produce sex pheromones whose principal components are 5,7-dodecadienals. *M. disstria* uses the (5*Z*,7*E*) isomer (Chisholm et al., 1980a) while *M. californicum* requires the (5*E*,7*Z*) form (Underhill et al., 1980). Doses (10 μg or less) of the synthetic pheromone components were effective as lures in attracting each species to field traps. Recently, Vu et al. (1980) found (5*Z*,7*E*)-5,7-dodecadien-1-ol in the pheromone of another lasiocampid moth *Dendrolimus spectabilis* Butler.

We report here the synthesis of all the four geometrical isomers of 5,7-dodecadien-1-ol and 5,7-dodecadienal.

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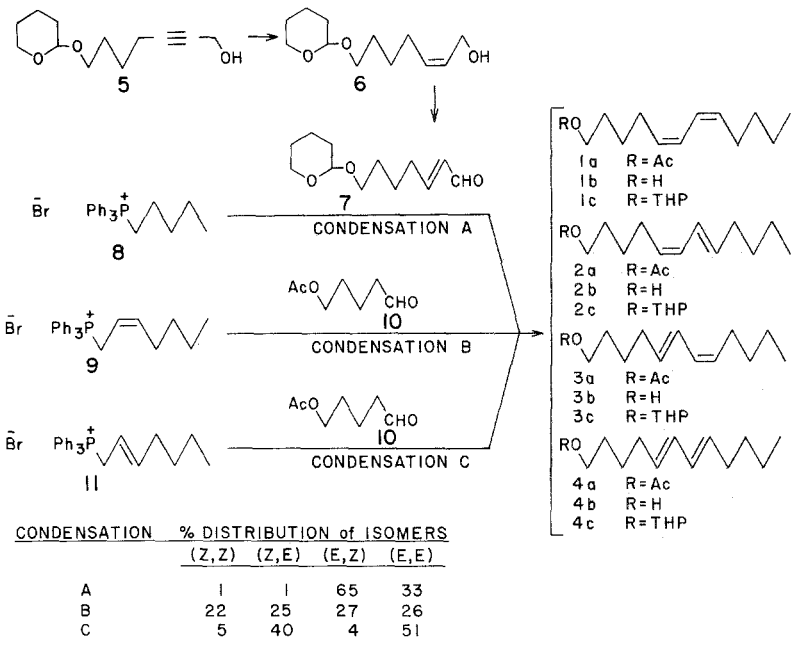

CONDENSATION	% DISTRIBUTION of ISOMERS (Z,Z)	(Z,E)	(E,Z)	(E,E)
A	1	1	65	33
B	22	25	27	26
C	5	40	4	51

FIG. 1. Synthesis route for all four geometrical isomers of 5,7-dodecadien-1-ol.

METHODS AND MATERIALS

All four geometrical isomers of 5,7-dodecadien-1-ol were obtained from three Wittig condensation reactions (Figure 1). Condensation A, pent-1-ylidene triphenylphosphorane (generated in ethyl ether by butyllithium from 8) and (*E*)-7-tetrahydropyranyloxy-2-heptenal (7) gave the four geometrical isomers 1c–4c in ratios 1:1:65:33. Condensation B, (*Z*)-2-heptene-1-ylidene triphenylphosphorane (generated from 9) and 5-acetoxypentanal (10) gave 1a–4a in about equal amounts. Condensation C, (*E*)-2-heptene-1-ylidene triphenylphosphorane (generated from 11) and 10 gave 1a–4a in ratios 5:40:4:51.

The mixed isomers from each of the three Wittig condensations were distilled after their acetate or THP groups had been removed. The geometrical isomers of the conjugated dienyl alcohols were resolved by elution from a 2.0 cm × 69 cm silver cation resin column by methanol at 20° (Houx et al., 1974). The products eluted from this column in the following order: (*E,E*), mixed (*Z,E*) and (*E,Z*), and (*Z,Z*); consequently, >98% isomerically pure 1b, 3b, and 4b were obtained. Mixed 2b (91%) and 3b (9%) (Figure I, condensation C) were quantitatively acetylated with hot Ac_2O/NaAc and the acetates (75 mg) separated on a 1.2 × 66 cm silver-loaded column (De Vries, 1963). The

column was wrapped with aluminum foil to exclude light and eluted with 70 ml of hexane, then by a series of hexane–benezene mixtures: 120 ml 5% benezene, 250 ml 17% benezene, 450 ml 19.5% benzene, 950 ml 20% benzene, 110 ml 23% benzene, 1390 ml 25% benzene, and 510 ml 50% benzene; total elution volume 5500 ml. Fraction 3720 to 4210 ml contained pure 2a (67 mg). Hydrolysis (methanolic KOH) gave 2b.

Acetylation of the dienyl alcohols with hot Ac_2O/NaAc gave quantitative yields of the corresponding acetates. Oxidation of the dienyl alcohols by pyridinium chlorochromate in CH_2Cl_2 produced the aldehydes (Corey and Suggs, 1975). Consistent IR and PMR spectra were obtained for all products. Mass spectra were obtained using a Finnigan 4000E GC-MS with a SP-2100 capillary column.

GC analysis was performed on a Hewlett-Packard gas chromatograph with flame-ionization detector equipped with the following columns: (1) 3mm OD × 1 m stainless steel packed with 5% SE-30 on AW DMCS Chrom W 80/100 mesh operated at 180° (used to monitor the general progress of the synthesis); (2) 3 mm OD × 4 m stainless steel packed with 3% Apiezon L on Supelcoport 80/100 mesh operated at 180° [geometrical isomers of conjugated dienyl acetates eluted from this column in the following order: mixed (*Z,E*) + (*E,Z*), (*Z,Z*), and (*E,E*)]; (3) 0.25 mm ID × 23 m Supelco SP-2100 glass capillary temperature programed 90–200° at 2° min (this column separated all four geometrical isomers of 5,7-dodecadienyl aldehydes, alcohols, and acetates, Table 1).

The starting compounds pentyltriphenylphosphonium bromide (8), (*Z*)-2-heptenyltriphenylphosphonium bromide (9), and (*E*)-2-heptenyltriphenylphosphonium bromide (11) were prepared by methods previously described (Chisholm et al., 1980b). 5-Acetoxypentanal was prepared by oxidation of 5-acetoxypentan-1-ol (Corey and Suggs, 1975).

7-Tetrahydropyranyloxy-2-heptyn-1-ol, (*5*). To a well-stirred solution of

TABLE 1. RELATIVE RETENTION TIMES OF GEOMETRICAL ISOMERS OF 5,7-DODECADIENYL ALDEHYDES, ALCOHOLS, AND ACETATES RELATIVE TO *n*-TRIDECYL ACETATE (1.000 = 33.2 min)[a]

Isomer	Aldehyde	Alcohol	Acetate
(Z,E)	0.549	0.721	0.876
(E,Z)	0.558	0.728	0.884
(Z,Z)	0.574	0.746	0.905
(E,E)	0.588	0.762	0.925

[a]On Supelco SP-2100 capillary column (0.25 mm × 23 m) temperature programed from 90° to 200° at 2°/min.

6-tetrahydropyranyloxy-1-hexyne (5.46 g, 30 mmol) in 75 ml ethyl ether at −10° was added butyllithium (34.5 mmol) in hexane. After 10 min tetrahydrofuran (33 ml) and paraformaldehyde (1.08 g, 36 mmol) were added, and stirring continued 45 min at 21° and 2.5 hr at reflux. The reaction mixture was cooled, poured into 90 ml of ice water, and the aqueous phase reextracted with 2 × 75 ml of ether and combined with the organic phase. This extract was washed with aqueous NH_4Cl, dried, and evaporated (Brandsma, 1971). Vacuum distillation (125–130°, 1 torr) gave 7-tetrahydropyranyloxy-2-heptyn-1-ol, (3.26 g, 15.4 mmol, 51.3%).

(Z)-7-Tetrahydropyranyloxy-2-hepten-1-ol, (6). Compound 5 (3.26 g, 15.4 mmol) was hydrogenated in ethanol (50 ml) over 40 mg of Lindlar catalyst (Lindlar, 1952). Upon complete disappearance of 5 the catalyst was removed and the solvent evaporated. The residue (3.2 g) was eluted from a silicic acid column (30 g) using hexane–benzene, 3:1. An unidentified contaminant (60 mg) emerged from the column first, followed by 6 (2.34 g, 10.9 mmol, 70.8%). Oxidation of 6 by pyridinium chlorochromate in CH_2Cl_2 gave *(E)*-7-tetrahydropyranyloxy-2-heptenal *7* (2.08 g, 9.8 mmol, 90%).

Wittig Condensation A; *(5E,7Z)-5,7-Dodecadien-1-ol* (3b). Butyllithium (12.5 mmol) in hexane was added to a well-stirred solution of 8 (4.13 g, 10 mmol) in 100 ml of dry ethyl ether. After 90 min at 25°, 7 (1.73 g, 8.2 mmol) was added dropwise over 5 min. After a final 80 min, water (40 ml) was stirred in until solution was attained. The ether phase was decanted and combined with the ether extract of the aqueous phase, then evaporated. The pentane-soluble part of the residue, a mixture composed primarily of 3c and 4c (1.72 g), was treated with paratoluenesulfonic acid in methanol (4 hr, 21°) to remove the THP group. The recovered alcohols were distilled (140–145°, 10 torr) affording 814 mg. Argentation chromatography gave 3b and 4b in 2:1 ratio.

Wittig Condensation B; *(5Z,7Z)-5,7-Dodecadien-1-ol* (1b). The procedure for 3b was used to couple 9 (10 mmol) and 10 (10 mmol). The pentane-soluble product contained dienyl acetate and alcohol in a 10:1 ratio by GC column 1. Column 2 showed mixed *(Z,E)* + *(E,Z)*, *(Z,Z)* and *(E,E)* in 2:1:1 ratio. The pentane was evaporated and the mixture of products distilled. The free alcohols (1.1 g, 6 mmol) recovered after hydrolysis of the acetates (methanolic KOH, 18 hr, 21°) were chromatographed on a silver cation resin column: 4b (216 mg, 1.20 mmol), 3b + 2b (426 mg, 2.34 mmol), and 1b (200 mg, 1.1 mmol) were recovered. GC analysis on column 3 showed 1b and 4b each >98% pure.

Wittig Condensation C; *(5Z,7E)-5,7-Dodecadien-1-ol* (2b). The procedure for 3b was used to couple 11 (10 mmol) and 10 (10 mmol). Alcohols recovered after argentation chromatography were: 4b (450 mg, 2.47 mmol); mixed 10:1 2b + 3b (378 mg, 2.08 mmol); 1b (36 mg, 0.20 mmol). Both 1b and 4b were 98% pure (GC column 3).

(*5E,7E*)-*5,7-Dodecadien-1-ol* (4b). Pure 4b was recovered by argentation chromatography from all three Wittig condensation reactions.

RESULTS

Mass spectra of 1a–4a showed virtually identical spectra with base peak *m/e* 79, M^+ 224, M—$[CH_3COOH]^+$ 164, and $[CH_3COOH]^+$ 60.

IR spectra (film) showed expected absorptions at 2950 (s), 1740 (s), 1360 (m), 1240 (S) and 1040 (M) cm^{-1}. The (*E,E*) isomers gave trans absorption near 988 cm^{-1}; and (*Z,E*) and (*E,Z*) isomer gave twin peaks near 950 and 980 cm^{-1}; the (*Z,Z*) isomer gave no absorption in the 900–1000 cm^{-1} region.

PMR spectra ($CDCl_3$) of all four acetate isomers follow. (*5Z,7Z*)-5,7-dodecadien-1-ol acetate, 1a, H-5 and H-8: δ5.46(m); H-6 and H-7: δ6.26(m). Analysis of the AA'BB' system after decoupling of H-4 and H-9 gave approximately $J_{5,6} = J_{7,8} = 11.0$ Hz (cis). $J_{6,7} = 13.0$ Hz, $J_{5,7}$ and $J_{6,8} = -1.3$ Hz, and $J_{5,8}$ 1.6 Hz. (*5Z,7E*)-5,7-Dodecadien-1-ol acetate, 2a, H-5: δ5.29 (d10.3, t7.4); H-6: δ5.97 (d10.3, d10.7); H-7: δ6.30 (d14.7, d10.7); H-8: δ5.69 (d14.7, t7.4). Some long-range couplings ($J < 1.5$ Hz) were also present. Decoupling of H-4 and H-9 transformed H-5 to a doublet, $J_{5,6} = 10.3$ Hz (cis); and H-8 to a doublet, $J_{7,8} = 14.7$ Hz (trans). Signals for H-6 and H-7 ($J_{6,7} = 10.7$ Hz) were little changed. (*5E,7Z*)-5,7-Dodecadien-1-ol acetate, 3a, H-5: δ5.60 (d14.7, t7.4); H-6: δ6.30 (d14.7, d10.7); H-7: δ5.91 (t10.7); H-8: δ5.29 (d10.7, t7.4). Some long-range couplings ($J < 1.5$ Hz) were also present. Decoupling of H-4 and H-9 transformed H-5 to a doublet, $J_{5,6} = 14.7$ Hz (trans); and H-8 to a doublet, $J_{7,8} = 10.7$ Hz (cis). Signals for H-6 and H-7 ($J_{6,7} = 10.7$ Hz) were little changed. (*5E,7E*)-5,7-Dodecadien-1-ol acetate, 4a, H-5 and H-8: δ5.59(m); H-6 and H-7: δ6.01(m). Analysis of this AA'BB" system (McCasland et al., 1968) after decoupling of H-4 and H-9 gave approximately $J_{5,6} = J_{7,8} = 14.9$ Hz (trans), $J_{6,7} = 10.0$ Hz, $J_{5,7}$ and $J_{6,8} = -0.5$ Hz, and $J_{5,8} = 0.6$ Hz.

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ALLELOCHEMICS AND NUTRITIONAL INDICES FOR LARCH SAWFLY, *Pristiphora erichsonii* (HARTIG): A Specialist Feeding on *Larix* spp.[1]

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Abstract—Nutritional indices for larch sawfly, *Pristiphora erichsonii* (Hartig), larvae fed single and tufted needles of four *Larix* spp. are reported. Larvae offered only single needles of *L. laricina*, *L. russica*, and *L. decidua* had lower relative growth rates than larvae fed tufted needles of the same species. There was no significant reduction in larval growth for larvae fed *L. kaempferi* single needles as compared to tufted needles. Abietic acid-treated foliage reduced consumption but did not lower relative growth rate. These findings are discussed with respect to the mechanism of preferential feeding of the larch sawfly and current hypotheses of host plant herbivore interaction.

Key Words—Larch sawfly, *Pristiphora erichsonii*, Hymenoptera, Tenthredinidae, *Larix laricina*, *Larix russica*, *Larix decidua*, *Larix kaempferi*, nutritional indices, feeding behavior, abietic acid, antifeedant allelochemics.

INTRODUCTION

Recent research has been directed at the role of allelochemicals in host plant resistance and host range determination (Beck, 1965; Chapman, 1974; Dethier, 1970; Rhoades and Cates, 1976). In most cases the presence of growth-reducing compounds renders a plant as a nonhost. Seasonal increase

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in allelochemical concentration renders the host increasingly unsuitable. Feeny (1976) suggested that "apparent" plants (vulnerable to discovery) are protected against herbivores by "quantitative" factors. Quantitative factors include: tough leaves, low leaf nutrients, and relatively large amounts of nonspecific chemicals such as tannins. He suggested that younger foliage of an apparent plant (oak trees) tended to be lower in quantitative defense factors. Rhoades and Cates (1976) proposed that within a given plant, mature tissue (apparent) would have a relatively greater amount of quantitative defense factors than younger tissue (unapparent). Our research (Wagner et al., 1979) does not entirely support this hypothesis. We report here our continuing research on the role of allelochemics in the larval feeding behavior of the monophagous larch sawfly, *Pristiphora erichsonii* (Hartig) (Hymenoptera: Tenthredinidae).

P. erichsonii is holarctic in distribution and a major defoliator of *Larix* spp. In North America its main host is tamarack, *Larix laricina* (DuRoi) K. Koch. *L. laricina* is a deciduous conifer that drops its needles each fall. Two morphological needle types exist: needles on current year's long shoots occur singly, whereas needles on 1-year-old or older short shoot stems occur in tufts (Figure 1). Larvae eclose from eggs laid in current season's growth by

FIG. 1. Single and tufted needles of tamarack.

parthenogenetic females, feed briefly on the single needles, then move to tufted needles where they feed and complete development. The basis for this preferential behavior is a group of resin acid compounds present in high concentration in the single needles (Ohigashi et al., 1980).

In addition to the immediate behavioral effect of these chemicals, our evidence suggests a more subtle chronic impact on larval growth and survival. We report on the extent to which these materials, that occur in natural single needles, affect parameters or larval growth.

METHODS AND MATERIALS

Larch sawfly larvae were collected from the field in Waupaca, Portage, and Langlade Counties, Wisconsin, and maintained in our laboratory according to methods described by Wagner (1977). Recently molted fifth instar larvae were used in all experiments. Larch sawfly exhibits 96–98% thelyotoky; consequently, the variability of sex is reduced. Foliage that was not to receive allelochemic treatment was collected from University of Wisconsin Arboretum—Madison, Dane County, Wisconsin. Foliage that was to receive allelochemic treatment was collected primarily from Columbia County, Wisconsin. All foliage was held for 20–24 hr at 100% relative humidity. Next, an aliquot of foliage for both studies was weighed, oven dried, and percent moisture determined.

A single twig was weighed and the stem placed through a hole in a medicine vial stopper that was capped over a vial containing distilled water. Twigs were of two types: (1) bearing single needles or (2) tufted needles (needles in tufts). The foliage and vial were placed in a 7 × 22-cm covered plastic box with water-soaked paper towels to maintain high relative humidity (Figure 2). One larva was placed on the needle-bearing twig and kept at 22°C under continuous light for 72 hr.

Biomass ingested, feces, and biomass gained, were determined gravimetrically on a dry weight basis (Sartorius 2432; accuracy 0.1 mg). Larval moisture content was determined independently by oven drying an aliquot of refreshly molted 5th instar larvae. The initial dry weights of foliage and larvae were estimated by multiplying the fresh weight by mean percent dry weight. Foliage not consumed, feces, and larvae at the end of the experiment were oven dried at 100°C to determine final dry weight. Total organic nitrogen (% dry weight) of foliage was determined by the micro-Kjeldahl technique (McKenzie and Wallace, 1954).

Foliage was treated with abietic acid (Pfaltz & Bauer Inc.) and crude foliage extracts according to methods described by Wagner et al. (1979). The solvent methanol (MeOH) was completely evaporated before the foliage was placed in the plastic cage.

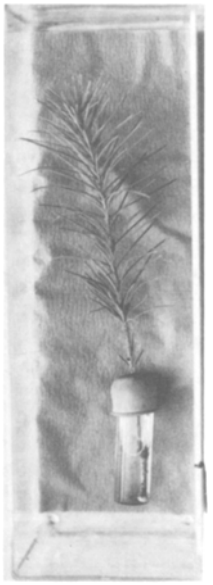

FIG. 2. Plastic box containing vial and foliage for feeding experiment.

Data was analyzed using one-way analysis of variance with Student Newman-Keuls multiple range test and Student's *t* test for unpaired values.

Nutritional indices calculated Waldbauer (1968):

$$\text{ACR: absolute consumptive rate} = \frac{\text{biomass ingested}}{\text{feeding duration}}$$

$$\text{RCR: relative consumptive rate} = \frac{\text{biomass ingested}}{\text{feeding duration} \times \text{mean larval wt}}$$

$$\text{RGR: relative growth rate} = \frac{\text{biomass accumulated}}{\text{feeding duration} \times \text{mean larval wt}}$$

$$\text{AD: approximate digestibility (\%)} = \frac{\text{biomass ingested - feces}}{\text{biomass ingested}} \times 100$$

ECD: efficiency of conversion of digested food to body substance (%) =

$$\frac{\text{biomass accumulated}}{\text{biomass ingested - feces}} \times 100$$

ECI: efficiency of conversion of ingested food to body substance (%) =

$$\frac{\text{biomass accumulated}}{\text{biomass ingested}} \times 100$$

$RGR = RCR \times ECI$

$ECI = AD \times ECD$

Units of measure: weight, mg; duration, days.

RESULTS AND DISCUSSION

Larch sawfly larvae offered only single needles of *L. laricina* had significantly lower larval growth (RGR) than those offered foliage from tufts (Table 1). Absolute consumptive rate is significantly lower in the case of single needles, which is consistent with the behavioral preference for tufted needles in nature. However, no significant difference in RCR was observed, which suggests reduced growth is not a result of lower consumption. The digestibility (AD) of single needles was higher than for tufted needles. It seems logical to conclude that either ECD or ECI is overriding the effects of greater digestibility. ECI is the product of AD and ECD; considering the higher AD for single needles, it would appear that lower ECD for larvae fed single needles was responsible for reduced larval growth.

Larvae fed *Larix russica* (Endl.) Sabine × Trautretter (Table 2) responded in precisely the same manner as those fed on *L. laricina*. The lower

TABLE 1. NUTRITIONAL INDICES FOR FIFTH INSTAR *P. erichsonii* FED TUFTED AND SINGLE NEEDLES OF TAMARACK, *Larix laricina*

Foliage (replicates)	ACR	RCR	RGR	AD	ECD	ECI	Moisture (%)
Tufted (10)	44.16	3.55	0.35	37.87	33.10	10.14	62.83
Single (9)	27.14	3.37	0.19	52.14	12.28	5.53	67.66
t value[a]	3.12	0.47	3.84	2.13	2.57	3.78	13.26
	***	NS	***	**	**	***	***

[a]Student's *t* test for unpaired values (NS, not significant; *, significant 0.10; **, significant 0.05; ***, significant 0.01). Designations apply for all following tables.

TABLE 2. NUTRITIONAL INDICES FOR FIFTH INSTAR *P. erichsonii* FED TUFTED AND SINGLE NEEDLES OF SIBERIAN LARCH, *Larix russica*

Foliage (replicates)	ACR	RCR	RGR	AD	ECD	ECI	Moisture (%)
Tufted (6)	37.60	2.62	0.40	34.12	53.97	15.50	63.57
Single (9)	23.99	2.47	0.26	45.47	26.14	11.31	68.56
t value	3.07	0.40	3.80	2.11	2.32	1.84	6.54
	***	NS	**	**	**	*	***

RGR is most likely the result of reduced efficiency of conversion of digested food (ECD); this reflects higher metabolic costs for larvae fed single needles.

Larix decidua Mill. fed larvae responded similarly to those fed *L. laricina* and *L. russica* with one major exception (Table 3). Larvae prefer tufted needles (higher ACR), but in relative terms they consumed more single needles (higher RCR for single needles). This might have been an attempt by larvae to compensate for lower food quality by increasing consumption. As in the cases of *L. laricina* and *L. russica*, the lower ECD was probably responsible for lower RGR of larvae fed single needles.

Nutritional indices for *P. erichsonii* larvae fed on *Larix kaempferi* (Lamb) Carr were somewhat in contrast to the other host species tested (Table 4). In this case RGR was possibly higher for single needle fed larvae. The level of significant was, however, very low (0.10). The ECD and ECI were significantly lower for larvae fed single needles, as is the case for all other host species tested. Here again the larvae may have been compensating for lower food value by consuming greater amounts of foliage.

Nutritional value of single needles versus tufted foliage, measured in terms of total organic nitrogen, was considered as a possible explanation for reduced growth rates. Nitrogen quantities present in the four *Larix* spp. are listed in Table 5. Tree species that produced higher RGR for *P. erichsonii*

TABLE 3. NUTRITIONAL INDICES FOR FIFTH INSTAR *P. erichsonii* FED TUFTED AND SINGLE NEEDLES OF EUROPEAN LARCH, *Larix decidua*

Foliage (replicates)	ACR	RCR	RGR	AD	ECD	ECI	Moisture (%)
Tufted (9)	46.15	2.91	0.39	30.74	55.17	13.65	57.68
Single (9)	35.71	3.39	0.26	35.22	26.45	7.64	59.07
t value	2.25	2.27	6.64	0.75	2.31	6.58	3.04
	**	**	***	NS	**	***	**

TABLE 4. NUTRITIONAL INDICES FOR FIFTH INSTAR *P. erichsonii* FED TUFTED AND SINGLE NFEDLES OF JAPANESE LARCH, *L. kaempferi*

Foliage (replicates)	ACR	RCR	RGR	AD	ECD	ECI	Moisture (%)
Tufted (8)	25.24	2.07	0.33	24.58	76.81	16.34	61.47
Single (7)	48.96	3.36	0.39	34.57	37.77	11.77	65.33
t value	4.02	4.80	2.06	2.12	2.63	3.84	7.48
	***	***	*	**	**	**	***

larvae fed on tufted foliage (*L. laricina, L. russica, L. decidua*) did not have a consistently higher nitrogen content. Therefore, nitrogen did not appear to be the limiting factor. Also, tissue water content is higher for single than tufted needles and is unlikely to be responsible for the reduced ECD values.

We established previously by a feeding preference bioassay that resin acids present in high concentrations in single needles significantly influence the feeding behavior (Wagner et al., 1979, Ohigashi et al., 1980). We theorized that resin acids also may be involved in decreasing the RGR and ECD. To test this we topically treated tufted *L. laricina* foliage with varying concentrations of abietic acid (one of four resin acids involved) and a crude MeOH extract of single needles. Treatment tests on larval growth and feeding efficiency were compared to the effects on larvae fed unaltered single and tufted foliage. The results (Table 6) show that both AGR and RCR for abietic acid-treated (15 mg/ml) tufted foliage are significantly lower than for untreated control and MeOH-treated foliage. This supports our previous results (Ohigashi et al., 1980), and suggests that the resin acid compound has a feeding-inhibitory effect. The success rate (surviving larvae consuming sufficient foliage enabling us to calculate nutritional indices) drops dramatically at a concentration above 3.75 mg/ml abietic acid. There is a slight reduction in RGR with

TABLE 5. PERCENT TOTAL ORGANIC NITROGEN CONTENT OF *Larix* SPP.

Species	Needle type	Nitrogen (%)	*t* value
Larix laricina	single	1.16	
	tufted	1.46	5.01 **
L. russica	single	2.85	
	tufted	2.75	1.13 NS
L. decidua	single	1.62	
	tufted	2.08	4.21 **
L. kaempferi	single	1.91	
	tufted	2.10	4.49 **

TABLE 6. NUTRITIONAL INDICES FOR ULTIMATE INSTAR *Pristiphora erichsonii* LARVAE FED TOPICALLY TREATED FOLIAGE OF *Larix laricina*

Foliage and treatment	Concentration	% Success[a]	ACR[b]	RCR	RGR	AD	ECD	ECI
Untreated tufted Foliage		100	44.16 a	3.55 ad	0.35 a	37.87 a	33.10 ab	10.14 bfg
MeOH		50	37.18 ac	3.47 acd	0.28 ab	31.33 a	30.53 ab	8.59 bfg
Abietic Acid +MeOH	1.875 mg/ml	100	36.36 abc	2.78 abcd	0.35 a	25.26 a	57.16 a	12.77 abcdefg
Abietic Acid +MeOH	3.75 mg/ml	50	28.43 abc	2.08 b	0.31 ab	21.71 a	70.82 a	16.77 acdefg
Abietic Acid +MeOH	7.5 mg/ml	40	34.27 abc	2.93 abcd	0.31 ab	25.64 a	45.04 ab	11.26 abcdefg
Abietic Acid +MeOH	15 mg/ml	50	21.74 b	2.11 b	0.31 ab	26.14 a	59.85 a	14.77 abcdefg
Single needle Crude extract	0.25 g N/ml	30	30.71 abc	2.35 bcd	0.37 a	28.95 a	57.03 a	16.15 defg
Untreated single needles		90	27.14 bc	3.37 abcd	0.19 b	52.41 b	12.28 b	6.20 b

[a]Success rate = surviving larvae that consumed sufficient foliage to calculated nutritional indices.
[b]Values followed by different letters statistically significant 0.05 level, SNK multiple range test.

increasing concentration of abietic acid, but this is not statistically significant. Consequently, abietic acid is probably not entirely responsible for the reduced growth we observe in larvae fed single needles. Approximate digestibility is not influenced by increasing concentration of abietic acid. ECD is significantly higher on abietic acid-treated foliage (1.875, 3.75, and 15 mg/ml) than on the untreated single needles. Also ECI is significantly higher for untreated tufted foliage and abietic acid-treated foliage (1.875, 3.75, 7.5, and 15 mg/ml) than for untreated single needles.

The relationship observed between moisture content and efficiency of conversion indices (ECI and ECD) for the larch sawfly is interesting in light of recent reports by Feeny (1975), Scriber (1977), and Reese and Beck (1978). Working with Lepidoptera, these researchers reported a positive correlation between moisture content and efficiency of growth. Scriber (1978, 1979) suggested that quantitative plant defense mechanisms (e.g., low leaf moisture content) may be metabolically more costly for insects to overcome than qualitative factors (e.g., allelochemics). For *P. erichsonii* feeding on *Larix laricina*, in spite of higher moisture content of single needles, larvae prefer and have a higher growth rate when fed tufted needles. We reported earlier an approximately tenfold greater amount of feeding-inhibitory resin acids in single needles than tufted foliage. This suggests that for *P. erichsonii*, an allelochemic is a significant factor affecting larval feeding efficiency and growth and might be an important index of larval feeding efficiencies.

Topical treatment of tufted foliage with abietic acid resulted in feeding inhibition, but did not significantly affect larval growth. The reason larval growth was not affected seems to be the corresponding increase in ECI and ECD. Reese and Beck (1976a,b) incorporated resorcinol and *p*-benzoquinone into an artificial diet of the black cutworm, *Agrotis ipsilon* (Hufnagel), and noted an increase in ECD; ECI remained the same. With both resorcinol- and *p*-benzoquinone-supplemented diets larval growth rate was reduced. Elliger et al. (1976) reported a significant growth reduction of the pine bollworm, *Pectinophora gossypiella* (Saunders), when larvae were fed on an artificial diet containing abietic acid. The reason growth rate was not reduced for *P. erichsonii* larvae fed high concentrations of abietic acid-treated foliage may be an anomaly attributed to the low success rate (see Table 6 footnote). It also may be that successful larvae were able to utilize the smaller amount of foliage consumed more efficiently. A third possible explanation could be that abietic acid actually has nutritional value for the larvae. Those larvae that became habituated to abietic acid could feed for a shorter duration and still maintain larval growth. This hypothesis is especially interesting since the structure of resin acid resembles that of common steroids. Elliger et al. (1976) suggested that the mechanism of resin acid action is antagonism between resin acid and steroids.

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CUTICULAR HYDROCARBONS OF ADULTS OF THE COWPEA WEEVIL, *Callosobruchus maculatus*[1,2]

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Abstract—The composition of the cuticular hydrocarbons of the cowpea weevil, *Callosobruchus maculatus* (F.), was determined by using combined gas chromatography–mass spectrometry. The hydrocarbons constituted 88% of the cuticular lipids and were composed of four homologous series of alkanes. Mono- and dimethyl branched-chain alkanes made up 83% of the hydrocarbon fraction.

Key Words—Hydrocarbons, *n*-alkanes, branched-chain alkanes, GC-MS, cuticular wax, insect, cowpea weevil, *Callosobruchus maculatus*, Coleoptera, Curculionidae.

INTRODUCTION

The composition of the epicuticular lipid layer in two holometabolous stored-product coleopterans, the black carpet beetle, *Attagenus megatoma* (F.), and the cigarette beetle, *Lasioderma serricorne* (F.), was found to change significantly in the transformation from the larval to the adult stage (Baker et al., 1979a,b). Qualitative and quantitative changes were especially noted in the hydrocarbon fractions. In both species, long-chain *n*-alkanes were predominant in the larval stages. In adults of *A. megatoma*, a homologous series of five alkenes was identified. 9-Pentacosene was the most abundant hydro-

[1]Coleoptera: Bruchidae.
[2]Mention of a proprietary product or company name in this paper does not constitute a recommendation of this product by the U.S. Department of Agriculture.

carbon and constituted 30.6% of the total hydrocarbon fraction. Branched-chain alkanes made up less than 2% of the total hydrocarbon fraction. In contrast, no alkenes were found in adults of *L. serricorne*, and branched-chain alkanes made up 83% of the total cuticular hydrocarbons. In this paper we report on the cuticular hydrocarbon composition of the adults of another coleopteran, the cowpea weevil, *Callosobruchus maculatus* (F.). *C. maculatus* is also a major pest of stored products and is found primarily in whole legume seeds.

METHODS AND MATERIALS

Extraction of Cuticular Lipids. Cultures of *C. maculatus* were maintained on blackeye peas held at 27° C and 60% relative humidity under a 12-hr photophase. Adults of both sexes (3–10 days old) were aspirated from the cultures and held at −20° C for 1 hr. The insects were arranged into replicates weighing 16.01 g and containing about 3020 adults each. The cuticular lipids were extracted with four 2-min rinses of hexane. The solvent was removed from the combined filtrates in tared flasks on a rotoevaporator, and the weight of the cuticular wax was determined. In later studies, the hydrocarbon composition of male and female *C. maculatus* adults was compared. Newly emerged (0–24 hr old) adults were sexed by the method of Utida (1972).

Isolation of Hydrocarbon Fraction. The cuticular lipid fraction was taken up in hexane and applied to a column of Florisil® (deactivated by adding H_2O, 7% v/w). The hydrocarbons were eluted with hexane (Carroll, 1961). The purity of the hydrocarbon fraction was checked by TLC analysis on silica gel by using a solvent system of hexane–diethyl ether–acetic acid (80:20:1, v/v) and by comparison with standard neutral lipids. The components were made visible with rhodamine 6 G in ethanol.

The presence of alkenes was checked by TLC on $AgNO_3$-treated silica gel plates and by bromination in carbon tetrachloride followed by GLC analysis. Branched-chain alkanes were determined by an overnight treatment of a 2,2,4-trimethylpentane solution of the hydrocarbon fraction with Linde molecular sieve 5A pellets and subsequent GLC analysis (O'Connor et al., 1962).

GLC and Mass Spectrometry. Alkanes were identified by GLC analysis on a 1.82 m × 3.2 mm stainless-steel column containing 2.36% OV-101 on 80/100 mesh Gas Chrom Q. Temperature programs were from 190° to 320° at 2° or 4°/min. Equivalent chain lengths were determined by linear plots of retention time vs. carbon number for standard *n*-alkanes and comparison with the unknowns. Peak areas were determined with a Hewlett Packard model 3380A integrator system.

Mass spectra were obtained with a Varian series 200 GLC interfaced with

a MAT CH5-DF mass spectrometer via a Watson-Bieman separator. The glass column contained 3.5% OV-101 on 100/120 mesh Gas Chrom Q. The temperature was programed from 200° to 320° C at 2° /min. The mass spectra were interpreted as previously described (Nelson and Sukkestad, 1970, 1975; Nelson et al., 1972).

RESULTS AND DISCUSSION

The cuticular lipids extracted from adult *C. maculatus* constituted a mean of 0.21% of the fresh weight or 11 μg per adult. The hydrocarbon fraction made up a mean of 87.9% of the cuticular lipid or 9.7 μg per adult. This percentage of hydrocarbon in the total surface lipid extract was similar to that found in the cockroaches *Periplaneta japonica* Karay and *P. americana* (L.) (85–90%) (Jackson, 1970), but much greater than that found in the grasshoppers *Melanoplus sanguinipes* (F.) and *M. packardii* Scudder (60–68%) (Soliday et al., 1974), *L. serricorne* (57%) (Baker et al., 1979b), newly emerged adults of *Sarcophaga bullata* Parker (53%) (Jackson et al., 1974), the scorpion *Paruroctonus mesaensis* (28–33%) (Hadley and Jackson, 1977), and the house cricket, *Acheta domesticus* (L.) (6%) (Hutchins and Martin, 1968).

Four series of alkanes from *C. maculatus* were identified by the GC-MS procedures (Figure 1, Table 1): (1) *n*-alkanes; (2) internally branched monomethylalkanes; (3) terminally branched monomethylalkanes; and (4) internally branched dimethylalkanes. No alkenes were present. A total of 29 major GLC peaks were analyzed, resulting in the identification of about 68 components or positional isomers. No significant differences were found between the hydrocarbon profiles of males and females.

The alkanes had a bimodal distribution. The first group made up 94% of the total hydrocarbons and consisted of *n*-alkanes and branched alkanes of 27–35 carbon atoms. The major component was GC peak 29-BA′, which was 33% of the total alkanes and consisted of a mixture of 5,9-, 7,11-, and 9,13-dimethylnonacosanes plus 3-methylnonacosane. The second group represented 6% of the total hydrocarbon fraction and consisted of monomethyl and dimethylalkanes composed of 42–49 carbon atoms. The major component of this group was GC peak 43-B, 11,15- and 13,17-dimethyltritetracontanes.

n-Alkanes from C_{27} to C_{31} made up 17% of the total hydrocarbon fraction in *C. maculatus*. *n*-Nonacosane was the predominant *n*-alkane (7.8%) with lesser amounts of *n*-octacosane and *n*-heptacosane. Branched-chain alkanes made up 83% of the total hydrocarbon fraction. The A series consisted of internally branched monomethylalkanes. These monomethylalkanes have been found in all species of insects studied that have methyl-branched alkanes.

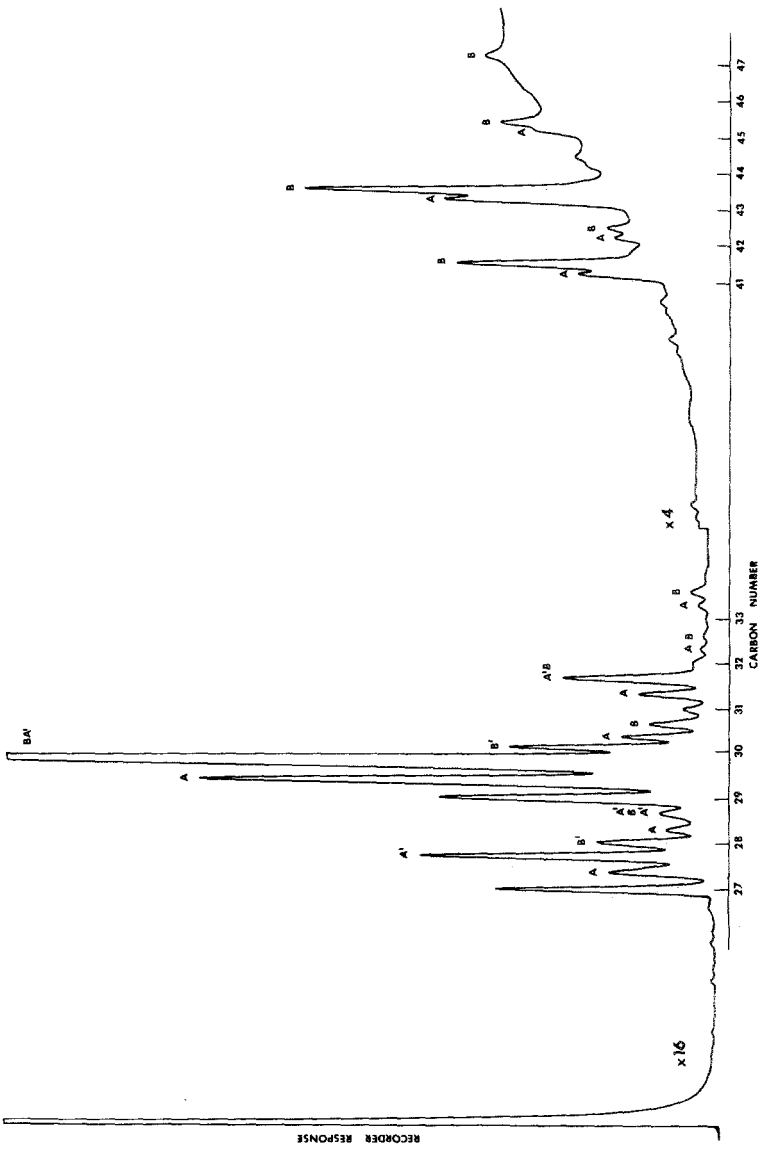

FIG. 1. Gas chromatogram of cuticular hydrocarbons of adults of *C. maculatus*. The sample was chromatographed on a 1.82 m × 3.2 mm stainless-steel column containing 2.36% OV-101 on 80/100 mesh Gas Chrom Q. Temperature program: 200–320° C at 2°/min.

TABLE 1. COMPOSITION OF CUTICULAR HYDROCARBONS ISOLATED FROM ADULTS OF COWPEA WEEVIL, *Callosobruchus maculatus*

GLC peak[a]	Components(s)[b]	Composition[d] ECL[c]	Composition[d] %	Amount per adult (ng)[e]
27	*n*-Heptacosane	27.0	5.2	500
27-A	5-, 7-, and 9-Methylheptacosanes	27.3	3.2	310
27-A′	3-Methylheptacosane	27.7	7.3	710
27B′ and 28	3,7- and 3,9-Dimethylheptacosanes and *n*-Octacosane	28.0	3.3	320
28-A	8-, 9-, 10-, 11-, 12-, 13-, and 14-Methyl octacosanes	28.3	1.3	120
28-A′BA′	4-Methyl-, 7,11-, 8,12-, and 9,13-dimethyl- and 3-Methyloctacosanes	28.7	1.7	160
29	*n*-Nonacosane	29.0	7.8	750
29-A	9-, 11-, 13-, and 15-Methylnonacosanes	29.4	17.3	1670
29-BA′	5,9- 7,11-, and 9-13-Dimethyl- and 3-methylnonacosanes	29.9	33.0	3190
29-B′	3,7-, 3,9-, 3,11-, and 3,13-Dimethylnonacosanes	30.1	4.4	420
30-A	12- and 14-Methyltriacontanes	30.4	1.9	180
30-B	6,10-, 8,12-, 9,13-, and 10,14-Dimethyl-triacontanes	30.7	1.4	130
31	*n*-Hentriacontane	31.0	0.4	40
31-A	11-, 13-, and 15-Methylhentriacontanes	31.3	1.4	130
31-A′B	7,11-, 9,13-, and 11,15-Dimethyl- and 3-methylhentriacontanes	31.6	3.5	340
32-?		32.0	T	
32-A	12-Methyldotriacontane	32.3	T	
32-B	10,14-Dimethyldotriacontane	32.6	T	
33-A	13-Methyltritriacontane	33.3	0.2	20
33-B	9,13- and 11,15-Dimethyltritriacontanes	33.6	0.5	50
35-A		35.3	T	
35-B		35.6	T	
41-A	11- and 13-Methylhentetracontanes	41.3	0.4	40
41-B	9,13- and 11,15-Dimethylhentetracontanes	41.5	1.5	140
42-A	12- and 13-Methyldotetracontanes	42.2	0.1	10
42-B	10,14- 11,15-, and 12,16-Dimethyldotetra-contanes	42.5	0.1	10
43-A	13-Methyltritetracontane	43.3	1.0	100
43-B	11,15- and 13,17-Dimethyltritetracontanes	43.5	2.2	210
44-B		44.4	T	
45-A	13-Methylpentatetracontane	45.2	0.5	50
45-B	11,15- and 13,17-Dimethylpentatetracontanes	45.4	T	
47-B	13,17-Dimethylheptatetracontane		T	

[a]For GLC peak nomenclature see Nelson et al. (1977).
[b]Structure determined by mass spectrometry.
[c]Equivalent chain length.
[d]Values are means of three replicates determined with a Hewlett-Packard model 3380A integrator system set in the log mode. *T* less than 0.1%.
[e]Values rounded to nearest 10 ng.

The A′ series consisted of the 3-methyl analogs of heptacosane, octacosane, nonacosane, and hentriacontane. No 2-methylalkanes were found. The sequential mass spectra of the 28-A′ peak indicated the presence of 4-methyloctacosane. 4-Methylalkanes are not common constituents of the alkanes of most insects (Jackson and Blomquist, 1976). However, they have been reported in *Lasioderma serricorne* (Baker et al., 1979b), *Formica nigricans* Emery (Bergström and Löfquist, 1973), *Iridomyrmex humilis* (Mayr) (Cavill and Houghton, 1973), and *Curculio caryae* (Horn) (Mody et al., 1975).

The B series of internally branched dimethyl alkanes consisted mainly of positional isomers with isoprenoid spacing. Dimethylalkanes with the methyl groups separated by 7 methylene units have been reported in *Locusta migratoria* (L.) (Lockey, 1976), *P. japonica* (Nelson et al., 1977), and *Heliothis virescens* (F.) (Coudron and Nelson, 1978). However, another series of dimethylalkanes with the methyl groups separated by 3, 5, 7, and 9 methylene units was also found in *C. maculatus*. This new series of dimethylalkanes, which elutes from the GC with *n*-alkanes, has recently been identified in *Solenopsis invicta* Byron and *S. richteri* Forel (Nelson et al., 1980), *L. serricorne* (Baker et al., 1979b), and *Musca domestica* (Nelson, unpublished).

It is not known with certainty how the degree of branching of long-chain alkanes affects the physical property of the cuticular lipid layer into which they are incorporated and any adaptive value that might be obtained. A better understanding of this functional relationship was a major reason for the recent intensive efforts in the analyses of cuticular hydrocarbons in insects (see reviews by Jackson and Blomquist, 1976; Lockey, 1976; Nelson, 1978). Other recent studies, however, have also indicated that cuticular alkenes and branched alkanes can act both individually (Carlson et al., 1971) and synergistically (Uebel et al., 1976) as pheromonal stimuli to elicit mating behavior. *C. maculatus* has a relatively high concentration of cuticular hydrocarbons. It is not known if any of these alkanes function as short-range or contact mating stimuli.

C. maculatus exhibits phase dimorphism when reared in crowded cultures at high temperatures (Utida, 1972). The flight form has a significantly higher amount of total lipid than the normal form (Nwanze et al., 1976). Since the adults do not feed, the increased lipid reserves in the flight form are thought to supply the energy required for dispersal and subsequent egg laying. Although no differences were found in the cuticular hydrocarbons between males and females of mixed but mainly flight forms, the hydrocarbon patterns of the two phases were not directly compared.

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INTERRUPTION OF RESPONSE OF *Dendroctonus brevicomis*[1] TO ITS ATTRACTIVE PHEROMONE BY COMPONENTS OF THE PHEROMONE[2]

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Abstract—The catch of the western pine beetle, *Dendroctonus brevicomis*, at an attractive source of racemic *exo*-brevicomin, racemic frontalin, and myrcene was reduced by surrounding the source with a grid of 48 stations releasing all three compounds together, or *exo*-brevicomin alone or myrcene alone. Each compound was released at the rate of 2 mg/24 hr/station. The catch at an attractive bolt cut from a tree being colonized by *D. brevicomis* was not reduced by *exo*-brevicomin, but was reduced by the combination of *exo*-brevicomin, frontalin, and myrcene in one of two tests. When a transect of traps was placed across a 0.81-hectare plot at six of the 48 stations releasing all three compounds, more beetles were caught at outer than at inner traps. More beetles were caught at unbaited traps on trees in a plot when the three compounds were released than when only *exo*-brevicomin or no compounds were released. A few trees were attacked by *D. brevicomis* in some of the plots. The antiattractant verbenone released from 48 stations at the rate of 4 mg/24 hr/station did not reduce the catch at an attractive tree bolt.

[1]Coleoptera: Scolytidae.

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Key Words—*Pinus ponderosa*, *Dendroctonus brevicomis*, western pine beetle, attractant, interruption, behavior, pheromone, Coleoptera, Scolytidae, *exo*-brevicomin, frontalin, myrcene, verbenone.

INTRODUCTION

Interruption of pheromonal communication as defined by Wood (1977, p. 372) as a method of population control has been attempted for several insect pests of agricultural crops, especially moths (Tette, 1974; Mitchell, 1975; Shorey, 1977). The purpose of those experiments was to prevent the male from finding the female for mating by permeating the atmosphere with the sex pheromone. Tette (1974) and Shorey (1977) proposed that one or more factors are involved: sensory adaptation of olfactory receptors, central nervous system habituation, and confusion of the searching male moths through competition between synthetic sources of attraction and female moths.

The western pine beetle, *Dendroctonus brevicomis* LeConte, aggregates on living pines, *Pinus ponderosa* Dougl. ex Laws. in response to a multicomponent pheromone (Wood, 1972; Wood and Bedard, 1977). This pheromone is composed of at least three compounds: the (+) enantiomer of *exo*- brevicomin, the (−) enantiomer of frontalin, and myrcene (Wood et al., 1976). Successful colonization of a tree occurs after the arrival of a sufficient number of beetles to overcome host resistance and create a suitable environment for brood development (Wood, 1972; Borden, 1974; Wood and Bedard, 1977). Death of a tree is believed to be due to construction of egg galleries in the tree's phloem–cambial tissues (Miller and Keen, 1960) and to the colonization of the xylem by pathogenic fungi, which are vectored by the beetles (Whitney and Cobb, 1972).

Colonization of a tree might be prevented by interrupting aggregation. This could best be done by interrupting females, which initiate entrance tunnels, produce *exo*-brevicomin, and release myrcene (Wood and Bedard, 1977). Tree protection could probably be enhanced by also interrupting males, which produce frontalin.

Most studies to date have investigated the possibility of using anti-attractants to protect trees. Bedard et al. (1980a,b) reduced the number of *D. brevicomis* trapped at natural and synthetic sources of attractant by releasing verbenone at the sources. Payne et al. (1977) reduced the number of *D. frontalis* Zimm. trapped on *Pinus taeda* L. by surrounding the trees with many sources of *exo*- and *endo*-brevicomin. Richerson and Payne (1979) reduced landing of *D. frontalis* on pines treated with *exo*- and *endo*-brevicomin and verbenone. Methylcyclohexenone was used to reduce the number of attacks by *D. pseudotsugae* Hopkins on live (Furniss et al., 1972) and felled *Pseudotsuga menziesii* (Mirb.) Franco (Furniss et al., 1974, 1977; Rudinsky et

al., 1974) and by *D. rufipennis* (Kirby) on windthrown *Picea sitchensis* (Bong.) Carr (Rudinsky et al., 1974).

In a study using an attractive pheromone, Vité et al. (1976) were not able to reduce the number of *D. frontalis* trapped on *P. taeda* under beetle attack by aerial applications of rice soaked with the mixture of frontalin and *alpha*-pinene.

This paper reports the results of field experiments to evaluate the interruptive effects of *exo*-brevicomin, frontalin, myrcene, and verbenone on the response of *D. brevicomis* to natural and synthetic sources of attractant. Some data from these experiments were reported earlier (Wood, 1977; Wood and Bedard, 1977).

METHODS AND MATERIALS

Experimental Design. The experiments were done during August and September 1972 and July 1973, near McCloud, Siskiyou County, California; and during July to September 1973, near Bass Lake, Madera County, California. In each of these areas two plots, at least 4.8 km apart, were installed in ponderosa pine stands. Response of *D. brevicomis* to traps baited with racemic *exo*-brevicomin (E), racemic frontalin (F), and myrcene (M) was monitored for several days prior to the experiments, and plots were located where similar numbers of beetles were trapped. Because these plots were not chosen randomly, results of these experiments may not apply to other areas. No trees currently under attack or with emerging beetles were within 100 m of the plots. Each 0.81-hectare plot was 90 m on a side with a 7×7 grid of 49 stations from which compounds were released. Stations were approximately 15 m apart and were at least 3 m from ponderosa pines to minimize the probability of beetles attacking trees near release stations (Vité and Pitman, 1970). The basic design of our plots was similar to those described by Gaston et al. (1967) and Shorey et al. (1967).

The center station was a source of either a natural or synthetic attractant. The natural attractant was a bolt (1 m long $\times$ 20–25 cm diam) cut from a ponderosa pine which had been baited 2–4 days earlier with EFM and was being colonized by *D. brevicomis*. Attractiveness of a baited tree was monitored with a sticky trap to catch responding beetles, and by chipping away the bark to assess the extent of gallery construction. The most attractive part of an attacked tree had female galleries less than 2 cm long, with males in about half of them (Wood and Bedard, 1977). Since a cut bolt rapidly loses its attractiveness (Vité and Crozier, 1968), it was replaced with a fresh bolt every two days during an experiment. Bolts were not exchanged between plots.

The synthetic attractant was a mixture of EFM, which was evaporated at the rate of 2 mg/24 hr/compound. E and M were each evaporated from a 3.5

mm ID × 52 mm glass tube, and F from a 2.2 mm ID × 62 mm glass tube. The tubes were placed inside an inverted glass jar with a perforated metal lid covered with aluminum foil. Release rates were measured by weight loss of five sample devices exposed in the field. The purity of each compound was: E = 98%, F = 95%, and M = 98%, as determined by gas–liquid chromatography. One percent "ethyl" antioxidant 330 was added to the M.

Two types of traps coated with Stickem Special were used to monitor the catch of *D. brevicomis* and *Temnochila chlorodia* (Mannerheim) (Coleoptera: Trogositidae) at the center station. *T. chlorodia*, a predator of *D. brevicomis*, is attracted to attacked trees (Stephen and Dahlsten, 1976) and to E alone (Bedard et al., 1969, 1980b; Vité and Pitman, 1969; Pitman and Vité, 1971). When a tree bolt was the source of attractant, it was covered with fine mesh aluminum insect screen and placed atop an iron pipe driven into the ground, so that the bottom of the bolt was approximately 0.9 m above the ground. A vane trap (Browne, 1978) was placed next to the bolt so that the bottom of the trap was also 0.9 m above the ground. The trap had two sticky panels in the same plane with a total trapping surface of 3 m^2 (2 m high × 1.5 m wide). When E, F, and M were the source of attractant, the trap was a cylinder of hardware cloth 30.5 cm high ×20.3 cm diam attached to a metal pipe stand (Bedard and Browne, 1969). The top of the trap was 1.5 m above the ground.

Candidate interruptants (treatments) were evaporated from each of the 48 stations surrounding the center trap. E, F, and M were released together and separately, as described above, at 2 mg/24 hr per compound per station. Verbenone was evaporated at the rate of 4 mg/24 hr/station from a small glass cup 10 mm ID × 5 mm inside an inverted glass jar like that used for EFM (Bedard et al., 1980a). One release device was attached to the top of a 150 × 1.3-cm white polyvinyl chloride pipe set vertically over a 91 × 0.95-cm hardwood dowel stake driven about 60 cm into the ground at each of the 48 stations. All replicates of each treatment were presented in a series discrete from other treatments (Table 1).

In 1972, at McCloud, the only trap in each plot was at the center. In 1973, at McCloud and Bass Lake, additional traps were placed on a transect of six stations, three on each side of the center trap. These traps were identical to the center trap when the source of attractant was EFM and were used to monitor the response of beetles to release stations. To monitor the landing of beetles on trees, we hung a 30.5 × 61-cm sticky trap 4.3 m high on a ponderosa pine near every other release station. There were 16 traps in each plot at McCloud and in one of the plots at Bass Lake, and 10 traps in the other plot at Bass Lake due to a scarcity of trees.

The center traps of the two plots in each area were baited with an attractant, and compounds were released from the surrounding 48 stations in

TABLE 1. EXPERIMENTS EVALUATING COMPOUNDS AS INTERRUPTANTS OF RESPONSE OF *D. brevicomis* AND *T. chlorodia* TO ATTRACTANTS[a]

Place	Date	Attractants	Treatments[b]
McCloud	1972	EFM	EFM, E, F, M
McCloud	1973	Tree bolt[c]	EFM
Bass Lake	1973	Tree bolt[c]	EFM, E, verbenone
Bass Lake	1973	EFM	EFM, E

[a]McCloud, Siskiyou Co., Calif., Aug. 3–Sept. 13, 1972, July 3–5 and 7–8, 1973; Bass Lake, Madera Co., Calif., July 18–Aug. 14 and Sept. 10–19, 1973.
[b]Treatments presented in a grid of 48 release stations 15 m apart surrounding the attractant. Racemic *exo*-brevicomin (E), racemic frontalin (F), and myrcene (M) released at rate of 2 mg/24 hr per compound per release station. Verbenone released at rate of 4 mg/24 hr per release station.
[c]One-m-long bolt cut from ponderosa pine being colonized by *D. brevicomis*.

the treated plot but not in the control plot. The treatment was randomly assigned to a plot the first day, then alternated daily between plots for the remainder of each experiment. Tree bolts and compounds were set out in mid-afternoon and removed the following morning. Beetles were then picked from traps and placed in labelled vials of solvent (Chevron-325) for later counting and sex determination (Tilden et al., 1979).

Statistical Analyses. A Wilcoxon signed-rank statistic for matched pairs (Lehmann, 1975) was used to test the null hypothesis of no treatment effect on catch at the center trap. The one-sided alternative hypothesis was that of a reduction in catch at the center trap due to the presence of a treatment. In matching treatment and control observations within days and across the two plots, we assumed that beetle populations in each plot were similar numerically and behaviorally.

The null hypothesis of no treatment effect on sex of *D. brevicomis* caught at the center trap was tested using a chi-square statistic (1 *df*) of independence for two dichotomous factors. Trap catches for each sex and treatment combination were summed over days for each plot.

The numbers of *D. brevicomis* caught on the six transect traps were analyzed as follows:

1. A Wilcoxon signed-rank statistic for matched pairs was used to test the null hypothesis of no treatment effect on catch against the one-sided alternative that presence of a treatment increased catch. Daily statistics were calculated by matching trap position relative to the center trap across plots and summarized to produce an overall test statistic (Hodges and Lehmann, 1962).

2. A Wilcoxon signed-rank statistic for matched pairs was used to test

the null hypothesis of no difference in catch between the two outermost and two innermost transect traps against the one-sided alternative of greater catch on the outer traps when the treatment was EFM. We matched an outer trap with an inner trap on each side of the center trap within plots and across days. Thus the daily data from a plot gave two matched pairs.

The numbers of beetles trapped on trees were not analyzed because the numbers were small, the numbers of traps in the two plots at Bass Lake were unequal, and one of the trees with a trap at McCloud became attacked.

Many statistical tests were made on the data from this study. Because this was an exploratory study, we did not attempt to maintain a low type-1 error rate (alpha) for the study as a whole. Rather, we maintained low error rates for the tests individually, in the hope that more often than not, these tests would indicate the true situation.

RESULTS

McCloud 1972. When the center attractant was EFM and the treatment was EFM, 97% fewer *D. brevicomis* were caught at the center trap in the treatment plot than in the control plot (Table 2). Smaller reductions in catch

TABLE 2. CATCH OF *D. brevicomis* AND *T. chlorodia* WITH AND WITHOUT A GRID OF 48 TREATMENT STATIONS RELEASING COMPOUNDS[a] SURROUNDING A SOURCE OF ATTRACTANT.[b]

			D. brevicomis		*T. chlorodia*
Attractant	Treatment	Days	Total of 2 plots[c]	Male proportion[d]	Total of 2 plots
EFM	None	4	469	0.47	15
EFM	EFM	4	15a	0.33	5
EFM	None	4	265	0.41	36
EFM	E	4	30a	0.33	7
EFM	None	4	191	0.42	1
EFM	M	4	93a	0.43	3
EFM	None	4	86	0.37	4
EFM	F	4	106	0.30	6

[a]Treatment release stations 15 m apart. Racemic *exo*-brevicomin (E), racemic frontalin (F), and myrcene (M) released at rate of 2 mg/24 hr per compound per station.
[b]McCloud, Siskiyou Co., Calif., Aug. 3–Sept. 13, 1972.
[c]Numbers followed by letter significantly different from control, based on Wilcoxon signed-rank statistic for matched pairs, (a) $P = 0.0625$, which is minimum P possible with sample size of four matched pairs.
[d]Two plots combined.

were apparent when treatments were E or M. These three reductions are significant at $P = 0.0625$, which is the smallest significance probability possible with a sample size of four matched pairs. There was no significant reduction in catch when the treatment was F ($P = 0.1875$). The proportions of males trapped were not significantly different, comparing treatment and control for each plot (largest chi square = 1.95; $P = 0.163$; eight tests at alpha = 0.01 each and overall alpha $\leqslant 0.08$).

Devices releasing EFM were mistakenly left at three stations near a corner of one of the plots on a Friday morning and were not discovered until the following Monday afternoon. Beetles began attacking four trees near these stations. The next treatment (E) was put out 15 days later. We checked both plots for attacked trees at the end of the experiments. In the plot with the four attacked trees we found an additional successfully attacked tree near the plot center and three unsuccessfully attacked trees near another corner. In the other plot we found two unsuccessfully attacked trees near the center, and three successfully attacked trees near a corner.

Fewer *T. chlorodia* were caught when treatments were EFM or E than at the controls, but these results were not significant (Table 2).

McCloud 1973. Fewer *D. brevicomis* were caught at the center trap when the center attractant was a tree bolt and the treatment was EFM than at the control (Table 3), but the difference was not significant ($P = 0.156$). The proportions of males trapped at the center of the plots were not significantly different, comparing treatment and control for each plot (chi squares = 0.282 and 1.832; *P*s = 0.596 and 0.176, respectively; two tests at alpha = 0.025 each and overall alpha $\leqslant 0.05$).

Significantly more beetles ($P < 1 \times 10^{-7}$) were caught at the six transect traps when the treatment was EFM than at the control (Table 3). Of the 330 beetles trapped when the treatment was EFM, 235 (71%) were caught at the two outer traps and 37 (11%) were caught at the two inner traps on either side of the center trap. This difference was significant ($P < 0.002$).

A tree with a trap near a corner of one of the plots became attacked on the second day of testing (the first day of the treatment). The trap caught beetles on that day and continued to catch beetles on the following three days of the experiment. Many of the beetles trapped on trees in both plots were caught on this tree: 54 of 63 on two days of the control and 66 of 101 on two days when EFM was released from the stations (Table 3).

T. chlorodia were caught at the plot center, at transect traps, and on trees (Table 3). Significantly ($P = 0.047$) more were caught on the six transect traps when the treatment was EFM than at the control.

Bass Lake 1973. Significantly ($P = 0.03125$) fewer *D. brevicomis* were caught at the tree bolt in the center of the plot when the treatment was EFM than at the control (Table 4). The number of beetles caught at a bolt was not

TABLE 3. CATCH OF *D. brevicomis* (*D. b.*) AND *T. chlorodia* (*T.c.*) WITH AND WITHOUT A GRID OF 48 TREATMENT STATIONS RELEASING COMPOUNDS[a] SURROUNDING AN ATTRACTIVE TREE BOLT[b,c]

				Trap locations					
				Center		Transect[d]		Trees[e]	
Species trapped	Attractant	Treatment	Days	Total of 2 plots	Male prop.[f]	Total of 2 plots[g]	Male prop.[f]	Total of 2 plots	Male prop.[f]
D.b.	Bolt	None	5	122	0.52	6	0.33	63	0.61
D.b.	Bolt	EFM	5	58	0.52	330a	0.52	101	0.72
T.c.	Bolt	None	5	13		3		2	
T.c.	Bolt	EFM	5	18		25b		8	

[a]Treatment release stations 15 m apart. Racemic *exo*-brevicomin (E), racemic frontalin (F), and myrcene (M) released at rate of 2 mg/24 hr per compound per station.
[b]McCloud, Siskiyou Co., Calif., July 3–5 and 7–8, 1973.
[c]One-m-long bolt cut from ponderosa pine being colonized by *D. brevicomis*.
[d]Transect of traps at six release stations, three on each side of center attractant.
[e]Traps 4.3 m high on 16 trees per plot.
[f]Male proportion of *D. brevicomis* trapped, two plots combined.
[g]Numbers followed by letter significantly different from control, based on Wilcoxon signed-rank statistic for matched pairs, (a) $P < 1 \times 10^{-7}$ and (b) $P = 0.047$.

TABLE 4. CATCH OF *D. brevicomis* WITH AND WITHOUT A GRID OF 48 TREATMENT STATIONS RELEASING COMPOUNDS[a] SURROUNDING A SOURCE OF ATTRACTANT[b,c]

			Trap locations					
			Center		Transect[d]		Trees[e]	
Attractant	Treatment	Days	Total of 2 plots[f]	Male prop.[g]	Total of 2 plots[f]	Male prop.[g]	Total of 2 plots	Male prop.[g]
Bolt	None	6	263	0.59	2	0.50	3	0.67
Bolt	EFM	6	15a	0.35	163b	0.47	26	0.46
EFM	None	5	141	0.54	17	0.76	6	0.50
EFM	EFM	5	7a	0.57	148c	0.53	39	0.54
Bolt	None	4	143	0.57	0		1	0
Bolt	E	4	123	0.51	1	1.00	6	0.33
EFM	None	4	107	0.46	3	0.33	1	1.00
EFM	E	4	15d	0.60	30	0.17	4	0.75
Bolt	None	7	214	0.59	1	1.00	1	1.00
Bolt	Verbenone	7	267	0.60	0		0	

[a]Treatment release stations 15 m apart. Racemic *exo*-brevicomin (E), racemic frontalin (F), and myrcene (M) at rate of 2 mg/24 hr per compound per station. Verbenone released at rate of 4 mg/24 hr per station.
[b]Bass Lake, Madera Co., Calif., July 18–Aug. 14 and Sept. 10–19, 1973.
[c]One-m-long bolt cut from ponderosa pine being colonized by *D. brevicomis*, or EFM at rate of 2 mg/24 hr per compound.
[d]Transect of traps at six release stations, three on each side of center attractant.
[e]Traps 4.3 m high on 10 trees in one plot and on 16 trees in another plot.
[f]Numbers followed by letter significantly different from control, based on Wilcoxon signed-rank statistic for matched pairs, (a) $P = 0.03125$, (b) $P = 2.9 \times 10^{-9}$, (c) $P = 9.2 \times 10^{-6}$, (d) $P = 0.0625$ (minimum P possible with sample size of four matched pairs).
[g]Male proportion of *D. brevicomis* trapped, two plots combined.

reduced when the treatment was E ($P = 0.3125$) or verbenone ($P = 0.65$). Significantly fewer beetles were caught at EFM in the center of the plot than at the control, when the treatment was EFM ($P = 0.03125$) or E ($P = 0.0625$, the minimum P possible with a sample size of four matched pairs). The proportions of males trapped at the center of the plots were not significantly different, comparing treatment and control for each plot (largest chi square = 3.986; $P = 0.046$; nine tests at alpha = 0.01 each and overall alpha $\leqslant 0.09$; one comparison not made due to zero catch).

Significantly more *D. brevicomis* were caught at the six transect traps when the treatment was EFM and the center trap was baited with a tree bolt ($P = 2.9 \times 10^{-9}$) or EFM ($P = 9.2 \times 10^{-6}$) than at the controls (Table 4). Of the 163 beetles caught at transect traps when the center was a bolt and the treatment was EFM, 85 (52%) were caught on the two outer traps and 42 (26%) were caught on the two inner traps. This was not a significant difference ($P = 0.0647$). Of the 148 beetles caught at transect traps when the center was EFM and the treatment was EFM, 98 (66%) were caught on the two outer traps and 12 (8%) were caught on the two inner traps. This difference was significant ($P = 0.0049$).

Few beetles were caught at the six transect traps when E or verbenone were the treatments (Table 4). When the center was EFM and the treatment was E, 25 of 30 beetles caught at transect traps were caught at one trap on one day. We have no explanation for this anomalous catch.

More *D. brevicomis* were caught at traps on trees when the treatment was EFM than at the controls (Table 4). Few beetles were caught at traps on trees when the treatments were E or verbenone.

T. chlorodia were caught at the plot center, at transect traps, and on trees, but the numbers were too few to analyze.

DISCUSSION

Our initial experiment (Table 2) indicated that response of *D. brevicomis* to a single source of EFM could be interrupted by surrounding it with other sources of EFM and E or M alone. Studies with individual compounds and their mixtures at various release rates are needed to more accurately determine their effects on the response of *D. brevicomis* to EFM.

The catch at an attractive bolt surrounded by 48 sources of EFM was reduced (but not significantly) at McCloud (Table 3) and was significantly reduced at Bass Lake (Table 4). This difference in results could be due to the presence of an attacked and attractive tree in one of the plots at McCloud, to differences in the attractiveness of the bolts, to differences in beetle populations in the two areas, to physical differences (especially meterological) among plots, or to a sample size at McCloud that was inadequate to detect a significant difference in numbers of beetles trapped.

E interrupted response to EFM, but not to the attractive bolt and EFM interrupted response to the attractive bolt (Table 4), which indicates that either F or M or both together with E are required for interruption. Vité et al. (1976) got similar results when they broadcast rice soaked with frontalure (an attractive mixture of frontalin and *alpha*-pinene) over a *D. frontalis* infestation. They interrupted response of beetles to frontalure-baited nonhost trees, but not to attractive host trees being attacked by the beetles. In fact, catch at host trees during treatment was greater than catch before treatment.

When verbenone was released at a source of attractant, it reduced the number of *D. frontalis* (Renwick and Vité, 1969) and *D. brevicomis* (Bedard et al., 1980a,b) trapped at the source. Vité and Renwick (1971) found that *exo*-and *endo*-brevicomin reduced the catch of *D. frontalis* at traps baited with frontalin and alpha-pinene. Payne et al. (1977) released *exo*- and *endo*-brevicomin from host trees in a 0.2-hectare plot within an active infestation of *D. frontalis*. They reduced the number of beetles trapped on host trees but not the number of beetles caught on unbaited traps placed away from host trees. They did not report, however, the spatial relationships among trapped beetles, chemical release device, trees from which beetles were emerging, and trees being attacked. Richerson and Payne (1979) released verbenone and *exo*- and *endo*- brevicomin from host trees of *D. frontalis*. While these treatments did not prevent beetle attack, fewer beetles were trapped on, and fewer egg galleries were excavated in, trees treated with brevicomin isomers (with or without verbenone) than in the control trees. The verbenone treatment alone did not affect either the trap catch or the number of egg galleries.

In our experiment, the catch at an attractive tree bolt surrounded by 48 stations releasing verbenone was not reduced (Table 4). Further testing at higher release rates and closer spacings might show an interruptive effect for verbenone when released near, but not directly at (Bedard et al., 1980a,b), a source of attractant.

The fact that the transect traps nearest the plot center caught fewer *D. brevicomis* than did traps near the perimeter, when the treatment was EFM, suggests that interruption was greatest at the center and least near the perimeter. But since we made no direct observations of beetle behavior in response to this treatment, we cannot designate its mode of action on behavior to explain this distribution of catch or the phenomenon we call interruption. Were the compounds acting as an arrestant, stimulant, attractant, or repellent (Dethier et al., 1960)?

Huddleston et al. (1977) were not able to reduce cotton square infestation in small plots within cotton fields by permeating the air with many sources of grandlure, a synthetic aggregative pheromone of the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae). In fact, they concluded that cotton square infestation increased in the plots because of the attraction of additional weevils into the plots. Similarly, *D. brevicomis* attacked several

trees in our plots when EFM was released from the 48 stations, probably because beetles were attracted into the plots and because of the proximity of pheromone release stations to trees.

For interruption to be effective, beetles should be dispersed over a large area, rather than attracted into a small area as they were in our study. Extending the application of compounds beyond the occurrence of host trees or decreasing the density of release stations near the edge of the treatment area might reduce tree mortality near the edges of large plots. The density of release stations within the treatment area should be uniform enough to avoid islands free of interruptants, which might become centers of tree mortality.

The success of the interruption strategy may also depend on the density of the beetle population (Johnson and Coster, 1978). At high population densities, it might not be possible to disperse the beetles widely enough to reduce attack densities on trees below the threshold of tree resistance (Smith, 1975; Wood and Bedard, 1977). Variables that should also be investigated are: other compounds involved in *D. brevicomis* aggregation, release rates of candidate interruptants alone and in various mixtures, density and height of release stations, and the duration of release of interruptants.

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ETHYL DECANOATE AS A MAJOR COMPONENT IN THE DEFENSIVE SECRETION OF TWO NEW ZEALAND ALEOCHARINE (STAPHYLINIDAE) BEETLES—*Tramiathaea cornigera* (BROUN) AND *Thamiaraea fuscicornis* (BROUN)

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Abstract—The defensive secretions of both *Tramiathaea cornigera* and *Thamiaraea fuscicornis* contain undecane, toluquinone, ethyl decanoate and smaller amounts of other esters. The presence of esters particularly ethyl decanoate as a major component sets these two beetles apart chemically not only among the staphylinids but also within the subfamily Aleocharinae.

Key Words—*Tramiathaea cornigera, Thamiaraea fuscicornis,* Aleocharinae, Coleoptera, Staphylinidae, defensive secretion, ethyl decanoate, esters, undecane, toluquinone.

INTRODUCTION

Many staphylinid beetles discharge their defensive secretions from pygidial glands near the tip of the abdomen (Schildknecht, 1970), but those belonging to the subfamily Aleocharinae exude their defensive secretions from tergal and posttergal glands (Pasteels, 1968, 1969). These glands are well developed in both the myrmecophilous Aleocharinae such as *Lomechusa strumosa* and the free-living species such as *Drusilla canaliculata* (Brand et al., 1973). Like

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other staphylinids, the Aleocharinae have a range of chemicals in their defensive secretions including quinones, hydrocarbons, and various aldehydes (Brand et al., 1973; Blum et al., 1971; Kistner and Blum, 1971; Kolbe and Proske, 1973), but this is the first report of esters in the defensive exudates of staphylinids. The Aleocharinae beetles *Tramiathaea cornigera* and *Thamiaraea fuscicornis* are endemic to New Zealand and belong to the tribe Schistogeniini.

METHODS AND MATERIALS

Adult *Tramiathaea cornigera* and *Thamiaraea fuscicornis* were collected from pitfall traps baited with rotting bananas in the Auckland area. By firmly holding freshly collected beetles near the elytra with a pair of forceps, they were induced to curve their abdomens up and discharge their defensive secretions. The secretions were collected on small squares of absorbent tissue paper which were immersed immediately in 2 ml of purified pentane. This method of collection did not harm the beetles and minimized the danger of contamination of the secretions by cuticular waxes. The samples were stored at −20°C, and analyses were performed as soon as possible to reduce the possibility of postcollection changes. Identical blank squares of tissue paper were similarly extracted with purified pentane and, when analyzed using gas chromatography, showed that no detectable contaminants were introduced from the paper.

The pentane solutions of the defensive secretions were chromatographed on 5 × 0.5-cm Florisil columns. The columns were successively eluted with 100% pentane, and with 5, 10, 20 and 50% (v/v) diethyl ether in pentane (Hutchins and Martin, 1968). The individual fractions were concentrated under a stream of nitrogen at room temperature. Care was taken not to concentrate the fractions to dryness. Each fraction and the total pentane extract was chromatographed on a Varian 2700 chromatograph. Each fraction was also analyzed by gas chromatography–mass spectrometry (GC-MS) on a Varian 2700 chromatograph coupled with a AEI MS30 spectrometer via a membrane separator (180°C) at 20 eV (low resolution) and 70 eV (high resolution). Helium was used as the carrier gas. The chromatograph columns used were 15% OV-17 on Chromosorb W-AW-HMDS (100–120 mesh), temperature programed from 80 to 200°C at 4°/min, and 10% Silar 10CP on Chromasorb W-AW-HMDS (100–200 mesh) isothermally at 110°C.

RESULTS

Individual samples of the defensive secretions of *Tramiathaea cornigera* and *Thamiaraea fuscicornis* were analyzed and gave similar results. Gas

chromatography of the defensive secretions of each of these beetles showed the presence of two major components (A and E), and five minor components (B, C, D, F, and G) (Figure 1). Further separation of each secretion on a Florisil column split the components into hydrocarbon, ester–aldehyde, and quinone fractions.

The hydrocarbon fraction, which eluted with pentane, was shown by gas chromatography to contain component A. The mass spectrum of this component gave a molecular ion at 156 and a fragmentation pattern typical of an *n*-alkane. The retention time of A was indistinguishable from that of authentic undecane, and when they were coinjected only a single GC peak was obtained. This was the most abundant compound in the defense secretions of both beetles.

The chromatography of the ester or aldehyde fraction which eluted with 5% ether–pentane showed the presence of components C, D, E, F, and G. High-resolution mass spectra of the second major peak, E, showed a molecular ion at *m/e* 200.1796 ($C_{12}H_{24}O_2$ = 200.1770). An ion at 155.1438 ($C_{10}H_{19}O$ = 155.1431) indicated a loss of C_2H_5O. Other peaks were observed at 171.1336 ($C_{10}H_{19}O_2$), 157.1228 ($C_9H_{17}O_2$), 143.1069 ($C_8H_{15}O_2$), 129.0927 ($C_7H_{13}O_2$), 115.0711 ($C_6H_{11}O_2$), 101.0559 ($C_5H_9O_2$). Each of these peaks differs by a CH_2 unit, which suggests a long-chain fragment. Assuming it to be an ethyl ester, the molecular formula suggests that the parent acid should be decanoic acid. Both the retention time and the mass spectrum of authentic ethyl decanoate corresponded with those of component E, confirming it to be ethyl decanoate.

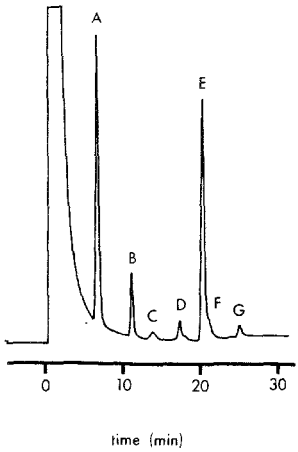

FIG. 1. Gas chromatogram of total extract of the defensive secretion of *Trameathaea cornigera* chromatographed on a 15% OV-17 on Chromasorb W-AW-HMDS (100–120 mesh) temperature programed from 80 to 200°C at 4°/min. A = undecane, B = toluquinone, C = ethyl octanoate, D = methyl decanoate, E = ethyl decanoate, F = unknown, G = ethyl dodecanoate.

The retention times of C, D, and G matched those of ethyl octanoate, methyl decanoate, and ethyl dodecanoate, respectively, both on the OV-17 and on the Silar 10CP columns. The polar Silar 10CP column enabled the separation of component F from the ethyl decanoate, but so far the identity of F is unknown. The available quantities of C, D, F, and G were insufficient for mass spectral analyses.

Quinones elute with 20% ether–pentane, and this fraction contained component B. The mass spectrum of B had a peak at *m/e* 122, and its fragmentation pattern and retention time were identical with that of toluquinone.

DISCUSSION

Ethyl decanoate has not previously been reported from the defensive secretion of any arthropod, although other esters have been reported in Hymenoptera (Gilby and Waterhouse, 1965), and certain Coleoptera (Weatherston and Percy, 1978). Esters are often present as acetates (Blum, 1978), such as nonyl acetate in the defensive secretion of the carabid beetle *Hellumorphoides* (Eisner et al., 1968) and hexyl acetate in the defensive secretion of nine bugs of the super family Coreoidea (Waterhouse and Gilby, 1964). Two of these bugs, *Amorbus rhombifer* and *Mictis caja*, also contain butyl butyrate in their defensive exudate (Waterhouse and Gilby, 1964). While these aliphatic esters are acetates or butyrates of long-chain alcohols, ethyl decanoate is an ester of ethanol and a long-chain acid. Esters of octanoic and nonanoic acids have been found in the ant *Camponotus clarithorax* (Lloyd et al., 1975), but these are either aromatic or branched aliphatic esters in contrast to those of *Tramiathaea cornigera* and *Thamiaraea fuscicornis* which are simple straight-chain aliphatic esters.

Blum (1978) suggests that often the esters in arthropod defensive exudates are present as minor components and act as wetting agents. But in these two aleocharine beetles, ethyl decanoate is a major component which, together with the other esters, masks the quinonoidal odor of toluquinone, giving the secretion an overall "fragrant" smell. In this case the ethyl decanoate is more likely to be a deterrent that merely a wetting agent.

However, the presence of ethyl decanoate as a major component of the defensive secretion is unique among the Staphylinidae and sets *Trameathaea cornigera* and *Thamearaea fuscicornis* chemically apart from the rest of the Aleocharinae. Esters could be a characteristic feature of the defensive secretion of beetles belonging to the tribe Schistogeniini or, for *Trameathaea cornigera* and *Thamearaea fuscicornis*, could be a consequence of the New Zealand environment.

Of the seven Aleocharinae investigated to date, *Lomechusa strumosa*

(Blum et al., 1971), *Drusilla canaliculata* (Brand et al., 1973), *Pella* sp. (Kistner and Blum, 1971), and *Zyras humeralis* (Kolbe and Proske, 1973) all belong to the tribe Zyrasini, whereas *Tramiathaea cornigera* and *Thamiaraea fuscicornis* are in the tribe Schistogeniini. Among these aleocharine beetles, some are free-living whereas others live in association with ant or termite populations. *D. canaliculata*, *Tramiathaea cornigera*, and *Thamiaraea fuscicornis* are all free-living and have a common component, undecane, in their defensive secretion which has not been reported from any other staphylinid. The defensive secretions of these three free-living Aleocharinae also have a "fragrant" smell as compared to the myrmecophilous species whose defensive secretion has a quinonoid odor as in *L. strumosa* or the odor of rancid butter as in *Z. humeralis* (Kolbe and Proske, 1971). Unfortunately the defensive secretion of *L. strumosa* is the only secretion of a myrmecophilous aleocharine that has been completely characterized chemically (Blum et al., 1971). Although chemical distinctions between the Aleocharinae cannot be made from the few species studied, there is a greater similarity between the aldehyde-rich defensive secretion of *D. canaliculata* of the tribe Zyrasini and the ester-rich defensive secretions of the Schistogeniini beetles than there is between the defensive secretions of the free-living *D. canaliculata* and the myrmecophilous *L. strumosa* which belong to the same tribe, Zyrasini. Thus among the Aleocharinae, the chemistry of the defensive secretion may not only depend on their taxonomic classification but also on their life-style.

Acknowledgments—We are grateful to Dr. G. Kuschel for identifying the beetles and clarifying the taxonomic position of the aleocharine beetles. We also appreciate the technical assistance given on the mass spectrometer by Vivienne Paterson.

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HOST LOCATION IN *Olesicampe monticola*, A PARASITE OF LARVAE OF LARCH SAWFLY *Cephalcia lariciphila*

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Abstract—In the laboratory, ovipositor probing in mated female *Olesicampe monticola* was elicited by fresh *Cephalcia lariciphila* frass but not by aged frass or freshly spun larval silk. Second instar sawfly larvae and *Larix* needles also elicited a low level of ovipositor probing. Extracts of frass in dichloromethane, but not hexane, were also active. No difference in response was found to frass from *C. lariciphila* larvae fed on three varieties of *Larix*, (*L. kaempferi*, *L. decidua* and *L.* × *eurolepis*).

Key Words—*Olesicampe monticola*, Hymenoptera, Ichneumonidae, *Cephalcia lariciphila*, Pamphiliidae, parasitism, kairomone, biological control.

INTRODUCTION

Host-habitat finding and host location (Doutt, 1964) in parasitic insects is directed by a variety of long- and short-range cues. Host location, when in the host habitat, may be directed by visual or olfactory cues. Monteith (1958) showed that movement provided a strong visual stimulus to *Drino bohemica* Mesn. when searching for its host, *Neodiprion lecontei* (Fitch). Weseloh (1976, 1977) showed that water-soluble chemicals (kairomones) stimulated the searching behavior of the gypsy moth [*Lymantria dispar* (L)] parasite, *Apanteles melanoscelus* (Ratzeburg). Jones et al. (1971) demonstrated that 13-methylhentricontane, from the frass and larvae of *Heliothis zea* (Boddie), triggered short-range host-seeking responses in its parasite *Microplitis croceipes* (Cresson). Tricosane, from female moth scales, attracted *Trichogramma evanescens* to the eggs of a wide range of lepidopterous species (Jones

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et al., 1973). There is therefore a wide variety of sources of cues available to insect parasites which are seeking their hosts.

Olesicampe monticola Hedwig is a parasite of the web-spinnning larch sawfly *Cephalcia lariciphila* (Wachtl.). The female parasite attacks sawfly larvae which are feeding, under a protective layer of silk, on larch (*Larix* spp.) needles. Female parasites, probably attracted to the host habitat by host plant volatiles (Longhurst, unpublished) explore the foliage, vigorously tapping it with their antennae. When larval silk is encountered antennal tapping ceases and the female probes with her ovipositor. Eggs are laid only in unparasitized larvae (Billany, unpublished observations). Development of the parasite takes place in sawfly prepupae in the soil, the parasite pupating in May before emergence in late May and June.

In this paper some of the cues used by *O. monticola* in locating the host larvae are examined, using the distinctive behavior of ovipositor probing to bioassay various stimuli.

METHODS AND MATERIALS

Parasite Rearing

Parasite pupal cocoons were collected from the soil at Margam Forest (OS Ref. ST 969 847 and 935 815) in late May and stored at 0–2°C until required. Adults were eclosed singly under plastic pots and the sexes held separately in 300 × 300-mm muslin cages. Males and females (1 : 1 sex ratio) were placed together in similar cages one day before they were needed for assay.

Bioassay

Bioassays were carried out in a 100-mm-diam glass Petri dish with a filter paper cover on the floor; fresh Petri dishes were used for each replicate. Single females were placed in the Petri dishes, left for 2 min to settle, and then observed for 2 min. The number of ovipositor probes in this period was recorded.

Presentation of Bioassay Stimuli

C. lariciphila Larvae. A second instar *C. lariciphila* larva was placed on a 13-mm-diam filter paper placed 20 mm from the Petri dish wall. A piece of wood, 5 mm long and extracted in hexane (BDH) and dichloromethane, was placed on a second 13-mm filter paper 20 mm from the opposite wall to act as a control for movement.

C. lariciphila Frass. Ten fresh (<6 hr old) fecal pellets from second and third instar larvae feeding on *L. kaempferi* were placed in the petri dish on a

13-mm filter paper 20 mm from the Petri dish wall. Ten fecal pellets from first instar *Neodiprion sertifer* (Geoff.) larvae (fed on *Pinus sylvestris*) were placed 20 mm from the opposite wall to act as controls. These pellets were roughly the same size and shape as *C. lariciphila* faecal pellets; they were dried at 20° C for three weeks before use. Experiments were also carried out with *C. lariciphila* frass that had been kept at 20° C for three days before use. For other experiments, frass from larvae fed on other varieties of larch was used.

Larval Silk. Silk was obtained by holding the end of the silk exudate in clean forceps and, as the larvae spun downwards, the silk was wound 10 times around a 13-mm filter paper. Silk was also collected from around feeding larvae for assay; this silk had been contaminated with frass.

Extracts of Frass. Extracts of larval frass were made in redistilled dichloromethane or hexane (0.5 g/ml). Aliquots (100 μl) were presented to females on 13-mm filter papers after allowing the solvent to evaporate (10 min).

Experiments

Experiments 1–13 were paired as follows in the Petri dish assay: (1) second instar larva against model larva; (2) second instar larva against *C. lariciphila* frass; (3) *C. lariciphila* frass against *Neodiprion* frass; (4) *C. lariciphila* frass against aged *C. lariciphila* frass; (5) *C. lariciphila* frass against *C. lariciphila* silk (from feeding larva); (6) *C. lariciphila* frass against *C. lariciphila* silk (freshly spun); (7) *C. lariciphila* frass against *Larix kaempferi* needles (3, 3 mm long); (8) extract of frass in dichloromethane against dichloromethane; (9) extract of frass in hexane against hexane; and (10) extract of frass in dichloromethane against extract of *L. kaempferi* needles in dichloromethane.

In experiments 3–10 the frass was collected from larvae fed on Japanese larch (*L. kaempferi*).

Experiment (11) *C. lariciphila* frass (*L.* × *eurolepis*) against *Neodiprion* frass; (12) *C. lariciphila* frass (*L. kaempferi*) against *Neodiprion* frass; (13) *C. lariciphila* frass (*L. decidus*) against *Neodiprion* frass.

The positions of the two treatments in the Petri dish were allocated at random for each replicate.

RESULTS

After mating female *O. monticola* can be observed searching through the larch foliage. When a suitable *C. lariciphila* larva is encountered, the female inserts her ovipositor into the larva where an egg is laid. (Billany, unpublished results).

In Petri dish assays second instar *C. lariciphila* larvae were found to

release more ovipositor probing than wooden controls for movement (Table 1, experiment 1). When *C. lariciphila* frass was assayed alongside larvae, it was found to elicit a significantly stronger reaction than the larvae. (Table 1, exp. 2). When the *C. lariciphila* frass was tested against *Neodiprion* frass, a control for size and texture (Table 1, exp. 3), the *C. lariciphila* frass elicited the same high level of ovipositor probing as in experiment 2. If the *C. lariciphila* frass (Table 1, exp. 4) was aged for 3 days, it no longer released such a high level of ovipositor probing as found with fresh frass.

Silk did not release ovipositor probing in the female parasite when it had been collected directly from the spinnerets (Table 1, exp. 6), although it was active (exp. 5) when collected from feeding larvae. This silk had frass particles on it, which were removed before assay, but which were probably responsible for the activity.

Experiment with larch needles (Table 1, exp. 7) indicated that the needles

TABLE 1. RESPONSE OF FEMALE *Olesicampe monticola* TO HOST-RELATED STIMULI

Experiment	Mean number of ovipositor probes/2 min			N	P (t test)
1	2nd instar *Cephalcia* larvae	vs.	Model control		
	2.7		0.0	5	<0.001
2	2nd instar *Cephalcia* larvae	vs.	*Cephalcia* frass		
	2.2		9.4	10	<0.01
3	*Cephalcia* frass	vs.	*Neodiprion* frass		
	8.6		0.0	10	<0.001
4	*Cephalcia* frass (fresh)	vs.	*Ceph.* frass (aged)		
	9.1		2.2	5	<0.001
5	*Cephalcia* frass	vs.	Silk from feeding larvae		
	8.4		6.6	5	NS
6	*Cephalcia* frass	vs.	*Cephalcia* silk (from spinnerets)		
	7.8		0.2	10	<0.001
7	*Cephalcia* frass	vs.	*Larix* needles		
	9.7		2.3	5	<0.01
8	*Cephalcia* frass (dichloromethane ext.)	vs.	Dichloromethane (Control)		
	5.4		0.0	5	<0.001
9	*Cephalcia* frass (hexane extract)	vs.	Hexane (control)		
	1.0		0.0	5	NS
10	*Cephalcia* frass (dichloromethane ext.)	vs.	*Larix* needles (dichloromethane)		
	4.8		0.8	5	<0.01

TABLE 2. RESPONSE OF FEMALE *Olesicampe monticola* TO FRASS FROM *Cephalcia lariciphila* LARVAE FED ON THREE VARIETIES OF LARCH

Experiment	Mean number of ovipositor probes/2 min			N	P^b
	Larch variety[a]		*Neodiprion* frass control		
11	Hybrid	6.5*	0.0	10	<0.001
12	Japanese	7.9*	0.0	10	<0.001
13	European	7.1*	0.0	10	<0.001

[a]Means followed by the same symbol between frass of different larch varieties are not significantly different at $P = 0.05$. One-way ANOVA followed by Newman-Keuls multiple-range test.
[b]P for t test between frass from a larch variety and its *Neodiprion* frass control.

were only slightly effective at releasing ovipositor probing when compared to frass. This may indicate that the active compounds are present in low concentrations in the host plant. Solvent extraction of frass (exp. 8 and 9) indicated that the active components in releasing probing were extractable in dichloromethane but not in hexane. Extracts of larch needles (Table 1, exp. 10) had a slight behavioral effect but not as great as that of frass extracts.

Three main varieties of larch are grown in Britain: Japanese larch, hybrid larch, and European larch. These three varieties show differences in the relative proportions of chemical constituents (Longhurst, unpublished results). In view of these differences frass was collected from larvae fed on the three varieties and tested against female parasites to establish if there were any differences in response. The number of ovipositor probes was not significantly different between frass collected from larvae fed on the three varieties of larch (Table 2).

DISCUSSION

After the parasite has entered the host habitat and mating has occurred, host location takes place. In *O. monticola* cues for ovipositor probing into host larvae are chemical ones contained in the frass. Although the host larva spins a silk web around itself, probably for protection, pellets of frass stick to the webbing thus providing a stimulus for ovipositor probing. No experiments were carried out to ascertain if frass from later instar (4 and 5) larvae was also active, although it is likely to be so as female parasites were observed probing through silk containing late instar larvae. As the larvae increase in size, the area of silk webbing containing trapped frass pellets increases and does not reflect the position of the *C. lariciphila* larvae. Old frass was found not to release ovipositor probing (Table 3, exp. 4). This would increase the probability of probes successfully encountering a host larva which would have fresh frass near to it.

In *Microplitis croceipes*, the parasite of the corn earworm, *Heliothis zea*, intense host searching behavior is released by 13-methylhentricontane (Jones et al., 1971). Silk webbing of *Heliothis virescens* (F.) larvae was effective in eliciting examination behavior by the parasite *Campolitis sonorensis* (Cameron) when collected from around the host but not when spun fresh from spinnerets (Wilson et al., 1974). A similar situation was found in *O. monticola* (Table 1, exp. 5 and 6) and in both cases was probably due to contamination of old silk by contact with the behaviorally active frass.

In contrast to *O. monticola*, *M. croceipes*, and *C. sonorensis*, *Apanteles melanoscelus*, the parasite of the gypsy moth (*Lymantria dispar*), responds mainly to water-soluble kairomones from the silk glands which are absorbed onto the silk web of the host (Weseloh, 1976).

C. lariciphila feeds on three varieties of larch in Britain. The needles contain differing relative proportions of chemical components, and frass extracts contain other chemicals, possibly detoxification products, again in differing proportions in the three varieties (Longhurst, unpublished results). Despite these variations, no significant differences were found in the response of *O. monticola* to frass from larvae fed on Japanese, hybrid, or European larch. This suggests that the presence of a chemical stimulus, rather than its relative concentration in the frass, is important in releasing ovipositor probing in *O. monticola.*

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FEEDING AND BORING BEHAVIOR OF THE BARK BEETLE, *Ips paraconfusus*,[1] IN EXTRACTS OF PONDEROSA PINE PHLOEM[2,3]

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Abstract—Male *Ips paraconfusus* Lanier bored and fed in cellulose powder substrates treated with solvent extracts of ponderosa pine phloem in preference to cellulose powder alone. Stimuli that elicit boring and feeding behavior occurred in the water extracts and the combined solvent extracts. No significant boring or feeding occurred in the methanol extract. There was a preference for, but no significant feeding in, the water partition of the ether extract. Feeding, but no preferential boring, occurred in the ether extracts.

Key Words—*Ips paraconfusus*, Coleoptera, Scolytidae, phloem, ponderosa pine, host selection, feeding stimulants, extracts, bark beetle.

INTRODUCTION

The bark beetle *Ips paraconfusus* Lanier utilizes the phloem tissue of various species of pine, particularly ponderosa pine [*Pinus ponderosa* (Laws.)] in California and southern Oregon for the development of all immature stages. The adult beetles colonize both living trees and recently felled logging debris. They do not colonize the firs (*Abies*). A recent study (Elkinton and Wood, 1980) has shown that a male *I. paraconfusus* accepts the host tree, ponderosa

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pine, but rejects the nonhost, white fir (*Abies concolor* Lindl.), only after it has bored into the phloem tissue of either of these two tree species. The preference for pine over fir occurs with phloem that has been ground into a powder as well as with unground tissue. These findings are consistent with the hypothesis that there are chemical stimuli in the phloem that elicit acceptance of the host and/or rejection of the nonhost. Most research on host selection with other insects has focused on such chemical stimuli. Several phagostimulants and feeding deterrents have been identified for other bark beetles (Baker and Norris, 1967; Gilbert et al., 1967; Baker et al., 1968; Levy et al., 1974; Doskotch et al., 1970, 1973). However, it is conceivable that the preference for pine over fir ground phloem was caused by textural differences in the ground up tissue of these two tree species. In this study we looked for evidence of chemical stimuli in extracts of ponderosa pine phloem that elicit boring and feeding behavior.

METHODS AND MATERIALS

Design of Experiments. Male *I. paraconfusus* were presented with a choice between pairs of alternative treatments consisting of ground ponderosa pine phloem or various solvent extracts thereof. These extracts were added to a substrate of powdered cellulose (Alphacel, ICN Pharmaceuticals Inc.) and packed firmly into No. 1 gelatin capsules with the short end removed. The two capsules containing alternative treatments were then glued parallel to each other about 2 cm apart onto a piece of 7-cm Whatman No. 5 filter paper and placed in a 9-cm plastic Petri dish. At the start of an experiment, one male beetle was placed in each Petri dish halfway between the 2 gelatin capsules, and the Petri dish cover was replaced. The initial orientation of beetles was noted and subsequent boring activity in the Alphacel media was observed periodically for the next 24 hr. At the end of 24 hr the location of the beetles was recorded and boring activity was determined by the presence or absence of frass on the filter paper in front of each gelatin capsule. All experiments began and ended at 1700 hr. At the end of the experiment, the beetles were stored at −40° C for subsequent dissection to determine the presence of ingested material in their hindguts.

Each experiment consisted of 35–40 replicate Petri dishes. All experiments were conducted in a growth chamber at a constant temperature of 20° C and a 16–8 hr light–dark cycle synchronized with the concurrent natural light period. The experiments were run during April and May 1978. Light came from four incandescent and two fluorescent bulbs at the top of the growth chamber above a sheet of white contact paper which transmitted at a uniform intensity of 700 lux onto the beetles. The walls of the chamber were white and all dark objects that might elicit visual orientation were covered with white paper.

The left-to-right arrangement of the two alternative gelatin capsules in each Petri dish was alternated sequentially to cancel the effects of any possible environmental gradients on the behavior of the beetles. Several drops of water were sprinkled on the filter paper in each Petri dish to maintain the relative humidity near 100% for the duration of the experiment.

Source of Phloem and Beetles. The ponderosa pine phloem came from a tree measuring 25 cm in diameter at the base. The tree was cut near Bass Lake, Madera County, California, in October 1977 and transported to Berkeley. The phloem tissue was immediately removed, frozen, and ground into a fine powder as described by Elkinton and Wood (1980).

The beetles used in these experiments emerged inside cages (Browne, 1972) from naturally infested ponderosa pine logging debris collected in April 1978 at Blodgett Experimental Forest, Eldorado County, California, or from near Bass Lake. After emergence, the beetles were held for no more than 6 days at 4 °C on moist paper towels in paper cartons until the start of the experiments.

Extraction of Phloem. One hundred grams of ground phloem (wet weight) were removed from the freezer and subjected to continuous soxhlet extraction with 500 ml of diethyl ether for 24 hr in a water bath at 50–53° C. The ether extract was vacuum evaporated to a volume of 150 ml and then partitioned three times in a separatory funnel with 50-ml aliquots of water. An emulsion layer formed at the interface of the ether and water fractions. The ether, water, and emulsion layer were collected separately. The emulsion layer was frozen at −10° C and melted at room temperature, causing separation into ether and water layers which were then added to their respective solvent fractions.

Following ether extraction, the ground phloem was vacuum evaporated at room temperature to remove the remaining ether and then soaked in a series of water washes for a total of 24 hr to yield a total of 1100 ml of water extract. This extract was filtered through GF/A glass fiber filter paper in a Buchner funnel and vacuum evaporated at room temperature to an approximate volume of 150 ml.

Following water extraction, the ground phloem was vacuum dried to remove the remaining water and soxhlet extracted under partial vacuum with 600 ml of methanol for 24 hr in a water bath at 65–72° C. The methanol extract was then vacuum evaporated at room temperature to a volume of 110 ml.

The water extract, the methanol extract, and the water partition of the ether extract were each centrifuged at 15,000 rpm at 0° C for 20 min to remove particulate matter. The supernatants were each vacuum evaporated to a volume of 40 ml and stored at −10° C.

After extraction the ground phloem tissue was vacuum dried at room temperature to remove all traces of solvent and then stored at −10° C. The entire sequence of solvent extraction is summarized in Figure 1.

Preparation of Extract Medium. To determine its water content, ground

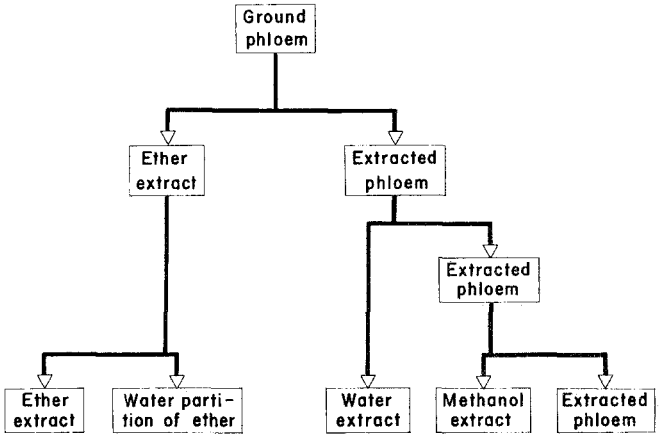

FIG. 1. Flow diagram of solvent extraction of ground ponderosa pine phloem. April–May 1978, Berkeley, California.

ponderosa pine phloem was freeze-dried for 76 hr and found to have a dry weight of 40% of the original wet weight. Consequently the solvent extracts of each 100 g wet weight of ground phloem was mixed with 40 g of Alphacel and 60 ml of water to achieve a concentration and water content equivalent, at least in theory, to that of the natural tissue. However, some losses may have occurred in the extraction process. The Alphacel and ground phloem treatments were mixed with 1% (by weight) of methyl *p*-hydroxybenzoate to inhibit the growth of mold and 5% powdered lubricating graphite to give all treatments a consistently dark grey color. This ensured that a choice between two treatments would not be based on obvious differences in color. Water was added back to the extracted phloem in the same proportion as the Alphacel.

The ether and methanol extracts were added to the Alphacel and then vacuum evaporated for 20 min at room temperature to remove the solvents. The Alphacel-alone treatments were also mixed with solvents, but without the extracts, and vacuum evaporated in the same way. In some experiments the water extract and/or the water partition of the ether extract were mixed into the water added to the Alphacel.

Ground ponderosa pine phloem had a pH of 4.45 as measured directly (no water added) with a Beckman pH meter. Alphacel, however, when mixed with water 2 parts to 3, had a pH of 3.5. Consequently, KOH solution was added drop by drop to all extract and Alphacel treatments until a pH of 4.45 ± 0.1 was attained.

After the Alphacel medium or ground phloem had been packed into the gelatin capsules, a hole 5 mm deep was made with a 100-μl pipette in the center of the exposed surface of the medium. This hole provided a site where the beetles could initiate boring.

Tabulation of Results. This study consisted of 12 experiments, each with two alternative treatments (Table 1 and 2). The four solvent extracts were presented separately or were mixed together in a treatment labeled "combined extract." The other treatments consisted of Alphacel without any extracts added and ground ponderosa pine phloem both before and after extraction. The number of beetles found on each treatment after 24 hr is listed in Table 1 and the number boring in each treatment is listed in Table 2. The numbers in Table 2 are, therefore, a subset of the numbers in Table 1. These numbers are

TABLE 1. NUMBER OF *I. paraconfusus* MALES IN CONTACT WITH PONDEROSA PINE PHLOEM AND CELLULOSE POWDER (ALPHACEL) TREATED WITH VARIOUS PHLOEM EXTRACTS OFFERED IN PAIRED TESTS. BERKELEY, CALIFORNIA, APRIL–MAY 1978

Experiment No.	Treatment A	No. of beetles on A	Treatment B	No. of beetles on B	Proportion on A (A/A + B)	Confidence interval for proportion on A[a]	
1.	Phloem	36	Alphacel	3	0.923	(0.717–0.983)*[b]	a[c]
2.	Combined extract	34	Alphacel	5	0.872	(0.654–0.961)*	ac
3.	Phloem	31	Combined extract	8	0.795	(0.568–0.919)*	acde
4.	Extracted phloem	13	Combined extract	25	0.342	(0.169–0.572)	b
5.	Phloem	20	Combined extract (3× conc.)	20	0.500	(0.294–0.706)	bc
6.	Water extract	26	Alphacel	13	0.667	(0.440–0.836)	ab
7.	Water extract (3× conc.)	35	Alphacel	6	0.854	(0.639–0.950)*	acd
8.	Ether extract	31	Alphacel	13	0.704	(0.489–0.856)	ab
9.	Ether extract (3× conc.)	17	Alphacel	25	0.405	(0.222–0.619)	be
10.	Water partition of ether	29	Alphacel	9	0.763	(0.532–0.901)*	ab
11.	Methanol extract	21	Alphacel	16	0.568	(0.344–0.766)	ab
12.	Water extract	13	Ether extract	17	0.433	(0.218–0.677)	ab

[a]Confidence intervals for binomial proportions cover simultaneously with a probability of 0.95.
[b](*) indicates that 0.5 is not contained within the confidence interval, and therefore the response to A is significantly different from the response to B.
[c]Any pair of treatments that shares the same letter is not significantly different by pairwise comparisons with a simultaneous error rate of $\alpha = 0.05$.

TABLE 2. NUMBER OF *I. paraconfusus* MALES BORING IN PONDEROSA PINE PHLOEM AND CELLULOSE POWDER (ALPHACEL) TREATED WITH VARIOUS PHLOEM EXTRACTS OFFERED IN PAIRED TESTS. BERKELEY, CALIFORNIA, APRIL–MAY 1978

Experiment No.	Treatment A	No. of beetles in A	Treatment B	No. of beetles in B	Proportion in A (A/A + B)	Confidence interval for proportion in A[a]	
1.	Phloem	36	Alphacel	2	0.947	(0.745–0.991)*[b]	a[c]
2.	Combined extract	28	Alphacel	3	0.903	(0.660–0.978)*	ac
3.	Phloem	31	Combined extract	7	0.816	(0.587–0.932)*	ac
4.	Extracted phloem	11	Combined extract	24	0.314	(0.114–0.544)	b
5.	Phloem	18	Combined extract (3× conc.)	18	0.500	(0.285–0.715)	bc
6.	Water extract	23	Alphacel	9	0.719	(0.467–0.882)	ab
7.	Water extract (3× conc.)	29	Alphacel	3	0.906	(0.668–0.979)*	ac
8.	Ether extract	29	Alphacel	13	0.690	(0.470–0.848)	ab
9.	Ether extract (3× conc.)	14	Alphacel	18	0.437	(0.226–0.647)	bc
10.	Water partition of ether	20	Alphacel	7	0.741	(0.466–0.903)	ab
11.	Methanol extract	17	Alphacel	16	0.515	(0.290–0.735)	bc
12.	Water extract	11	Ether extract	13	0.458	(0.243–0.702)	ab

[a]Confidence intervals for binomial proportions cover simultaneously with a probability of 0.95.
[b](*) indicates that 0.5 is not contained within the confidence interval and, therefore, the response to A is significantly different from the response to B.
[c]Any pair of treatments that shares the same letter is not significantly different by pairwise comparisons with a simultaneous error rate of $\alpha = 0.05$.

also expressed as proportions along with a confidence interval for each proportion. The confidence intervals are constructed with an error rate of $\alpha = 0.0087$ for each interval. This assures that all 12 intervals cover simultaneously with an error rate of $\alpha = 0.05$ (Miller, 1966). If the proportion of beetles found on or boring in treatment A (Tables 1 and 2) is significantly greater than 0.5, we conclude that there is a preference for A over B. This occurs when 0.5 is less than the lower cutoff point of the confidence intervals. A preference for B over A occurs when 0.5 is greater than the upper cutoff point.

Differences between treatments were analyzed by Pearson's chi-square test of hypothesis that all treatments have the same proportion. When this hypothesis was rejected, indicating differences among treatments, simultaneous pairwise comparisons (Miller, 1966, Chap. 6, Sect. 2.2) were used to determine which treatments differed.

Of the beetles that bored in the various substrates from all 12 experiments we conclude that feeding occurred if the hindguts contained solid materials (Table 3). *I. paraconfusus* males emerging from their brood trees have no such material in their guts (Elkinton et al., 1980). Only those beetles that bored in just one of the two alternative treatments are included in this list. Thus, we can identify with certainty which treatment the beetles ingested. When a particular treatment such as Alphacel alone was used in more than one experiment, the number of beetles that bored in that treatment were combined from all experiments in which the treatment occurred.

RESULTS

It is clear that *I. paraconfusus* males remained in contact with and bored into both ground phloem and the combined solvent extract more often than untreated Alphacel (Tables 1 and 2). On the other hand, the ground phloem

TABLE 3. HINDGUT CONTENTS OF MALE *I. paraconfusus* THAT BORED FOR 24 HR IN PONDEROSA PINE PHLOEM AND CELLULOSE POWDER (ALPHACEL) TREATED WITH PHLOEM EXTRACTS. BERKELEY, CALIFORNIA, APRIL–MAY 1978

Treatments	No. of beetles with guts		Proportion with empty guts	Confidence interval[a] for proportion with empty guts	
	Empty	Full			
Alphacel	56	8	0.875	(0.715–0.951)	a[b]
Phloem	8	72	0.100	(0.039–0.234)	b
Extracted phloem	7	7	0.500	(0.198–0.802)	ab
Combined extract	12	19	0.387	(0.188–0.632)	bcd
Combined extract (3× conc.)	1	11	0.083	(0.008–0.492)	bc
Water extract	14	1	0.933	(0.572–0.993)	a
Water extract (3× conc.)	7	18	0.280	(0.106–0.561)	bcd
Ether extract	3	16	0.158	(0.036–0.482)	bcd
Ether extract (3× conc.)	3	9	0.250	(0.058–0.642)	bcd
Water partition of ether extract	12	6	0.667	(0.349–0.882)	ac
Methanol extract	11	4	0.733	(0.379–0.925)	ad

[a]Confidence intervals for binomial proportions cover simultaneously with a probability of 0.95.
[b]Any pair of treatments that shares the same letter is not significantly different by pairwise comparisons with a simultaneous error rate of $\alpha = 0.05$ (Miller, 1966, Chap. 6, Sec. 2.2).

was preferred to the combined extract (Exp. 3). However, when the concentration of the combined solvent extract was tripled (Exp. 5), the beetles then bored into the ground phloem and the combined extract in equal numbers. When the choice was offered between the combined extract at the 1× concentration and the ground phloem after extraction with all solvents (Exp. 4), 24 beetles bored in the extract compared to only 11 in the phloem (Table 2), but this apparent difference is not statistically significant. However, the phloem after extraction (Exp. 4) elicited significantly less boring activity than before extraction (Exp. 3).

Some of the individual solvent extracts elicited boring activity (Exp. 6–12, Tables 1 and 2). In experiment 6 with water extract at 1× concentrations, 26 beetles were found on the extract vs. 13 on the Alphacel (Table 1), although this did not represent a significant difference. However, when the concentration of the water extract was tripled (Exp. 7), there was a significant boring response in favor of the extract.

With the ether extract at 1× concentrations (Table 1, Exp. 8), 31 beetles were found in contact with the extract vs. only 13 in the Alphacel. Although this did not represent a statistically significant preference for the ether extract at $\alpha = 0.05$, it was significant at $\alpha = 0.10$. Tripling the concentration of the ether extract (Exp. 9) did not produce a significant response in favor of the ether as it did with the water extract.

The number of beetles on the water partition of the ether extract (Table 1, Exp. 10) was also significantly greater than the number on Alphacel alone. There was no significant response in favor of the methanol extract (Tables 1 and 2, Exp. 11). Nor was there any preference exhibited when the beetles were given a choice between the water and ether extracts (Tables 1 and 2, Exp. 12). The preference for the combined extract over Alphacel alone was clear-cut, (Table 1 and 2, Exp. 2), whereas the preferences for the individual extracts were barely or not significant, except for the water extract at 3× concentration.

Only a small proportion of beetles fed in the Alphacel (8/64), whereas most of the beetles fed in the phloem (72/80) (Table 3). The feeding response to the various extracts may be compared to these two extremes. In comparison to Alphacel, significantly more beetles fed in the combined extract at 1× and 3× concentration, the ether extract at 1× and 3× concentration, and the water extract at 3× concentration. The proportions of beetles that fed in these five treatments were not statistically different from the proportion that fed in phloem.

The proportions feeding in the methanol extract, the water partition of the ether extract, and the water extract at 1× concentration were not statistically different from the proportion that fed in Alphacel. The feeding response to the phloem was significantly greater than any of these three extracts.

It is clear from the analysis of gut contents that feeding behavior generally coincides with the boring behavior already described. There was significant boring and feeding behavior in the water (3×) and combined extracts and in the phloem. There was no significant feeding or boring behavior in the methanol extract. However, beetles were in contact with the water partition of the ether extract preferentially over Alphacel (Table 1, Exp. 10), but only 6/18 fed in it. In contrast 16/19 fed in the ether extract at 1× and 9/12 at 3× concentration (Table 3), but there was no preferential contact or boring activity in these extracts over Alphacel.

DISCUSSION

The results demonstrate clearly that there are soluble chemical stimuli in ponderosa pine phloem that elicit boring and feeding behavior. These stimuli may largely account for host selection. It is not yet clear, however, how boring and feeding behavior interact to produce host acceptance. Boring behavior does not invariably lead to acceptance, for *I. paraconfusus* will often bore in the nonhost, white fir, before subsequently rejecting it (Elkinton and Wood, 1980). On the other hand, boring behavior always precedes acceptance, because the beetles must bore through the outer bark prior to reaching the phloem and before selection occurs (Elkinton and Wood, 1980). Very few beetles in these experiments remained in contact with any of the treatments without initiating boring. The small number of beetles (16%) that did not bore (the difference between Table 1 and Table 2) were found equally on the phloem, the phloem extracts, and the untreated Alphacel substrates. Consequently, it was only the beetles that exhibited boring behavior (Table 2) that caused the observed preferential distribution of beetles (Table 1) in favor of phloem and certain phloem extracts vs. Alphacel.

The results of this study demonstrated that a strong boring preference for a particular extract is usually accompanied by feeding in that extract. However, beetles were distributed in favor of the water partition of the ether extract (Table 1, Exp. 10) but fed very little in that treatment. Dethier (1953) has described host selection as a sequence of behavior that consists of orientation to the host, initial biting response, and sustained feeding. Here we had evidence of "preference" and prolonged boring activity but little feeding. Throughout the entire series of experiments there were individual beetles who bored extensively but did not feed. Perhaps the stimuli that elicit boring and those that elicit feeding are different. Such a conclusion is suggested by a previous study (Elkinton and Wood, 1980), where boring in the outer bark occurred in both host and nonhost without feeding.

We must also recognize the possible important role of deterrent stimuli in the host selection of this insect. The feeding and boring stimuli which were

evident in this study may be compounds that occur widely in host and nonhost plants. Selection of the host pines may depend on the absence of deterrent stimuli that are present in nonhost trees. Several workers have emphasized the importance of such deterrent stimuli, and deterrent receptors have been identified in some insects (Gupta and Thorsteinson, 1960; Jermy, 1966; Schoonhoven, 1972).

Stimuli that elicit feeding behavior were present in both the water and ether extracts. The occurrence of stimuli in both these solvents suggests that more than one compound may elicit this behavior, as relatively few compounds would be soluble in both. Any compound that was extracted by the ether and was soluble in water would also have gone into the water partition of the ether at least to some degree. Although beetles were distributed on this fraction preferentially, they fed very little on it. In other studies of insect host selection, the phagostimulants in the host plant have been shown, almost without exception, to involve several compounds rather than just one (see reviews by Schoonhoven, 1968, 1972; Hedin et al., 1974).

If there were two or more active compounds in these extracts, their activity could have been either additive or synergistic. With synergy we might have expected little response to the individual components but a strong response to the combined extracts. Since there was no significant difference between the response to the combined solvent extract and to those of three of the individual extracts (water, methanol, and ether), synergy was not apparent in this study. However, a series of concentrations of these extracts needs to be tested before firm conclusions can be made about synergy.

Since the methanol extraction was preceded by extractions with water and ether, the lack of activity in the methanol extract could be expected. Many of the compounds removed by the two prior extractions might otherwise have come out with the methanol, which is intermediate in polarity between ether and water.

Although the dry weight of the extracted phloem was equal to the dry weight of the Alphacel to which the extracts were added, we might have expected the concentration of compounds in the Alphacel to be less than that of the phloem tissue because of unavoidable losses during extraction and concentration. First of all, extraction was probably not complete. There was evidence, although inconclusive, that some activity remained in the phloem after extraction. Second, some of the activity may have been destroyed by heating of the solvents during soxhlet extraction. Finally some of the volatile components (e.g., terpenes) may have been lost in the vacuum evaporation of solvents. Consequently, it is not surprising that the response to the water and combined solvent extracts increased when the concentration was tripled. The most puzzling result was the apparent decrease in response to the ether extract as the concentration was tripled. This decrease was not statistically significant and thus awaits corroboration, but we might explain it as follows: The

amount of chemical stimulus available to a beetle as it bites into the phloem may only be a small proportion of the compound present in the tissue. The compound may be bound up in the living tissue such that very little is actually tasted by the beetles. The solvent extraction, on the other hand, might remove most of the compounds and, when mixed with Alphacel, it might be more available and thus at a higher effective concentration than in the living tissue. With the 3× ether extract, we may have attained concentrations at which certain ether-soluble compounds become deterrents. A high proportion of the beetles, however, did feed on this extract (Table 3).

Whatever the losses or changes that may have occurred during extraction, there was no significant or apparent difference in the boring or feeding response in the unextracted phloem and the combined extracts at 3× concentration. This result suggests that we had successfully extracted most of the stimuli that elicit boring and feeding behavior in the natural tissue.

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Journal of Chemical Ecology, Vol 7, No. 1, 1981

Obituary

On June 26, 1980, Dr. Ada S. Hill died after a brief illness. For the past decade, she has been a research associate at the New York State Agricultural Experiment Station, Geneva, New York, with Dr. Wendell L. Roelofs in pheromone research. In that time, she has identified the pheromone blend of over 25 lepidopterous species. She has developed a number of international cooperative projects and was a devoted friend to scientists around the world, who are saddened by her untimely death. A memorial has been established with the Experiment Station library to fund a continuing subscription to the *Journal of Chemical Ecology*. Contributions may be sent to Dr. Ada S. Hill Fund, c/o Librarian, New York State Agricultural Experiment Station, Geneva, New York 14456 U.S.A.

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Announcement

CHEMICAL SIGNALS III

The Third International Symposium on Chemical Signals in Vertebrates will be held April 11–13, 1982 at the Sarasota-Hyatt Hotel, Sarasota, Florida in conjunction with the Annual Meeting of the Association for Chemoreception Sciences (AChem S) which will follow on April 14–16, 1982 at the same hotel.

For further information contact Dr. D. Müller-Schwarze or Dr. R. M. Silverstein at State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210. Telephone: 315-470-6801 or 6743 or 6852.

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THE NASONOV PHEROMONE OF THE HONEYBEE *Apis mellifera* L. (HYMENOPTERA, APIDAE). Part II. Bioassay of the Components Using Foragers

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Abstract—The Nasonov pheromone of the honeybee comprises seven components, (*Z*)-citral, nerol, geraniol, nerolic acid, geranic acid, and (*E,E*)-farnesol. Bioassay of individual components showed each attracted foraging bees. A mixture of components in proportions present in the honeybee was as attractive as the natural secretion, and each component contributed to the attractiveness of the mixture. Honeybees responded anemotactically to the source of Nasonov odor. The presence of footprint pheromone enhanced the attractiveness of the synthetic Nasonov mixture. Nasonov and footprint pheromones may prove useful in attracting honeybees to crops needing pollination.

Key Words—Honeybee, *Apis mellifera*, Hymenoptera, Apidae, pheromone, Nasonov pheromone, footprint pheromone, bioassay, anemotaxis, geraniol, nerobic acid, (*Z*)-citral, geranic acid, (*E,E*)-farnesol.

INTRODUCTION

The Nasonov gland of the worker honeybee produces a volatile pheromone that attracts other workers. The pheromone has been reported to comprise geraniol, nerolic acid, and possibly (*Z*)- and (*E*)-citral and geranic acid (Boch and Shearer, 1962, 1964; Weaver et al., 1964; Butler and Calam, 1969). More recently, Pickett et al. (1980) confirmed the presence of these five components, identified nerol and (*E,E*)-farnesol in the secretion, and determined the average amounts of components present per insect.

Workers release the Nasonov pheromone when foraging, either at a source of water (Free and Williams, 1970) or artificial food, such as a dish of sugar syrup (von Frisch, 1923; Free and Williams, 1972). Although earlier, some of the components had been assayed for attractiveness to foraging

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honeybees (Free, 1962; Weaver et al., 1964; Boch and Shearer, 1964; Shearer and Boch, 1966; Butler and Calam, 1969; Waller, 1970), a mixture based on the amounts of the components in the natural pheromone had not been tested.

We have now tested the attractiveness, to foraging honeybees, of each Nasonov component (as identified by Pickett et al. (1980) and of a mixture of components in the amounts present in the gland, determined the contribution of each component to the attractiveness of the mixture, and compared the attractiveness of the synthetic mixture with that of the natural secretion.

Honeybees, foraging at a dish of sucrose syrup, without exposing their Nasonov glands, deposit a substance which attracts other foragers to the dish (Lecomte, 1957). Butler et al. (1969) also found that a substance, capable of attracting foragers to a feeding place, was deposited by crawling bees at their hive entrance and called the substance "footprint" pheromone. We have now compared the relative attractiveness of and interactions between Nasonov and footprint pheromones to foragers.

METHODS AND MATERIALS

Bioassay Technique. A modification of Butler and Calam's (1969) bioassay was used. Honeybees were trained to visit two glass Petri dishes (90 mm diameter) containing 50% sucrose syrup. The dishes were placed 300 mm apart along the diameter of a circular sheet of glass (600 mm diameter) supported on a turntable, 300 mm above ground level, on a large lawn. A perforated zinc cover enclosing the sides and top of each dish prevented contact with the dish but allowed foragers that landed on the cover to reach the syrup below. About 15 min before a test, sufficient syrup was put into the dishes to ensure that about 200 bees were visiting them.

Immediately before a test, the sheet of glass was replaced by a similar clean one and the dishes of syrup by two similar clean glass Petri dishes with perforated zinc covers. Liquid paraffin (1 ml) containing the test chemicals was distributed on a wad of five sheets of filter paper (70 mm diameter) in one dish (A); as a control, liquid paraffin (1 ml) either pure or containing other chemicals (later specified) was put on filter paper in the other dish (B). Liquid paraffin served as a diluent and slowed evaporation of test chemicals.

Because the orientation of honeybees to the test dishes was affected by the alignment of the dishes to wind direction, the diameter of the glass circle along which the dishes were placed was maintained crosswind during each test by rotating the glass sheet on its support as necessary.

For each test, the honeybees that landed on each dish during ten 1-min intervals were counted. After each 1-min count, the positions of the two dishes were reversed to prevent the bees from learning to associate an odor with dish

position, and the zinc covers were replaced by clean ones to prevent any footprint pheromone left by foragers from inducing others to land.

Each test was analyzed by a χ^2 test, and a dish was considered to be significantly ($p < 0.05$) more attractive than the other if more honeybees landed on it in 8 or more of the 10 counts. Unless otherwise stated each test was repeated ten times. The honeybees landing on the dishes were not seen to expose their Nasonov glands. Not more than two tests were conducted on the same site on the same day.

Chemicals. The preparation and purification of chemicals used for bioassay have been described by Pickett et al. (1980). The synthetic mixture of components (*Z*)-citral, 1:(*E*)-citral, 1:nerol, 1:geraniol, 100:nerolic acid, 78:geranic acid, 11:(*E*,*E*)-farnesol, 44, was dissolved in liquid paraffin so that each ml of solution contained the approximate absolute amounts of the components of 10, 20, 100, or 1000 Nasonov glands of foraging honeybees (see Pickett et al., 1980) unless specified otherwise.

Extraction of Natural Nasonov Pheromone from Glands. Two methods were used to obtain natural pheromone from honeybees captured as they left their hives to forage and killed by chilling at 5° C: (1) a "wipe" of a gland was obtained by squeezing the abdomen to expose the Nasonov groove and wiping it with a small piece of filter paper; (2) a whole gland was excised after pulling the sting apparatus and gut from the abdomen. Analysis of extracts by gas chromatography (Pickett et al., 1980) showed that the recovery of geraniol from wipes was 75% of that from glands.

Five, 20, or 40 wipes of glands were placed on the filter paper of the test dishes, and liquid paraffin (1 ml) was dripped onto them. In one test, the glands were washed before testing by dripping hexane (1 liter) onto them to remove the Nasonov pheromone.

Deposition of Footprint Pheromone on Zinc Covers. Footprint pheromone was obtained by inducing honeybees to walk on perforated zinc covers. Ten Petri dishes containing sucrose syrup and with perforated zinc covers were placed on the test table and bees were allowed to feed from them for about 15 min. The dishes of sucrose were then replaced with clean empty ones with clean zinc covers, and bees were allowed to land, walk on, and deposit footprint pheromone on the covers for 15 min. These covers contaminated with footprint pheromone were used immediately in tests.

RESULTS

Effect of Odor of Synthetic Nasonov Mixture on Recruitment of Foragers. The attractiveness to foragers of dishes containing 10, 20, 100, or 1000 gland equivalents of synthetic Nasonov mixture (in liquid paraffin) and liquid paraffin alone was compared.

TABLE 1. EFFECT OF ODOR OF SYNTHETIC NASONOV MIXTURE ON RECRUITMENT OF FORAGERS

Dish	No. of gland equivalents of synthetic Nasonov mixture in dish	No. of tests out of 10 in which A or B received significantly more visits	Total no. bees/test that landed on A or B (mean of 10 tests)
A	10	1	124
B	0	0	88
A	20	9	212
B	0	0	72
A	100	9	195
B	0	0	80
A	1000	10	155
B	0	0	70

In only one test did 10 gland equivalents receive more visits than liquid paraffin alone, but 20, 100, or 1000 gland equivalents received more visits than liquid paraffin in all or 9 of the 10 tests (Table 1).

The duration of the attractiveness of synthetic Nasonov mixture (100 gland equivalents) was determined by testing the same dishes containing the mixture at 2-hr intervals. Between tests, the dishes were kept uncovered in a laboratory at about 20° C. The synthetic mixture remained more attractive than liquid paraffin alone for at least 4 hr (Table 2). The rate of loss of geraniol (200 μg/1 ml liquid paraffin) from a wad of filter paper in a Petri dish kept at 20° C in a laboratory was monitored by gas chromatography. After 0.5, 4.0, and 6.0 hr, 100%, 65%, and 59%, respectively, of the geraniol remained (analysis procedure as for excised glands, Pickett et al., 1980).

TABLE 2. DURATION OF EFFECT OF ODOR OF SYNTHETIC NASONOV MIXTURE ON RECRUITMENT OF FORAGERS

No. gland equivalents of synthetic Nasonov mixture in dish		Time (hr) after first test	No. of tests out of 10 in which A or B received significantly more visits		Total no. bees (mean of 10 tests) that landed on	
A	B		A	B	A	B
100	0	0	10	0	238	86
		2	10	0	218	45
		4	8	0	123	47
		6	0	0	107	127
		8	0	0	59	68

TABLE 3. EFFECT OF PRESENCE OF ALL ISOMERS OF FARNESOL AND OF RELATIVE PROPORTIONS OF NEROLIC AND GERANIC ACIDS IN A SYNTHETIC NASONOV MIXTURE ON RECRUITMENT OF FORAGERS

Chemicals in dish		No. of tests out of 10 in which A or B received significantly more visits		Total no. of bees that landed on A or B (mean of 10 tests)	
A	B	A	B	A	B
Complete synthetic Nasonov mixture containing (*E*,*E*)-farnesol	Complete synthetic Nasonov mixture containing all 4 isomers of farnesol	0	0	155	167
Complete synthetic Nasonov mixture with nerolic and geranic acids in natural proportions (78 parts nerolic: 11 parts geranic, see section on chemicals)	Complete synthetic Nasonov mixture with nerolic and geranic acids in unnatural proportions (4 parts nerolic: 4 parts geranic)	1	0	182	182

TABLE 4. EFFECT OF ALIGNMENT OF TEST DISHES (EACH CONTAINING 100 GLAND EQUIVALENTS OF SYNTHETIC NASONOV MIXTURE) IN RELATION TO WIND DIRECTION ON RECRUITMENT OF FORAGERS

Dish	Alignment	No. of tests out of 10 in which A or B received significantly more visits	Total no. of bees/test that landed on A or B (mean of 10 tests)
A	Crosswind	0	160
B	Crosswind	0	162
A	Upwind	9	249
B	Downwind	0	39

The Nasonov pheromone contains only the *E,E* isomer of farnesol. To determine whether other isomers of farnesol diminished attraction, a synthetic mixture containing only the *E,E* isomer was compared with one containing the same amount of the *E,E* isomer and the other three isomers. The presence of other farnesol isomers did not influence attraction (Table 3).

A synthetic Nasonov mixture with nerolic and geranic acids in natural proportions (nerolic acid–geranic acid, 78:11) was compared with one containing less of both acids (nerolic acid–geranic acid, 4:4). The former was more attractive than the latter in one test only (Table 3).

Effect of Wind Direction on Orientation of Foragers to Source of

TABLE 5. EFFECT OF INDIVIDUAL NASONOV COMPONENTS ON RECRUITMENT OF FORAGERS

Chemicals in dish		No. of tests out of 10 in which A or B received significantly more visits		Total no. of bees that landed on A or B (mean of 10 tests)	
A	B	A	B	A	B
(*E*)-Citral (200 μg)	Nil	9	0	170	66
Geranic acid (200 μg)	Nil	9	0	191	84
Nerolic acid (200 μg)	Nil	7	0	163	98
Geraniol (200 μg)	Nil	4	0	165	105
Nerol (200 μg)	Nil	3	0	81	52
(*Z*)-Citral (200 μg)	Nil	3	0	118	99
(*E,E*)-Farnesol (200 μg)	Nil	1	0	101	91

Synthetic Nasonov Odor. Honeybees probably orient to an odor source by flying upwind towards it guided by the odor plume (Butler & Fairey, 1964).

To determine whether the number of honeybees that landed on two test dishes was affected by their alignment to wind direction, the attractiveness of two dishes, each containing 100 gland equivalents of synthetic Nasonov mixture, was compared in two sets of tests. In one, with the diameter of the glass circle along which the dishes were placed in line with the wind, one dish was upwind and one downwind; in the other, the diameter was crosswind.

When crosswind, similar numbers of honeybees landed on the two dishes; when along the wind, more landed on the upwind dish in most tests (Table 4). Therefore, dishes were placed crosswind in all other tests, which were confined to periods when a light steady wind was blowing (<3 m/sec).

Effect of Individual Nasonov Components on Recruitment of Foragers. The attractiveness of dishes containing individual Nasonov components (in liquid paraffin) and dishes containing liquid paraffin only was compared.

(*E*)-Citral and geranic acid were more attractive than liquid paraffin in most tests (Table 5); nerolic acid was also very attractive. Geraniol, nerol,

TABLE 6. EFFECT OF ABSENCE OF A COMPONENT FROM SYNTHETIC NASONOV MIXTURE ON ITS RECRUITMENT OF FORAGERS (100 GLAND EQUIVALENTS OF MIXTURE USED IN EACH TEST)

Chemicals in dish		No. of tests out of 10 in which A or B received significantly more visits		Total no. of bees/test that landed on A or B (mean of 10 tests)	
A	B	A	B	A	B
Complete synthetic Nasonov mixture	Mixture minus (*E*)-citral	8	0	161	53
	Mixture minus geranic acid	8	0	219	111
	Mixture minus (*E,E*)-farnesol	8	0	198	96
	Mixture minus nerolic acid	5	0	144	69
	Mixture minus nerol	5	0	160	71
	Mixture minus geraniol	4	0	170	99
	Mixture minus (*Z*)-citral	2	0	140	104

TABLE 7. COMPARISON OF EFFECT OF EXCISED NASONOV GLANDS OR WIPES OF GLANDS WITH SYNTHETIC NASONOV MIXTURE ON RECRUITMENT OF FORAGERS

Materials in dish		No. of tests out of 10 in which A or B received significantly more visits		Total no. of bees/test that landed on A or B (mean of 10 tests)	
A Natural pheromone	B Synthetic mixture (no. of gland equivalents)	A	B	A	B
20 wipes	0	10	0	133	66
40 wipes	0	10	0	105	15
20 wipes	20	0	2	162	160
40 wipes	20	9	0	256	108
20 glands	0	10	0	245	16
5 glands	20	3	0	181	175
20 glands	20	10	0	253	69
20 washed glands	0	0	0	176	174

(*Z*)-citral, and (*E,E*)-farnesol were more attractive than liquid paraffin alone in fewer than half of the tests.

Effect of Absence of a Component from Synthetic Nasonov Mixture on Its Recruitment of Foragers. To determine whether each component contributes to the full attractiveness of the synthetic Nasonov mixture, a dish containing the complete mixture (100 gland equivalents) and a dish containing the mixture from which a component had been omitted were compared.

Each mixture with a component missing was less attractive than the complete mixture, although the magnitude of the effect depended on the component omitted (Table 6). Omission of geranic acid, (*E*)-citral, or (*E,E*)-farnesol diminished attraction in most tests; omission of nerolic acid, nerol, geraniol, or (*Z*)-citral diminished attraction in fewer tests.

Comparison of Synthetic Nasonov Mixture and Natural Nasonov Pheromone on Recruitment of Foragers. Dishes containing synthetic Nasonov mixture and dishes containing either the excised Nasonov glands or filter paper wipes of the glands of foraging honeybees were compared.

Natural pheromone from excised glands or wipes was more attractive than liquid paraffin alone in all tests (Table 7).

The synthetic mixture and natural pheromone presented as wipes of glands were almost equally attractive. Twenty wipes were slightly less attractive than 20 gland equivalents of synthetic mixture in two tests; 40 wipes were more attractive.

However, the synthetic mixture was less attractive than the natural pheromone presented as excised glands. Twenty excised glands were more attractive than 20 gland equivalents of synthetic mixture, and five excised glands were as attractive in most tests. Glands from which the pheromone had been washed were not attractive; therefore glands were not visually attractive.

Comparison of Synthetic Nasonov Mixture and Footprint Pheromone on Recruitment of Foragers. Foragers were induced to walk on and deposit footprint pheromone on perforated zinc covers at the feeding site as described.

Dishes with footprint covers were visited more than dishes with clean covers both when the dishes were empty and when they contained synthetic Nasonov mixture (Table 8), indicating that a volatile substance attractive to foragers had been deposited on the footprint covers by honeybees walking over them and that this substance enhanced the attractiveness of Nasonov pheromone. Empty dishes with footprint covers and dishes containing synthetic Nasonov mixture (20 gland equivalents) but with clean covers were about equal in attractiveness.

To determine the duration of attractiveness of footprint pheromone, footprint covers were prepared and kept in a laboratory at 20° C for 1, 2, 4, 6, and 8 hr before testing. Some covers remained attractive for at least 6 hr (Table 9).

TABLE 8. COMPARISON OF EFFECT OF SYNTHETIC NASONOV MIXTURE AND FOOTPRINT PHEROMONE ON RECRUITMENT OF FORAGERS

No. of gland equivalents of synthetic Nasonov mixture in dish and type of cover		No. of tests out of 10 in which A or B received significantly more visits		Total no. of bees that landed on A or B (mean of 10 tests)	
A	B	A	B	A	B
0 + footprint	0 + clean	9	0	194	89
20 + footprint	20 + clean	9	0	286	79
0 + footprint	20 + clean	2	1	167	140

DISCUSSION

Each of the seven components of the Nasonov secretion identified by Pickett et al. (1980) attracted foraging honeybees individually and, although tests on different days and under different conditions of wind speed, etc., could not be compared strictly, some components appeared relatively more attractive than others.

Geraniol, the major component of the secretion was previously found to attract foragers (see Free, 1962; Boch and Shearer, 1964; Shearer and Boch, 1966; Butler and Calam, 1969). Weaver et al. (1964), Shearer and Boch (1966), and Butler and Calam (1969) found a mixture of (*Z*)-citral and (*E*)-citral attractive to foragers but, whereas we found (*E*)-citral considerably more

TABLE 9. DURATION OF EFFECT OF FOOTPRINT PHEROMONE ON RECRUITMENT OF FORAGERS

Type of cover		Time (hr) of test after preparation of covers	No. of tests out of 3 in which A or B received significantly more visits		Total no. of bees (mean of 10 tests) that landed on	
A	B		A	B	A	B
Footprint	Clean	1	3	0	209	62
Footprint	Clean	2	2	0	173	95
Footprint	Clean	4	1	0	134	94
Footprint	Clean	6	1	0	202	162
Footprint	Clean	8	0	0	163	164

attractive than (*Z*)-citral, Shearer and Boch (1966) found (*Z*)-citral and (*E*)-citral equally attractive. Boch and Shearer (1964) and Butler and Calam (1969) found a mixture of geranic and nerolic acids attractive but, whereas Shearer and Boch (1966) found geranic acid had no apparent attraction, we found geranic acid more attractive than nerolic acid. Both nerol and (*E*,*E*)-farnesol, the two most recently identified components of the Nasonov secretion, proved attractive in our tests. Farnesol (isomers unspecified) only attracted one forager to a feeder dish in 1½ hr when tested by Weaver et al. (1964).

The *E* isomers, (*E*)-citral, geraniol, and geranic acid, were more attractive in our tests than their corresponding *Z* isomers, (*Z*)-citral, nerol, and nerolic acid. Although *Z* isomers would be expected to be more volatile than *E* isomers, electrophysiological responses of excised worker antennae to *E* isomers were also greater than to the *Z* isomers (Williams et al., 1982). The consistent attractiveness of (*E*)-citral and geranic acid is interesting because we have recently demonstrated that a highly specific enzyme converts geraniol to (*E*)-citral and geranic acid in the gland (Pickett et al., 1981).

The full biological activity of the natural secretion had not previously been accounted for (Boch and Shearer, 1964; Shearer and Boch, 1966; Butler & Calam, 1969), probably because the components of the secretion had not all been identified and the mixtures tested did not contain the components in their natural proportions. The most active mixture tested consisted of citral (isomers not specified) and geraniol, but with proportionally more citral than in the natural secretion (Butler and Calam, 1969).

A synthetic mixture of the seven components in their natural proportions has now been found to attract foragers, and each component is important for full effect. Again, (*E*)-citral and geranic acid were the most attractive components. Isomers of farnesol not in the pheromone and unnatural proportions of nerolic and geranic acids in the synthetic mixture had little effect on its attraction to foragers. The synthetic mixture was as attractive as the natural pheromone on filter paper wipes, probably indicating that all the components of the secretion have now been identified. Excised glands were about four times more attractive than the synthetic mixture, possibly either because the glands and surrounding cuticle contained other attractants, for example, footprint pheromone, or because between excision and testing, geraniol was converted enzymically to the more attractive (*E*)-citral and geranic acid (Pickett et al., 1981).

The difference in attractiveness of the dishes according to their alignment to wind direction indicated that bees were orienting anemotactically to the odor source. Bees approached the test table upwind with their antennae extended forward. When the two dishes containing pheromone were cross-wind, equal numbers of bees landed on each, probably because any bees flying into overlapping zones of odor plumes downwind of the dishes had an equal chance of being attracted to either dish. But when the two dishes were along

the wind the upwind dish attracted more bees, probably because its odor plumes overlapped with that of the downwind dish, so that there was no behaviorally significant difference in the concentration of attractant in the two dishes. Bees flew past the downwind dish within the combined odor plumes and landed on the upwind dish. Wall and Perry (1978) similarly found that when lines of three pheromone traps for the pea moth (*Cydia nigricana*) were placed along the wind, the upwind trap caught more moths than either the central or downwind traps. Clearly, pheromone sources may mutually interact if their spacing is less than their range of attraction. Little is known of the range of attraction of the Nasonov pheromone. Although Butler (1970) thought that it operated over a short range only, probably not more than 100 mm, our results indicate that it is at least 300 mm, the distance between the two dishes at the feeding table. We are investigating this further.

Many workers (Ribbands, 1955; Lecomte, 1957; Butler et al., 1969; Ferguson and Free, 1979), have reported that honeybees landing on a substrate, without exposing their Nasonov glands, leave behind a substance that attracts other foragers, yet few who have bioassayed the Nasonov components have considered this either when planning their experiments or interpreting their results. We have now shown that footprint pheromone enhances the attractiveness of the Nasonov pheromone. Little is known of its chemical composition, production, or biological role; this is now being investigated. It may contain a colony-specific element (Kalmus and Ribbands, 1952), although if present, it is only of secondary importance, because footprint gauzes prepared at one feeding table were attractive to bees trained to forage at another site.

We demonstrated that the synthetic Nasonov mixture and natural footprint pheromone are both attractive individually and additively to foragers trained to visit a particular site for food. Work continues to discover whether untrained foragers, scouts, or recruits can also be attracted to pheromone sources in the field and to plants needing pollination.

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EXAMINATION OF NORTH AMERICAN BISON SALIVA FOR POTENTIAL PLANT GROWTH REGULATORS

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Abstract—A series of laboratory bioassays were utilized to test for the presence of potential plant growth factors in saliva from a large native ungulate, the North American bison (*Bison bison* L.). Whole saliva enhanced *Avena* coleoptile growth at high pH, whether alone or in combination with indoleacetic acid (IAA). However, this enhancement was a result of salts in the saliva (primarily $NaHCO_3$) rather than of other compounds acting hormonally, enhancing IAA activity, or inhibiting IAA oxidase activity as possibly occurs with some insect salivas. Additionally, the absence of detectable cytokinins in the saliva was indicated by its failure to enhance cucumber cotyledon expansion. This suggests that biochemical control of plant growth by salivary compounds following grazing is probably not an important component of this ruminant's interactions with its food plants, as has been suggested for some herbivores.

Key Words—Saliva, bison, *Bison bison*, *Avena sativa*, oats, Cucumus sativus, cucumber, *Avena* coleoptile, indoleacetic acid, plant growth regulator, herbivore.

INTRODUCTION

Much of the recent research on biochemical interactions between plants and herbivores has dealt with the effects of secondary plant chemicals on herbivores (Levin, 1976; Cates and Rhoades, 1977; Rosenthal and Janzen, 1979). Another biochemical aspect of plant–herbivore interactions concerns the way in which herbivores may affect growth of their host plants by injecting plant growth-regulating compounds into plant tissues during feeding. For

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example, there have been a number of reports of naturally occurring plant growth regulators, such as cytokinins (Englebrecht et al., 1969; Engelbrecht, 1971), auxins (Miles and Lloyd, 1967; Miles, 1968a), or compounds which interact with them (Hori, 1974; 1975; 1976), in relatively high concentrations in the salivary systems of a variety of herbivorous insects. Miles' (1968b) literature review suggests that certain of these compounds might account for some of the toxic reactions of plants following insect feeding. Although their potential role in plant–herbivore interactions has received little attention, there is also evidence that a number of compounds of animal origin, such as steroid and protein gonadatropin-like compounds, have plant growth-regulating properties (Leshem, 1967; Leshem and Lunenfeld, 1968; Leshem et al., 1969; Stowe and Hudson, 1969; Geuns, 1978; Loeys and Geuns, 1978).

Little research regarding potential effects of saliva from vertebrate herbivores on plant growth has been conducted, and the few available results are variable. Results from a preliminary experiment (Reardon et al., 1972) indicated that bovine saliva, when applied to the cut leaf surfaces of defoliated sideoats grama (*Bouteloua curtipendula*) plants, enhanced forage and root yield relative to that of similarly clipped control plants. However, in later experiments, bovine saliva did not significantly affect growth of this species (Reardon et al., 1974) except when plants were grown in sand with very low fertility (Reardon and Merrill, 1978). Nevertheless, because thiamine, a reported component of bovine saliva (Reardon et al., 1972), apparently enhanced plant growth under some conditions, these investigators suggested that it was an active compound in this plant–animal association and that the grazing animal was necessary to maintain production of native rangelands (Reardon and Merrill, 1978). By contrast, Johnston and Bailey (1972) found that bovine saliva had no effect on growth or tillering of two species of *Festuca*. Similarly, we (Detling et al., 1980) recently reported that a single application of saliva from another ruminant, the North American bison (*Bison bison* L.), had no measurable effect on metabolism or growth of blue grama (*Bouteloua gracilis*), regardless of intensity of defoliation or plant nitrogen status.

In spite of the apparent lack of effect of bison saliva on blue grama (Detling et al., 1980), it might be argued that one or more plant growth regulators are actually present, but that they are effective at different concentrations or frequencies of application, at different phenological stages of plant development, or in different plant species. In this paper, we report the results of a series of experiments to determine whether saliva from this native North American herbivore contains any of a variety of plant growth factors capable of influencing oat coleoptile elongation or cucumber cotyledon expansion.

METHODS AND MATERIALS

All experiments utilized saliva collected between 10:00 AM and noon from two 7-year-old esophageally fistulated bison (a female and a castrated male) maintained at the Pawnee Site, USDA Central Plains Experiments Range near Nunn, Colorado. Saliva samples, usually 1 liter from each animal, were kept on ice during transport (1 hr) to Colorado State University where they were centrifuged at 12,000 g for 1.5 hr at 4° C to remove major microbial and particulate contaminants. Supernatants were lyophilized, and the resulting residues were weighed and kept frozen until immediately prior to their use in an experiment. Results of an earlier experiment indicated that amylase activity, and therefore likely activity of other complex organic molecules, was not affected by these collection, handling, and storage procedures (Detling et al., 1980). Prior to growth studies, residues were dissolved in 10 mM Tris HCl, pH 8.6, to give various concentrations of solids relative to their concentration in whole saliva. Maintenance of this high pH during all experiments was necessitated by the strong buffering capacity and high pH (8.6–8.8) of the saliva. Of the 11.38 g/liter of soluble salts in the saliva sample we analyzed, Na^+, and K^+ (determined by atomic absorption) accounted for 3.03 and 0.24 g/liter, respectively, and HCO_3^- (determined titrimetrically) accounted for 7.17 g/liter.

The first group of experiments, patterned after those of Hori (1974, 1975, 1976), were designed to determine whether plant growth factors capable of enhancing oat coleoptile elongation are present in bison saliva. Oat (*Avena sativa* L. cv. Victory) seedlings were grown in vermiculite for 92 hr at 25° C in darkness. After excision of the apical 3 mm of each coleoptile, a subapical section of 7 mm was removed for growth studies. All cutting was done in dim red light. Sections were rinsed twice with distilled water during 4 hr of preincubation darkness. Initial lengths of 20–25 sections then were measured with a dissecting microscope and ocular micrometer, and all subsequent length changes were determined relative to these initial lengths. Each experimental unit consisted of 10 sections incubated at 25° C for 24 hr in 5.5 ml of 10 mM Tris HCl, with or without saliva and IAA, in 9-cm plastic Petri dishes.

The second group of experiments utilized a cucumber cotyledon expansion assay (Narain and Laloraya, 1974; Green and Muir, 1978) to test for the presence of cytokinins in the saliva. Cotyledons from 3 to 4-day old, dark-grown cucumbers (*Cucumus sativus* L. cv. Marketer) were utilized in all cases. Fresh weights of 20 cotyledons were determined immediately following their excision, and subsequent weight changes were determined by comparison with these initial weights. Cotyledons were incubated in darkness for 3

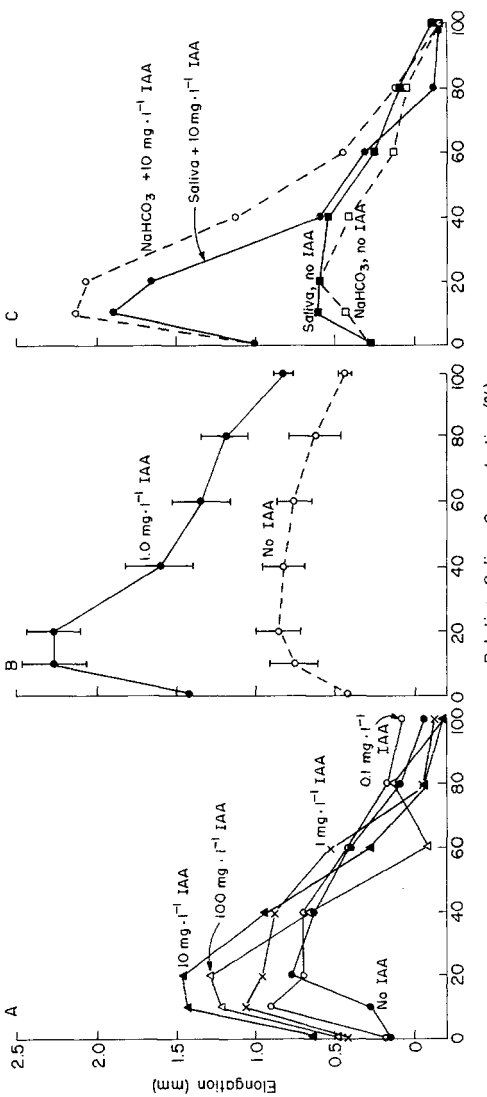

FIG. 1. (A) Promotion of coleoptile elongation by bison saliva at various concentrations of IAA. The concentration of lyophilized solids representing the 100% relative saliva concentration was 14 mg/ml. Each value is the mean of 10 coleoptile sections. (B) Promotion of coleoptile elongation by the 0 to 500-dalton fraction of bison saliva with and without 1 mg/liter IAA. Two liters of saliva yielded 12 g of solids in the 0 to 500-dalton fraction following fractionation by ultrafiltration. Thus, the 100% relative saliva concentration here was 6 mg/ml of incubation solution. Each value is the mean ± SE of 10 sections. (C) Similarity of saliva and $NaHCO_3$ effects on coleoptile elongation at 0 and 10 mg/liter IAA. Each value is the mean from two experiments, each with 10 coleoptile sections per treatment. There were 17 mg of lyophilized solids per ml incubation medium in the 100% saliva concentration. The $NaHCO_3$ concentrations used were calculated relative to the HCO_3^- concentrations in the whole saliva used; thus, a 100% $NaHCO_3$ concentration here was 12.5 mg $NaHCO_3$/ml incubation medium (148 mM).

days in Petri dishes containing 3 ml of a solution of 20 mM KCl, 10 mM Tris HCl (pH 8.4), and reconstituted bison saliva at concentrations of 0–40% of that of whole saliva. Four Petri dishes, each containing 10 cotyledons, were used for each treatment. After the 3-day incubation period, fresh weight of cotyledons was determined by weighing in groups of 5. Sensitivity of the cotyledons was confirmed with an additional treatment involving the addition of 0.017 mM zeatin.

RESULTS AND DISCUSSION

Although coleoptile elongation was relatively small at pH 8.6, bison saliva did enhance elongation, the optimum concentration being 10–20% that of whole saliva (Figure 1A). Elongation was further enhanced by the addition of IAA, the optimum concentration of which was about 10 mg/liter (57 μM). Addition of 2% (w/v) sucrose to saliva with and without 1 mg/liter IAA yielded growth curves (data not shown) similar to those without sucrose in Figure 1A, suggesting that saliva does not act primarily as an energy source.

To determine the active salivary component(s), we first tested thiamine because of its reported growth regulation of sideoats grama (Reardon et al., 1972, 1974; Reardon and Merrill, 1978). At 1 mg/liter, alone or with various concentrations of saliva, this vitamin did not promote elongation (data not shown). We then separated reconstituted saliva by ultrafiltration into

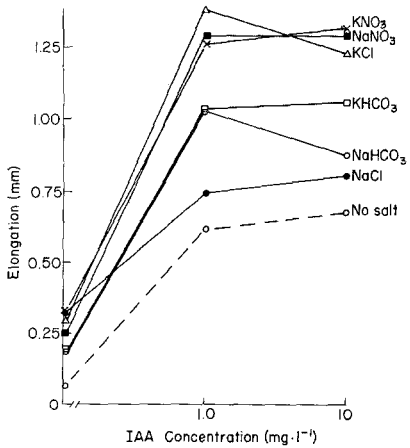

FIG. 2. Stimulation of coleoptile elongation by sodium and potassium salts (31 mM) at various IAA concentrations. Each salt value represents the mean from three or four experiments, each with 10 sections per treatment; no salt values are means from seven experiments.

fractions of <500, 500–2000, 2000–10,000, and >10,000 daltons. Only the <500-dalton fraction was consistently active. When solids in this fraction were varied relative to their abundance in whole saliva, dose–response curves with and without 1 mg/liter IAA (Figure 1B) were similar to those produced by unfractionated saliva (Figure 1A). Because of the large amounts of $NaHCO_3$ in saliva from both cattle (Johnston and Bailey, 1972) and bison, we investigated its effects with and without IAA. Data in Figure 1C indicate that $NaHCO_3$, when varied according to its content in saliva, caused dose–response curves with overall shapes almost identical to those caused by whole saliva and the <500-dalton fraction. The optimum $NaHCO_3$ concentration was 10–20% of its concentration in saliva. This suggested that the primary constituent of bison saliva causing elongation or oat coleoptiles was $NaHCO_3$ and that this salt probably can account for all such activity.

To distinguish between Na^+ and HCO_3^- effects, we tested various concentrations of other monovalent salts of Na^+ and K^+ at 0, 1, and 10 mg/liter IAA. All salts stimulated coleoptile growth with or without IAA (Figure 2). The optimum concentration of each was near 30 mM (data not shown), and the optimum IAA concentration was similar to that with saliva. Since bison saliva contains much more Na^+ than K^+, and since $NaHCO_3$ is active with or without sucrose, activity of the saliva probably results largely from $NaHCO_3$ but partly from $KHCO_3$.

Although we are unaware of other studies on elongation of coleoptile sections at such high pH values, growth activity of salts on coleoptile elongation was demonstrated over 40 years ago (Thimann and Schneider, 1938). More recently, Oertli (1975) developed a theoretical basis for effects of

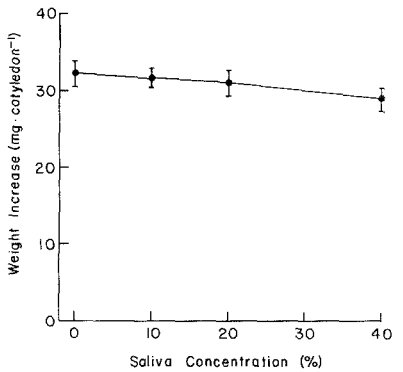

FIG. 3. Change in fresh weight of cucumber cotyledons at various concentrations of bison saliva. Each point is the mean (± 1 standard error) of four replicates each with 10 cotyledons. Two replicates utilized saliva from a male and two utilized saliva from a female. Data from the four replicates were pooled since no significant differences in growth were found between cotyledons in male and female saliva.

KCl, NaCl, and RbCl, on elongation of barley coleoptiles. His calculations and experimental data showed that approximately bell-shaped growth curves should be and were produced when concentrations of these salts were varied. Promotion apparently resulted from absorption of salts that maintained the osmotic potential and turgor of the growing cells, while inhibition at high concentrations resulted mainly from retarded uptake of water. Dose-response curves with saliva (Figure 1A and B), $NaHCO_3$ (Figure 1C), and other monovalent salts of Na^+ and K^+ generally agree with those of Oertli (1975). Therefore we infer that primary effects of bison saliva and its bicarbonate salts on coleoptile elongation can be explained by effects on the water potential according to Oertli's equations, although effects of absorbed ions on enzymes (Rains, 1976), and for nitrate, on nitrogen metabolism may contribute to such effects. Our results suggest that bison saliva does not contain detectable amounts of IAA or complex organic compounds capable of either promoting IAA activity or inhibiting IAA oxidase activity such as those Hori (1974, 1975, 1976) reported present in a variety of insect salivas.

Results of the cucumber cotyledon expansion assay (Figure 3) indicate additionally that bison saliva contains no detectable cytokinin, since excised cotyledons failed to respond to saliva. By contrast, cotyledons receiving the zeatin check treatment gained more than twice as much weight (to 73.1 $\pm$ 1.5 mg/cotyledon) as controls during the same period.

The absence of measurable quantities of any plant growth factors in bison saliva in this study is consistent with our earlier observations (Detling et al., 1980) that growth and metabolism of blue grama was unaffected by addition of raw saliva collected from the same animals. This suggests that injections of plant growth-regulating salivary compounds into wounded plant tissues during grazing is probably not a significant factor in natural interactions between this large ungulate and its food plants.

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TOLERANCE OF ACRIDIDS TO INGESTED CONDENSED TANNIN

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Abstract—Four species of Acridoidea were fed on wheat leaves with and without the condensed tannin, quebracho. In no case was it deleterious to survival and growth at levels below about 10% dry weight on the food. Similarly, consumption, digestibility, and utilization of food were unaffected at up to 10% dry weight. At higher concentrations, however, the consumption and the efficiency of conversion of digestion were reduced, although digestibility was little affected. The possible mechanisms for such tolerance are discussed, and contrast made with insects which are very sensitive to ingested condensed tannin.

Key Words—Condensed tannin, quebracho, Orthoptera, Acrididae, digestibility, *Schistocerca*, *Locusta*, *Zonocerus*, *Chortoicetes*, peritrophic membrane.

INTRODUCTION

The condensed tannins are a heterogeneous group, but they share the characteristic of being potent protein precipitants (Haslem, 1966). They are flavonoid derivatives with an average molecular weight of 1200–1500 daltons (Swain, 1977). Their presence in plants is considered to be a primitive character, being distributed in the primitive plant groups which are vascular and in angiosperms which are woody, but being infrequent among the higher herbaceous angiosperms (Bate Smith and Metcalfe, 1957). When present at all, concentrations in leaves vary with the growth stage, but commonly reach levels of 1–5% (e.g., Bate Smith, 1973; Feeny and Bostock, 1968), although sometimes levels are as high as 20%, particularly in old leaves (Bate Smith, 1977).

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Ingestion of such compounds is believed generally to impair digestion by formation of complexes with food protein and with digestive enzymes (e.g., Feeny, 1970; Swain, 1977). Further, it has been proposed that such compounds may be expected in plants which are long-lived, widespread, conspicuous, or otherwise "apparent" to potential herbivores, which are thereby deterred from attacking them (Levin, 1971; Feeny, 1975; Rhoades and Cates, 1976). The present work is a study of the effects of an ingested condensed tannin on growth and development of four different acridids.

METHODS AND MATERIALS

Insects. Species used were *Schistocerca gregaria* (Forskål), *Locusta migratoria migratorioides* (R. & F.), *Chortoicetes terminifera* (Walker) and *Zonocerus variegatus* (L.). They were tested in groups for survival and growth, with and without condensed tannin. For the first two species 50 late first or early second instar nymphs from stock cultures were placed in a 64-liter cage and reared in a standard manner (Hunter-Jones, 1966). For each experiment there were three replicates of both the tannin treatment and the control treatment. For the latter two species, 20 late first instar individuals were reared in 11 liter cylindrical Perspex cages (for details of conditions see Bernays, 1978). Three pairs of experiments were run for each species. Counts of numbers present in each instar were made at regular intervals and adults were weighed within 24 hr of ecdysis.

In a separate experiment, consumption (C), approximate digestibility (AD), and efficiency of conversion of digested food (ECD) were measured on individual insects over the whole of the last nymphal instar, in constant light and at a constant temperature of 30° C. Each insect was kept in a separate jar (350 ml) and each day feces and uneaten food were removed, the insect weighed, and weighed amounts of food given to each individual. Calculations were made on a dry weight basis (Waldbauer, 1968). In some instances, fecal samples were analyzed for condensed tannin (Swain and Hillis, 1959).

Food. In all cases the food was young wheat leaves taken approximately 14 days after germination, freshly cut and weighed. Each day, samples were dried so that the dry weight of the food given on that day could be calculated. The condensed tannin used was quebracho. In the survival experiments with *S. gregaria* and *L. migratoria* the material was purified and kindly supplied by T. Swain (ARC, Kew). For other experiments, the quebracho (Harshaw Chem. Co., Glasgow) was purified by washing thoroughly on a Sephadex column (Sigma, LH-20-100) with 50% methanol and then extracting with acetone. This extract was evaporated and the condensed tannin crystallized out. This was redissolved in 70% ethanol for application to wheat leaves. The leaves were dipped in the solution and the surface liquid allowed to evaporate in a cool air stream. The amount of tannin actually applied to the surface of

leaves was regularly estimated by testing the dried, treated wheat leaves by the method of Swain and Hillis (1959). Concentrations varied from day to day by up to ±30% of the mean value, but weekly means were more consistent. A mean value of approximately 10% dry weight was used in experiments with *S. gregaria* and *L. migratoria*, of nearly 11% for *C. terminifera*, and just over 11% for *Z. variegatus*. This gave tannin–protein ratios of approximately 0.2.

Protein Digestion. Apart from the study of approximate digestibility, simple experiments were carried out to investigate the effects of the tannin on protein utilization (and hence digestion). The amounts of protein present in the food and feces were estimated by measuring total nitrogen (N) in the samples by micro-Kjeldahl analysis. To correct the fecal N values for waste N, uric acid concentrations in the feces were estimated. Ground dry feces was first extracted in methanol to remove excess tannin, which was found to interfere with the reaction. It was then extracted with 0.6% lithium carbonate and treated with uricase to oxidize the uric acid to allantoin. The decrease in absorbance at 292 nm is proportional to the uric acid concentration, so that from this the uric acid concentration in the feces could be calculated.

Tannin Distribution. The distribution of tannin in the insect after ingestion for three days was examined in the following ways. First, to see if the tannin passed through the peritrophic membrane, 20 *S. gregaria* nymphs were dissected and the midgut carefully opened so that the peritrophic membrane and its contents could be removed without rupture. Then the unwashed midgut epithelium was extracted and tested for the presence of condensed

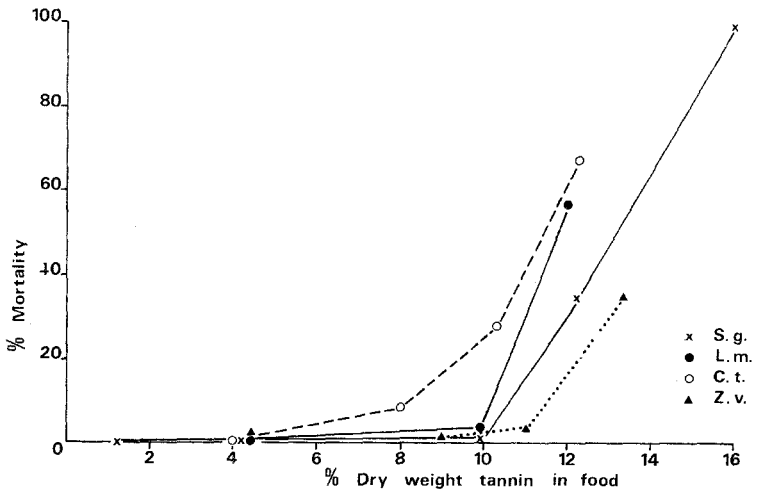

FIG. 1. Survival of insects with increasing concentrations of condensed tannin in the food. X = *Schistocerca gregaria*, ● = *Locusta migratoria*, ○ = *Chortoicetes terminifera*, ▲ = *Zonocerus variegatus*.

tannin (Swain and Hillis, 1959). Finally, fecal samples from 10 separate insects were dissected to separate the peritrophic membrane which normally surrounds the fecal pellets. The peritrophic membranes and their contents were then separately extracted and the amounts of condensed tannin associated with each were determined.

RESULTS

Survival and Growth. Survival and growth of nymphs of the four species of acridid are not affected at all by concentrations of condensed tannin below 10% dry weight. At higher concentrations, survival and the weights of the surviving adults were reduced (Figures 1 and 2). *C. terminifera* is apparently more sensitive, and *Z. variegatus* less so, than *S. gregaria* or *L. migratoria*, although the differences are not very great. Actual values and the variation at approximately 10% dry weight of tannin are shown in Table 1. This is the level at which deleterious effects begin to emerge.

Food Consumption and Utilization. At approximately 4% and 10% dry weight of condensed tannin, food consumption, digestibility, and utilization of digested food are unaffected (Table 2). At higher concentrations, however, the consumption is markedly reduced in the two species tested, approximate digestibility is slightly affected in *L. migratoria* only, while the efficiency of utilization of digested food is also greatly reduced in both species.

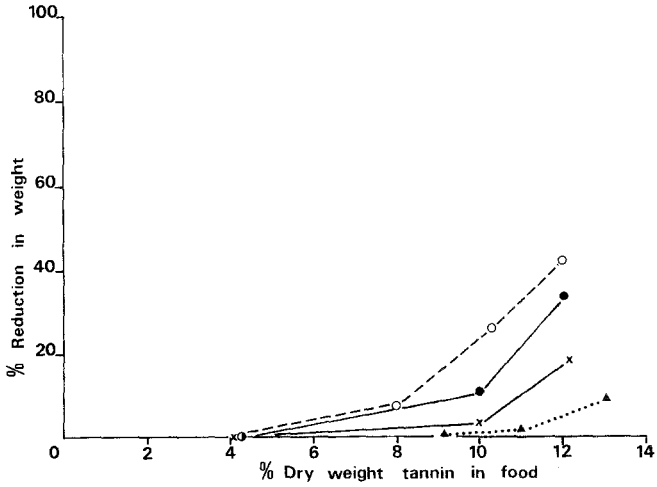

FIG. 2. The percent reduction in adult weights of insects reared on food with different concentrations of condensed tannin compared with control insects having no condensed tannin. For key to symbols see Figure 1.

TABLE 1. OVERALL PERFORMANCE OF FOUR SPECIES WHEN TANNIN IS ADDED TO FOOD

Species	Mean % mortality (%)			Development time (days)[b]		Adult weights[c]			
						Treated		Control	
	Tannin[a]	Treated	Control	Treated	Control	♂	♀	♂	♀
S. gregaria	10	35	34	33	32	1198 ± 111	1435 ± 162	1193 ± 106	1507 ± 105
L. migratoria	10.2	20	23	30	29	806 ± 113	1047 ± 138	908 ± 141	1163 ± 149
C. terminifera	10.8	52	40	35	29	187 ± 21	236 ± 32	240 ± 19	285 ± 26
Z. variegatus	11.2	35	35	90	81	510 ± 56	590 ± 47	566 ± 40	649 ± 44

[a] Percent on dry weight basis.
[b] Time for 50% of survivors to reach the adult stage.
[c] Mean values (mg) ± SE.

TABLE 2. CONSUMPTION (C), APPROXIMATE DIGESTIBILITY (AD), EFFICIENCY OF CONVERSION OF DIGESTED FOOD (ECD), AND WEIGHT INCREASE DURING LAST NYMPHAL INSTAR WITH DIFFERENT CONCENTRATIONS OF CONDENSED TANNIN IN DIET[a]

Species	Treatment	C[b]	AD	ECD	% weight increase	Instar length (mean days)
S. gregaria	controls	654 ± 24	34 ± 4	33 ± 5	76 ± 18	12.4
	4% tannin	650 ± 27	38 ± 4	37 ± 6	78 ± 16	12.2
	10% tannin	645 ± 19	35 ± 4	31 ± 5	71 ± 12	12.6
	[c] controls	623 ± 31	36 ± 5	68 ± 8	98 ± 9	
	[c] 16% tannin	343 ± 40[d]	32 ± 6	31 ± 7[d]	50 ± 9[d]	
L. migratoria	controls	591 ± 41	44 ± 2	28 ± 4	116 ± 13	11.4
	4% tannin	596 ± 30	45 ± 2	26 ± 3	110 ± 10	11.5
	10% tannin	637 ± 17	42 ± 2	22 ± 5	92 ± 10	12.8
	[c] controls	517 ± 35	41 ± 3	71 ± 13	114 ± 7	
	[c] 15% tannin	204 ± 30[d]	30 ± 2	17 ± 3[d]	31 ± 10[d]	
C. terminifera	controls	702 ± 41	38 ± 4	36 ± 4	100 ± 7	7.9
	8% tannin	643 ± 32	31 ± 5	30 ± 6	90 ± 7	8.5
Z. variegatus	controls	741 ± 16	46 ± 3	18 ± 2	102 ± 6	19.4
	4% tannin	751 ± 21	53 ± 4	16 ± 4	99 ± 11	21.0
	10% tannin	736 ± 15	50 ± 6	18 ± 4	102 ± 9	19.6

[a] 10–20 in each treatment. Mean values are given ±SE.
[b] C in amount eaten/100 mg initial wt.
[c] Experiment run for 10 days only and terminated before ecdysis; hence high ECD levels in controls.
[d] Control and treated values significantly different (t test, $P < 0.001$).

TABLE 3. NITROGEN (N) CONCENTRATIONS IN FOOD AND FECES OF INSECTS FED WITH AND WITHOUT TANNIN, TOGETHER WITH FECAL VALUES CORRECTED FOR URIC ACID CONTENT, AND PERCENTAGE UTILIZATION OF DIETARY N AFTER CORRECTION[a]

	Treated			Control		
	Total N (g/100 g)[b]	Total N minus uric acid N (g/100 g)	N utilization (%)	Total N (g/100 g)[b]	Total N minus uric acid N (g/100 g)	N utilization (%)
Leaves	7.6 ± 0.1			7.8 ± 0.2		
S. gregaria feces	5.0 ± 0.4	3.2	58	5.2 ± 0.3	3.3	58
L. migratoria feces	5.4 ± 0.5	3.7	51	5.8 ± 0.6	3.8	51

[a] Six determinations in each case.
[b] Mean values ±SE.

Digestion of protein was apparently unaffected by the presence of condensed tannin in the food. From the levels of N in the food and feces, the percentage uptake of dietary N was estimated at 36% and 34% in control *S. gregaria* and *L. migratoria*, respectively, while the utilization in the presence of 10% dry weight tannin was 40% and 31%, respectively. These values are increased if the N of fecal uric acid is subtracted from the total measured fecal N, but there is still no effect from the presence of tannin (Table 3).

Fate of Condensed Tannin. Quantitative measurements of condensed tannin in feces suggest that the bulk of it is passed out with the feces (Table 4). Thus when food contains 10% tannin, if the AD is 40% (the overall mean in all species), then the expected value in the feces is 14%. None of the measured values fell below 14%. No positive reaction was found for condensed tannin in the tissues of the midgut. It is thus assumed that the peritrophic membrane prevents the passage of condensed tannin through to midgut epithelium. This may be partly a simple filtration process, but the peritrophic membrane may also play another role in selectively adsorbing tannin since high proportions (always 30%) of fecal tannin were always associated with the peritrophic membrane.

DISCUSSION

Concentrations of condensed tannin below 10% had no effects on the growth or survival of the acridids but were deleterious at concentrations at and above this. The effects were similar in the four species tested, but it is interesting that the polyphagous species *S. gregaria* and *Z. variegatus* were a little less sensitive than the two graminivorous species *L. migratoria* and *C. terminifera*, which will encounter little or no condensed tannin in their natural diets.

Studies on individual insects showed that consumption, approximate

TABLE 4. CONDENSED TANNIN IN FECES AFTER FEEDING ON WHEAT WITH APPROXIMATELY 4% AND 10% CONDENSED TANNIN[a]

	4% tannin in food		10% tannin in food	
	Amount in feces (mg/100 mg)	% in peritrophic membrane	Amount in feces (mg/100 mg)	% in peritrophic membrane
S. gregaria	6 ± 1	48	15 ± 3	41
L. migratoria	6 ± 1	44	14 ± 3	33
C. terminifera	6 ± 1	43	14 ± 3	32
Z. variegatus	7 ± 1	40	15 ± 2	37

[a] Mean ±SE for 10 insects fed on test diets for 48 hrs.

digestibility, and efficiency of conversion of digested food were little affected at 10% dry weight or below. At higher concentrations, however, consumption in both *S. gregaria* and *L. migratoria* was markedly reduced as was the ECD. Approximate digestibility was little effected in *L. migratoria* and not at all in *S. gregaria*, even at these high levels. The utilization of nitrogen was also unimpaired by the addition of up to 10% dry weight of condensed tannin in the diet. These results suggest that the poor growth and survival at higher concentrations are due largely to a decrease in consumption. This in turn may lead to an increase in the level of locomotor activity (Ellis, 1951) which will thus be a major contributory factor in the reduced efficiency of conversion of digested food to body substance, since much of the absorbed material will be expended as energy. The insects are apparently reducing food intake at tannin concentrations which have little deleterious effect, at least on digestive functions, since the AD at 15% dry weight was little affected, although consumption was approximately halved.

The absence of any marked effect on digestion is unexpected. This may result partly from the very high level of protein in the diet, amounting to approximately 50% dry weight of the wheat leaves. A separate element of tannin tolerance may relate to the peritrophic membrane. The fact that this contains such a high proportion of the fecal tannin suggests that it may be of benefit by selectively adsorbing tannin so that the effective gut concentration is reduced. This can only take place behind the foregut, but even during digestion in the foregut fluid movement to and from the midgut (Baines, 1979) will ensure that tannin will also be present in the midgut soon after the start of feeding. It is also possible that some tannin is adsorbed onto the cellulose in the wheat so that the effective concentration in the food is reduced (Swain, personal communication).

Larval Lepidoptera examined so far are much more sensitive to the presence of condensed tannin. Relatively small quantities of oak leaf condensed tannin (1%) in an artificial diet severely restricted growth of the larvae of *Operophtera brumata* (Feeny, 1968), and extremely low levels of cotton condensed tannin (0.1%) in artificial diet restricted growth of *Heliothis armigera* (Chan et al., 1978). Both artificial diets were rich in protein so that these caterpillars, at least, are very sensitive. The distribution of the tannin throughout the diet in these experiments may have resulted in more thorough complexing of dietary tannin compared with the present work, but the deleterious effect is very great. In both species, condensed tannin at concentrations of less than 3% dry weight is considered to account for the relative resistance of particular ages and varieties of host plant (Feeny, 1970; Chan et al., 1978). In respect of the artificial diets used for the caterpillars above, it is difficult to invoke the protein-binding theory (i.e., reduction in available protein for growth, as suggested by Feeny, 1968) since the tannin–

protein ratio is so low that only a small fraction of the protein in the diet will be unavailable. Such a theory could be relevant in a natural situation, however, if protein levels are relatively low. Thus, the oak tree in September in Britain has protein levels of only about 12%, and the added presence of over 5% tannin could certainly make protein availability a limiting factor for growth (Feeny, 1970). For caterpillars at least, it is likely that there is some sensitivity to condensed tannin which is not explained by the protein-binding mechanism, unless the tannin selectively binds with the digestive enzymes. It is also possible that it is a potent antifeedant, since the above studies with artificial diet did not separate reduced food intake from other deleterious effects. Whatever may be the cause of the excessive sensitivity, it appears to be absent in acridids.

Levels of 10% dry weight of tannin had very little effect on acridids, and it follows that, providing protein levels are relatively high, condensed tannin in the food plant is unimportant, particularly as levels commonly do not exceed 5% dry weight in green leaves (Long, 1971), although in some species they certainly reach much higher levels, and have possibly been greatly underestimated in the past (Bate Smith, 1977). The combination of high tannin levels together with very low protein levels is more likely to have deleterious effects on digestion and growth, by severely restricting protein availability. Experiments with *S. gregaria*, however, using an artificial diet containing 18% protein and 18% tannic acid (i.e., hydrolyzable tannin) showed no effect on approximate digestibility and growth. Even when the protein level was reduced to 9% and the tannic acid kept at 18%, differences between control and test insects were not measurable after the first 1–2 days (Bernays and Chamberlain, 1980). There must be extraordinary resistance to the binding of tannin with food protein or enzymes in the gut of the locust since it is usual for a given weight of tannin to precipitate an approximately equal weight of protein (Goldstein and Swain, 1965; Feeny, 1969), at least in the prevailing gut pH of 6.5–7 (van Sumere et al., 1975). A similar resistance to both condensed tannin and hydrolyzable tannin occurs in the beetle *Paropsis atomeria* Ol. which feeds and grows well on *Eucalyptus* species with protein–tannin ratios of less than 1 and with protein concentrations of less than 10% (Fox and Macauley, 1977).

It is possible that primitive insects acquired some resistance to condensed tannin at an early stage in evolution, since the initial radiation occurred when plants were already very rich in such compounds (Bernays, 1978). The exact nature of the apparent difference between the Acridoidea, which appear to retain such resistance to tannins, and the more recently evolved Lepidoptera, which do not, remains to be found.

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ELECTROCARDIOGRAPHIC TECHNIQUE FOR STUDYING OLFACTORY RESPONSE IN THE ROCK HYRAX *Procavia capensis* L. (MAMMALIA: HYRACOIDEA).

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Abstract—Rock hyraces were exposed to the dorsal gland and urinary odors of conspecifics in a test chamber constructed such that electrocardiographic biopotential could be picked up through the plantar surfaces of the feet. Both heart rate acceleration and deceleration were observed. Female hyraces responded more strongly to odors than males, but no reponse was observed towards the odor of the dorsal gland of sexually inactive males. Electrocardiography allows a more sensitive measurement of response to odor stimuli than has hitherto been reported, and the technique may have a wide applicability in studies on other mammalian species with naked foot soles.

Key Words—Dorsal gland, electrocardiography, olfactory perception, rock hyrax, *Procavia capensis*, sexual status, urine, odor.

INTRODUCTION

The rock hyrax *Procavia capensis* L. is a diurnal, rabbit-sized herbivorous mammal with a distribution range stretching from the Cape of Good Hope to northern Syria. Hyraces are gregarious and live in groups of up to several hundred individuals. Typically they inhabit rocky outcrops and mountainous regions. All individuals bear a medial dorsal skin gland which is surrounded by, and normally covered by, a tuft of contrastingly colored hair. In South Africa the tuft is normally black, but in central and east Africa it is pale yellow

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or off-white in color (Sale, 1970). During aggressive interactions and when hyraces are mildly alerted to possible danger, the tuft is erected, exposing the bare surface of the gland. When two hyraces meet they frequently sniff the dorsal gland of each other. In an adult male the gland measures about 5 × 8.5 mm and has a markedly convex surface, while those of females and juveniles are smaller and flatter. The apocrine glandular tissue which comprises the gland occupies seven or eight lobules and the secretion is ducted to the surface through fine channels. The secretion is watery in color and consistency and is comprised of various long-chain fatty acids, sterols, triterpenes, a paraffin, and at least two alcohols (Lederer, 1950). It has the distinctive odor of hyraces. Histological examination of the dorsal gland reveals that it is maximally active in both sexes during the mating season (autumn in South Africa), and it has been suggested that the odor of the secretion plays a part in courtship and reproductive behavior (Sale, 1970; Hvass, 1961).

The object of the present study was to develop a technique by which the hyrax's reaction to the dorsal gland secretion odor could be monitored. Hesterman et al. (1976) have drawn attention to the usefulness of heart rate monitoring as a tool in the study of odor perception by mammals. In their study on young rabbits, an ECG transmitter was attached to a test subject and electrodes were implanted under the skin of the sternum. Other studies, on birds (Wenzel and Sieck, 1972) and reptiles (Cowles and Phelan, 1958), have necessitated subjecting the test animal to an amount of stress and physical discomfort. Such handling trauma may be relatively unimportant for studies on laboratory stock, for animals born and reared under laboratory conditions quickly attune to minor shock. For wild-caught animals, however, the induced trauma may upset the sensitivity of the monitoring technique and render the results of little value. We describe here a procedure for assessing the effect of species-specific odors, and particularly dorsal gland odor, on the heart rate of the rock hyrax which seems to overcome the problems associated with handling and which could be modified for use on other mammalian species.

METHODS AND MATERIALS

All experiments were carried out on a captive colony of hyraces which was kept in an outside enclosure measuring approximately 5 × 5 m. Protection against rain was provided by a roof canopy which covered half the enclosure, but otherwise the animals were subject to natural lighting and weather conditions. Although all the animals had been born in captivity, none was capable of being handled without first being restrained with a pole lassoo or by being anesthetized with xylazine (6 mg/ml) and phencyclidine (20 mg/ml) in sterile water. Anesthesia for over 1 hr, without apparent side effects, could be induced by injecting at a dose rate of 0.25 ml/kg.

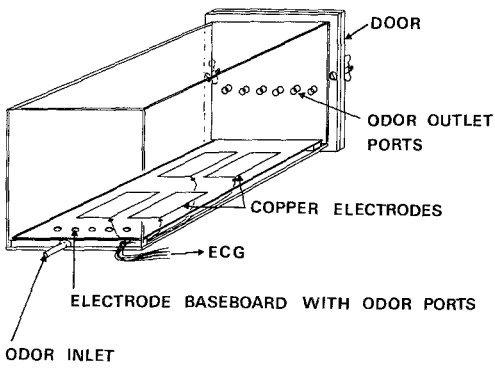

FIG. 1. Diagramatic sketch of the test chamber. It is made from welded transparent Perspex with a removable electrode bearing baseboard. Approximate size of the smallest chamber: 22 × 10 × 10 cm; intermediate, 27 × 12 × 12 cm; largest, 42 × 18 cm (cylindrical).

Test Procedure. Recording of heart responses was made when the subject was inside a specially built chamber (Figure 1). This was a rectangular box-shaped structure made of transparent Perspex 5 mm thick, with an air inlet at one end and a series of exhaust holes at the other. Four copper strips attached to the floor of the chamber served as electrodes. Hyraces do not attempt to escape from enclosed spaces, and the test subject usually remained calmly within the chamber with its four paws on the electrode plates. Heart rate biopotential could be picked up from any two electrodes. If an individual was sufficiently small to be able to turn round within the chamber, it usually did so and disturbed the recording. To overcome this, three sizes of chamber were used and the subject was put into the most appropriate size. The chamber was placed on the laboratory bench and covered with towels to exclude the light. The temperature in the laboratory varied between 16°C and 19°C. No test subject was used in more than one experiment with any one odor stimulus on any one day. Experiments were conducted between 0830 and 1800 hr daily, between July and September 1979.

Compressed air was driven through the chamber at a rate of 0.75 liters/min for the smallest chamber and 1.0 liter/min for the larger chambers. Prior to the introduction of test odors, air flowed through the apparatus for approximately 30 min. Upon first entering the chamber, the subject's heart rate was as high as 150 beats/min, but within 1 min this had fallen to an average of 90–110 beats/min. This rate was maintained until the start of testing.

Dorsal gland secretion odors were taken directly from an anesthetized donor because it was found that the high volatility of the secretion rendered its translation from a donor to a flask impractical. A plastic vial (1.8 cm internal diameter; 5.0 cm long) with its base removed was positioned over the gland.

An airstream from the compressor at the same flow rate entered the cap of the vial via a glass tube. Gentle massaging of the skin surrounding the gland caused some expression of the secretion whose vapors were swept out of the vial and into the test chamber via a three-way tap. Tests with this arrangement in a water bath indicated that there were neither breaks nor surges in the flow when the tap was operated. It was not possible to control either the odor intensity or the amount of secretion expressed.

In trials using urine as the odor stimulus, the donor animal was placed in a metabolic cage overnight and the urine removed in the morning. It was pipetted into a small wash bottle and connected to the three-way tap in place of the dorsal gland vial.

The electrocardiograph was a standard medical model (Birtcher Corp., model 350), and the leads connected to the electrode plates were RA and LA. The chart speed was 2.5 cm/sec.

The measurement of cardiac response used throughout the study was the mean interbeat interval (Schell and Catania, 1975) calculated from 20 successive beats (1) immediately before the introduction of the odor, and (2) immediately after the odor had entered the chamber. It was found that if a time-based method of analysis were used, in which the number of beats per standard unit time was counted, no changes in heart rate of less than about 10% could be detected.

RESULTS

Table 1 shows the number of experiments in which the subject's heart rate showed a greater than 5% change in the interbeat interval. The difference between the total female and total male response to all three odor stimuli is statistically significant [χ^2 exact test (Yates correction) $P < 0.001$]. The difference between the female and the male response to the dorsal gland odor of sexually active males is also statistically significant ($P < 0.01$ and > 0.001, respectively), but the difference between their responses to dorsal gland odors from sexually inactive males is insignificant ($P > 0.05$). Urine odor of sexually active males elicits a statistically significant response ($P < 0.001$).

Although the data in some of the cells of Table 1 are few, there is a suggestion that the young of both sexes are more responsive than the older classes, but statistical comparison is not possible.

DISCUSSION

The data in Table 1 indicate that the electrocardiographic technique successfully revealed sex-related differences in the hyraces response to certain odors. These data parallel those obtained from behavioral studies on black-

TABLE 1. NUMBERS OF TESTS IN WHICH SUBJECTS SHOW A 5% CHANGE IN HEART INTERBEAT INTERVAL UPON EXPOSURE TO VARIOUS ODORS[a]

	Dorsal gland secretion							
	Sexually active male		Sexually inactive male		Urine of sexually active male		Totals	
Subject	No.	(n)	No.	(n)	No.	(n)	No.	(n)
Female								
1st year	8	13	0	1	2	3	10	17
2nd year	5	10	1	2	—	—	6	12
3rd year	3	5	0	2	1	1	4	8
All females	16	28	1	5	3	4	20	37
Male sexually inactive	3	13	0	4	0	2	3	19
Male sexually active	0	2	0	4	0	2	0	8
All males	3	15	0	8	0	4	3	27

[a] Female subjects are grouped into three categories according to their age. All third-year and several second-year individuals were thought to be pregnant. Male subjects are grouped into two categories. Sexually inactive males weighed less than 3 kg and were either 1 or 2 years old. Sexually active males weighed around 5–7 kg and were probably 3 or more years old. To ensure that their dorsal glands were maximally active, they were given weekly injections of testosterone propionate (depot.) at a rate of 2 mg/kg. Histological examination confirmed the state of activity of the dorsal gland. No. = number of subjects responding. (n) = number of subjects tested.

tailed deer *Odocoileus hemionus* (Müller-Schwarze, 1980) and short-tailed voles *Microtus agrestis* (Stoddart, 1980); in both these species females have been shown to respond to particular odors more dramatically than males.

In the great majority of experiments (65.6 %), the hyrax heart rate showed a deceleration upon the introduction of an odor stimulus. At its most dramatic this took the form of an apparently suspended heart beat (Figure 2), and this reduced the rate by up to 16%. More usually the interbeat interval was slightly lengthened, reducing the rate by 8–10%. In a minority of experiments the heart rate accelerated, but this was never as dramatic as the most obvious decelerations. There is an abundant psychophysiological literature devoted to the interrelationship between heart rate and sensory acuity and, although almost all of this relates to acoustic and visual experimentations only, it is apparent that a deceleration of heart rate accompanies an orienting response in which the subject pays close attention to the stimulus. Orienting response occurs only at low or moderate stimulus intensity and at high intensities, or if the stimulus has a rapid build-up time, the subject shows a defensive or startling reaction which causes the heart rate to accelerate (Graham and

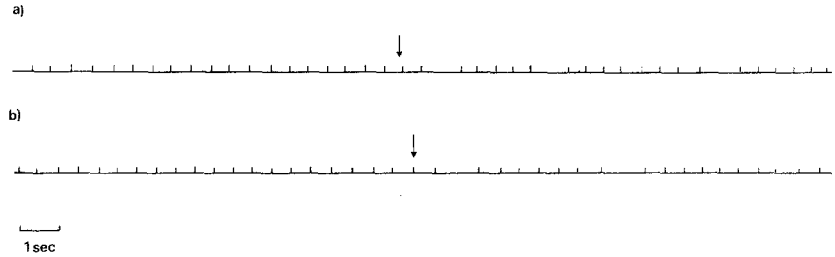

FIG 2. Two examples of heart rate deceleration in rock hyrax, *Procavia capensis* L., exposed to the odor of conspecific dorsal gland secretions. (a) Subject: 1st-year female; stimulus: dorsal gland secretion from sexually active male. (b) Subject: 2nd year female; stimulus: as in (a). Arrow denotes introduction of odor into the chamber. Time bar: 1.0 sec. The trace reads from left to right.

Clifton, 1966). As it was not possible to either monitor or control the intensity or concentration of the odor stimulus in this study, it was considered desirable to analyze all the experiments in terms of both response types.

The accelerated heart rates reported above are in clear distinction to the findings of Hesterman et al. (1976), who observed the effect of conspecific odors on heart rate of nestling rabbits, *Oryctolagus cuniculus*, 3–12 days old. In their study, acceleration was never observed, but it is not known whether this was related to the state of maturity of the peripheral and central neural mechanisms in the young rabbits or whether it was an artifact of the time-based analysis they employed. Using telemetry, Hesterman et al. (1976) counted the number of beats in 1.2-sec time periods and, while this was adequate to show up substantial changes, it is likely to have obscured slight changes. Electrocardiography using an ECG or oscillograph allows the experimenter to choose the sort of analysis best suited to the particular heart rate of his subjects.

Although the objective of the study was to develop a satisfactory technique for monitoring the effects of odor perception by hyraces, the data provide some information about the suggested function of the dorsal gland. The secretions from sexually inactive males evoked little response from females and no response from males. The urine of sexually active males evoked a female response but again no male response. Thus it appears that the level of sexual activity of the male donor governs female responsiveness, and the data do not contradict the notion that the gland may play a role in courtship or other reproductive behaviors. It is felt that the technique described here is adequate to enable an intense study of the dorsal gland function to be undertaken.

Rock hyraces are particularly good subjects for the demonstration of this technique because they normally live in confined spaces and so will sit quietly in a test chamber for long periods, and they have bare foot soles. It is unlikely

that the technique would be as successful for rabbits and other mammals with hairy feet, but it might have wide applicability for studies on insectivores, primates, rodents, and other mammalian species with naked foot soles.

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ATTRACTION RESPONSES OF THE AMERICAN COCKROACH TO SYNTHETIC PERIPLANONE-B

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Abstract—Synthetic periplanone-B has been shown not only to be a sex excitant to male *Periplaneta americana* by bioassay in the laboratory but also an attractant pheromone by field tests in a rice storage house in Taipei. During both summer and winter months, it attracted significantly more adult males into the traps used in the experiments than adult females. There is a statistically significant increase in the sex ratio (male–female) of the trapped adults with increase in periplanone-B used. An attempt has been made to explain the trapping of females and nymphs in addition to males by the chemicals used in our tests.

Key Words—Sex pheromones, cockroach, *Periplaneta americana*, Orthoptera, Blattidae, periplanone-B, margarine oil, sex ratio, control.

INTRODUCTION

Substantial progress has been made in the biochemistry of cockroach sex pheromone since Bowers and Bodenstein (1971) showed that *d*-bornyl acetate and β-santalal at proper concentration can elicite sexual response from the male American cockroach. Tahara et al. (1975) isolated germacrene-D from *Erigeron annus* (L.) Pers. (a plant of the Compositae family) and showed that it could induce in the male American cockroach sexual behavior similar to that elicited by natural female sex pheromone. Takahashi et al. (1978) prepared germacrene-D and its chemical derivatives, which they showed were only weak sexual stimulants to male American cockroach. Based on the combined results from gas chromatography–mass spectrometry, hydrogenation, infrared, and NMR analyses, Persoons et al. (1979) published data to show that the natural female cockroach sex pheromone had two components, periplanone-A and periplanone-B, and proposed for per-

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iplanone-B the tentative formula (1*Z*,5*E*)-1,10(14)-diepoxy-4(15,5-germacradien-9-one. We have also demonstrated that the crude sex pheromone extracted from the female American cockroach could be used as an attractant to the males in a heavily infested rice storage room (Chow et al., 1976). The structural configuration of periplanone-B has been worked out by Adams et al. (1979) and substantiated by chemical synthesis by Still (1979). Synthetic (±)-periplanone-B is now available for large-scale testing. The aim of this paper is to report on our tests of this synthetic product on local populations of *Periplaneta americana* (L.).

METHODS AND MATERIALS

The synthetic (±)-periplanone-B used in the experiments reported here was kindly supplied by Dr. Still of the Chemistry Department, Columbia University, who prepared it as previously described (Still, 1979). A stock solution was prepared by dissolving 5 μg of racemic periplanone-B in 50 μl of mineral oil. Working solutions for laboratory experiments were made by diluting selected quantities of the stock separately with appropriate volumes of *n*-hexane. The working solutions were placed in polyethylene capsules for bioassay and field experiments.

In laboratory tests the capsules were dropped directly into 30 × 30 × 30-cm wooden boxes each containing 50–60 adult male *P. americana*. The quantities of periplanone-B tested ranged from 0.001 to 1.0 μg. In our bioassay, a male is considered to have exhibited positive sexual response if it (1) increases its searching activity for female cockroach, or (2) stands still but flutters its wings, or (3) shows homosexual behavior. The tests were conducted in the dark under red light for 6 hr followed by a 6-hr observation under daylight. The former is designated "under dark condition" and the latter, "under light condition."

Field tests were carried out between March 1979 and January 1980, in Hwa-shan barn, the largest rice storage house in Taipei city. Seventy-two traps, transparent plastic devices patented under (65) Tai-Chua-Yang-Tze No. 24676, Taiwan (see Figure 2), each containing a polyethylene capsule with its specific quantity of periplanone-B, were placed along the building's edge most often frequented by American cockroaches. The traps were allowed to remain for 4–6 days; at the end of the period, they were replaced by new ones, and the old traps were taken to the laboratory. The trapped cockroaches were paralyzed by CO_2, counted, and separated into nymphs, adult males, and adult females.

Traps were placed in room 4 of the storage house between March and July 1979. Later they were placed in room 8, August 1979 to January 1980, because room 4 was too open and some of the traps were inadvertently

destroyed by workmen. Before moving the field tests from room 4 to room 8, a preliminary survey was made of both rooms simultaneously, by placing four control traps, which contained only margarine oil and no pheromone, in each room for two weeks. At the end of the period the sex ratios of the cockroaches caught in the two sets of traps were analyzed and the F test applied. No significant difference in sex ratio was found (observed F value 0.162; theoretical F value 3.46). The absence of sex ratio difference in the two populations would allow us to analyze data from rooms 4 and 8 in a similar way.

RESULTS AND DISCUSSION

In laboratory tests under dark condition, the isolated males in the wooden boxes responded positively to all three levels of sex pheromone: 0.001, 0.01, and 0.1 μg/capsule. This is comparable with our results (Chow et al., 1976) obtained with crude sex pheromone extract.

Under light condition there was no response from males to capsules containing 0.001 μg of synthetic sex pheromone. When a capsule with 0.01 μg of the same chemical was dropped into the box, 25% of the males responded positively. Figure 1 shows a typical pseudocopulation behavior of a male in response to 0.01 μg of sex pheromone. When capsules with 0.1 μg of periplanone-B were presented to the males, most of them responded strongly. The sexual response was very similar to that found under dark condition, which in turn was very similar to that described by Adams et al. (1979), although the quantity used in our tests was higher. The use of a polyethylene capsule in our tests as a container of the pheromone to retard the evaporation of the chemical, resulting in lower concentration of the pheromone available to the cockroach in the wooden box, may account for the difference. Hawkins and Rust (1977) showed that there was a cyclic response of males to various concentrations of crude sex pheromone: darkness would bring the sex pheromone effect to its maximum. The fact that 0.01 μg (and more) of synthetic periplanone elicited positive male sexual response under both dark and light condition, while 0.001 μg did not under light condition, might imply that darkness lowered the sexual response threshold of the male.

The results of the fields tests are summarized in Table 1. The left half of this table contains data from 22 traps recovered from 36 placed in room 4 of Hwa-shan storage house. Three levels of synthetic periplanone-B were used as attractant—0.1, 0.5, and 1.0 μg/capsule. Without the pheromone (control) the traps caught on the average one third as many males as females, (column 1). With increased pheromone (columns 2 to 4), the ratio of males to females increased from 0.36, through 1.24, to 2.36. There was no similar

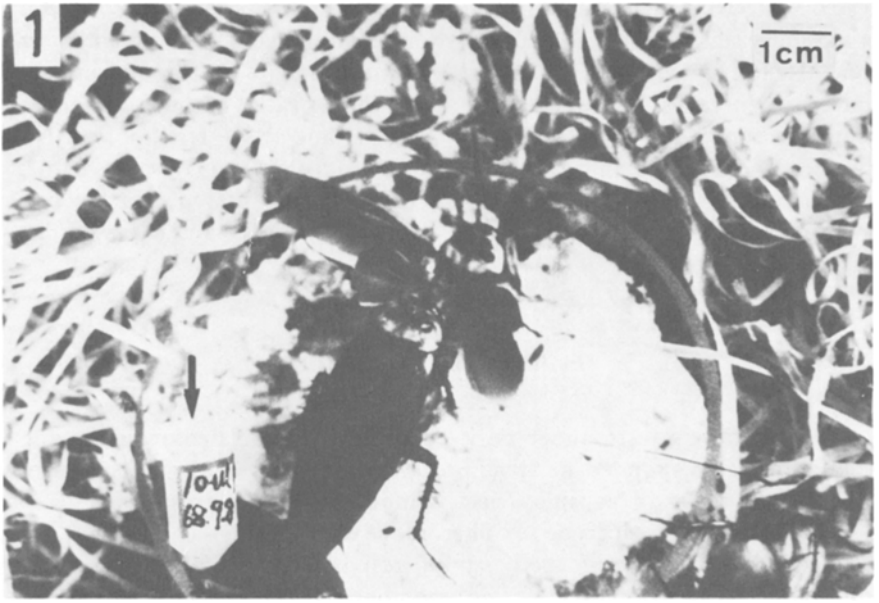

FIG 1. The sex response of male cockroaches to polyethylene capsule (arrow) which contained 0.01 μg synthetic (±)-periplanone-B.

increase in the number of nymphs or females caught as the concentration of pheromone was increased.

The right half of Table 1 (columns 5 to 8), shows the data obtained from 32 traps recovered from room 8 of the rice storage house. In these tests 1 g of margarine oil was added to each polyethylene capsule. The male–female ratio of adults caught increased when 0.5 or 1.0 μg of periplanone was used (columns 7 and 8), but showed no increase over that of the control when the traps had only 0.1 μg pheromone per capsule. In each case, the male–female ratio was lower than the corresponding ratio found in room 4, and the average number of males caught was also lower. The average number of adults (both males and females) caught in room 8 by pheromone-containing traps was also lower than that in room 4. This may be due to one or both of the following reasons. First, the last part of the experiment in room 8 was done during the cold time of the year. There are reports that the American cockroach is less active during the winter months (Cornwell, 1976; Hawkins and Rust, 1977). Second, room 8 was twice as large as room 4, and the active range of periplanone-B is not as large as most pheromones of lepidopterous insects (Shorey et al., 1977; Roelofs, 1978). It was possible that the actual quantity of pheromone reaching the cockroach was less in room 8 than in room 4.

In examining the sex ratio of cockroaches caught, the original data in

TABLE 1. NUMBERS OF *Periplaneta americana* CAUGHT PER TRAP BY SYNTHETIC PERIPLANONE-B AND PERIPLANONE-B PLUS MARGARINE OIL[a]

	Periplanone-B												Periplanone-B + margarine oil											
	(1) Control 0 μg			(2) 0.1 μg			(3) 0.5 μg			(4) 1.0 μg			(5) Control 0 μg			(6) 0.1 μg			(7) 0.5 μg			(8) 1.0 μg		
Replicate	F	M	N	F	M	N	F	M	N	F	M	N	F	M	N	F	M	N	F	M	N	F	M	N
1	18	5	38	2	0	8	1	3	15	1	3	14	6	0	20	9	4	25	2	2	32	16	8	10
2	1	0	37	8	0	43	4	0	30	11	9	14	0	3	16	7	3	26	3	0	10	1	1	8
3	1	1	80	4	3	16	5	8	37	0	12	4	4	3	15	3	1	0	2	9	90	1	12	30
4	0	1	6	0	2	5	0	2	2	2	7	8	1	3	8	1	1	5	2	1	154	2	3	60
5	1	0	0				2	7	2	0	1	38	3	0	6	0	1	9	1	2	30	1	1	55
6							0	1	4	0	1	2				5	1	5	1	0	5	0	2	149
7							13	10	52							3	0	16	0	0	60	1	4	30
8																6	0	62	1	0	18	0	2	19
9																3	1	34	0	0	52	0	1	3
N		5			4			7			6			5			9			9			9	
$\bar{X}$	4.2	1.4	32.2	3.5	1.3	18	3.6	4.4	20.3	2.3	5.5	13.3	2.8	1.8	13	4.1	1.3	20.2	1.3	1.6	50.1	2.4	3.8	40.4
S	7.7	2.1	31.9	3.4	1.5	17.3	4.6	3.9	19.8	4.3	4.6	13.1	2.4	1.6	5.8	2.9	1.3	19.4	1	2.9	47.3	5.1	3.8	45.3
M/F		0.33			0.36			1.24			2.36			0.64			0.32			1.17			1.55	

[a] F = female; M = male; N = nymph.

Table 1 were transformed before applying the F test, by taking log [(No. ♂ + 1/No. ♀ + 1) × 10] in order to avoid the use of zeros. Table 2 shows the result. The effect of periplanone-B on the male–female ratio of the trapped cockroaches was significant at the 1% level, whereas the other factors were not. These results agree with the recent finding of Bell et al. (1977). Boeckh et al. (1970) and Nishino and Takayanagi (1979) showed that the middle region of male antenna produced greater EAG response to female sex pheromone and compounds like germacrene-D than did the female antenna. This phenomenon might be at the root of the higher male–female ratio we observed with the use of sex pheromone.

The average number of nymphs caught, as shown in the right half of Table 2, was 33.2 individuals per trap. This lends support to our previous finding (Chow et al., 1978) that margarine oil was a good food attractant to cockroach nymphs. The average number of nymphs caught by the control traps (i.e., traps without periplanone-B but only 1 g of margarine oil each) was 13 per trap, while the experimental traps averaged 37 nymphs. However, more comparative studies are needed to establish the hypothesis that synthetic sex pheromone attracts *P. americana* nymphs.

In summary, synthetic periplanone-B has been shown to be a sex pheromone to the male *Periplaneta americana* by bioassay in the laboratory under both dark and light conditions. In field tests, both during the summer and winter months, increased quantities of periplanone-B attract more males than females. There is an indication that this synthetic chemical may also be a food attractant to cockroach nymphs.The fact that in our experiment both nymphs and females were trapped leads us to speculate that synthetic periplanone-B may be more than just a sexual attractant to male cockroach or

TABLE 2. TEST OF SIGNIFICANCE OF RESULTS TRANSFORMED BY [(No. ♂ + 1/No. ♀ + 1) × 10] FROM TABLE 1.

Sources of variation	Degrees of freedom	Sum of squares	Mean squares	F (observed)	F (theoretical) 5%	F (theoretical) 1%
Treatment	7	3.0525	0.3691			
Margarine oil	1	0.1899	0.1899	1.1981	4.08	7.31
Periplanone-B	3	2.8152	0.9384	5.9205[a]	2.84	4.31
Margarine oil + periplanone-B	3	0.0474	0.0158	0.10	2.84	4.31
Error	46	7.2928	0.1585			
Total	53	10.3453				

[a] Highly significant.

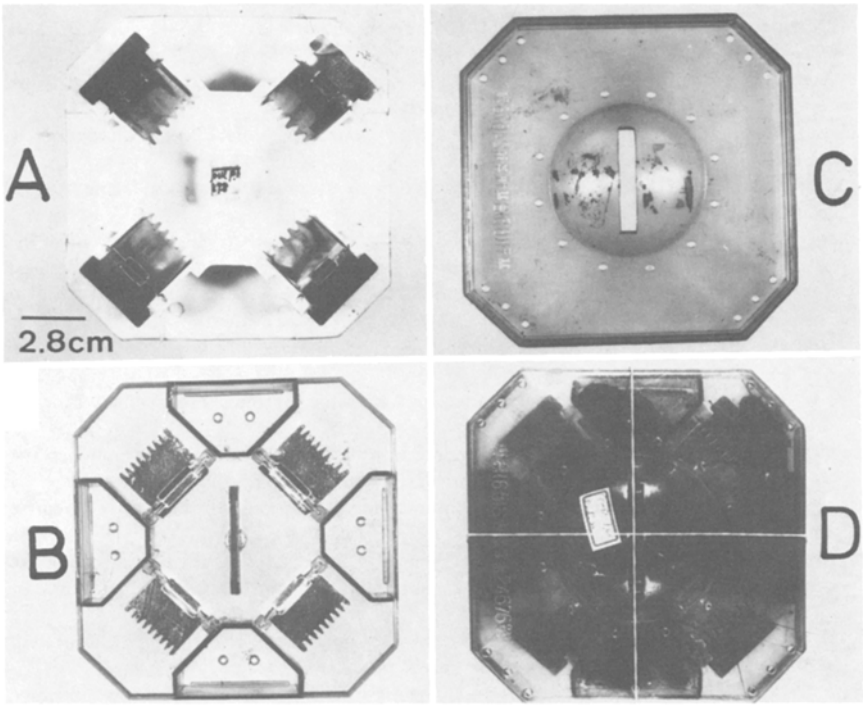

FIG. 2. Different parts of the transparent roach trap used in this experiment. (A) bottom part; (B) middle part; (C) top part; (D) 3 parts mounted together, many roaches were trapped within.

that the trapped males may produce aggregation and/or seducin pheromone (Burk and Bell, 1973; Stinson, 1979) which in turn attracted females and nymphs into the trap.

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APHID FEEDING DETERRENTS IN SORGHUM
Bioassay, Isolation, and Characterization[1]

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Abstract—Improvements in a synthetic diet for use in a bioassay to screen for feeding deterrents against *Schizaphis graminum*, greenbug, are reported. Feeding on the synthetic diet was highly pH dependent with maximum feeding occurring at about pH 8.0. The bioassay was used as a guide in the isolation of feeding deterrent substances from aphid-resistant lines of sorghum (*Sorghum bicolor*). The major greenbug feeding deterrents isolated from sorghum leaves were *p*-hydroxybenzaldehyde (ED_{50} 0.13%), dhurrin (ED_{50} 0.16%), and procyanidin (ED_{50} 0.08%).

Key Words—*Schizaphis graminum*, Homoptera, Aphididae, greenbugs, *Sorghum bicolor*, feeding deterrents, bioassay, aphids, host plant resistance, *p*-hydroxybenzaldehyde, dhurrin, procyanidin, luteolin-7-glucoside.

INTRODUCTION

The identification of constituents in sorghum [*Sorghum bicolor* (L.)] which are responsible for resistance to grasshopper (*Locusta*) feeding has been extensively investigated by Bernays and coworkers (Woodhead and Bernays, 1978, and references cited therein.) It was established that resistance in young plants was mainly due to the cyanohydrin glucoside, dhurrin. More mature sorghum had lower dhurrin levels but increasing amounts of phenolic acids. These simple phenolic acids were the main contributors to sorghum resistance towards *Locusta* in older plants.

[1]Reference to a company and/or product named by the department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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The greenbug [*Schizaphis graminum* (Rondani)] is an economically important pest on sorghum and some other small grains in the midwestern United States. Some lines of sorghum are relatively resistant to the greenbug. The resistance appears to include tolerance, nonpreference, and antibiosis. All three of these resistance factors can occur in the same line or in different sorghum varieties (Schuster and Starks, 1973) and can vary with temperature and the nutritional state of the plant (Schweissing and Wilde, 1979).

The identification of substances causing insect resistance in sorghum may aid plant geneticists and plant breeders in the development of greenbug-resistant (GBR) hybrids. A breeding program could then take advantage of appropriate chemical analysis to determine levels of antibiotic compounds for greenbug resistance in small grains.

Previous work to determine greenbug resistance in barley implicated a role for benzyl alcohol (Juneja et al., 1972, 1973). It appeared to act by lowering the reproductive rate of the aphids (biotype C). The presence of benzyl alcohol in barley has not been confirmed (Starks, 1979). In addition, Todd and coworkers have measured the effect of a series of commercially available phenolic compounds on the growth and reproduction of greenbug (biotype B) feeding on a synthetic diet containing the test substance as the bioassay (Todd et al., 1971).

Four biotypes, A, B, C, and D, of the greenbug are now recognized (Starks and Burton, 1977). Biotypes A and B are known to differ in their feeding habits and, for the most part, do not appear to feed on sorghum (Saxena and Chada, 1971a,b). Much less appears to be known about the feeding habits of biotypes C and D. At the present time biotype C is the most destructive pest on sorghum. The feeding habits of greenbug biotypes are complex and varied. Thus one cannot be sure that cells encountered during the extensive probing preceding feeding do not play a role in the acceptability of sorghum to the greenbug. For biotype C, it is not known if feeding takes place in the mesophyll parenchyma or only in the phleom tissue. The latter case is more likely because of the large amounts of honeydew production. (Lowe, 1967).

This paper describes the development of a bioassay precedure which can be used to evaluate feeding deterrency of plant constituents towards the greenbug as well as its use to qualitatively determine some of the chemical factors which may contribute to greenbug resistance in sorghum.

METHODS AND MATERIALS

Bioassay. A stock greenbug colony, biotype C, was maintained on an aphid-susceptible sorghum line, NC 70X. The colony was kept under light 24 hr/day so that the culture consisted only of apterous aphids.

Small (35-ml) polystyrene catsup cups were used as test chambers for the bioassay. For each test, 50–75 aphids of all ages were transferred from the colony by brushing carefully off the plant with an artist's fine camel-hair paint brush into each test cup. When it was necessary to collect an especially large number of aphids, advantage could be taken of their alarm pheromone. Aluminum foil was spread around the base of the plant, and the aphids were exposed to an open vial of an essential oil fraction rich in β-farnesene (Wientjens et al., 1973; Nault and Bowers, 1974). The aphids would drop off the plant onto the foil and could be shaken off into the test chambers as required.

The polystyrene test chambers had tight-fitting plastic snap-on caps. The caps had circular holes (about 1.5 cm) punched with a cork borer for the placement of the diet container. The diet container was made from a soft polyethylene vial cap which fitted snuggly into the hole prepared with the cork borer in the snap-on lid. A thin sheet of Parafilm® was stretched across the vial cap to create a sealed diet chamber. Diet containing the test materials was added by injection with a syringe through the top side of each of six such polyethylene caps. Three of the six diet containers were stored in a freezer. The remaining three filled diet containers were placed by a snug fit into the hole bored in the lid of the test chamber with the Parafilm membrane facing the interior of the test chamber toward the aphids.

The tests were maintained for 24 hr at 24°C but without control of photoperiod or humidity. Usually a good indication of feeding deterrent activity could be obtained by visual inspection in 2–3 hr. After 24 hr, the number of aphids feeding and the number wandering were counted and compared with the appropriate controls. One week later, the identical set of the further three tests was run on diet which had been stored in the freezer. The results of the six replicates were then averaged.

It was often practical to prepare a large number of diet containers at one time, store them in the freezer, and run them at a rate governed by the supply of aphids and the capacity of the processing system.

In the initial stages of data collection, after the bioassay technique had been worked out, the controls routinely ran 70–80% feeding. As the work progressed over a period of months the controls slowly rose to around 95% feeding. The reasons for this improvement are not exactly clear, but one contributing factor may be an increase in cleanliness in handling the diet to avoid bacterial contamination. Diets with high bacterial contamination, which had clearly gone bad, were avoided by the insects.

Initially, three different aphid diets were explored: Mitsuhashi (Singh, 1977), Cress and Chada (1971), and the Akey-Beck diet (Akey and Beck, 1971). No substantial differences in the diets were observed when used to maintain aphids for 24–48 hr. As a result, subsequent development work was confined to the Akey-Beck diet.

The Akey-Beck diet developed for the pea aphid was used as previously described (Akey and Beck, 1971) with several slight modifications. One was that the ferric chloride is added separately after all of the other metal salts. After cooling, the ascorbic acid was added and the pH adjusted to 8.0 with 4 M KOH. The diet was then filtered through a Millipore® filter and stored in a freezer. Just before use, the diet was diluted with an equal volume of 1% potassium tribasic phosphate to bring the pH up to 8.7. Substances to be tested were added to the diet at appropriate levels. Substances which dissolved in the diet with difficulty were sonicated to effect solution or at least dispersion.

Isolation of Procyanidin. Concentrated methanol extracts of freshly harvested and ground GBR sorghum were treated with decolorizing charcoal to remove waxes and chlorophyll. Solvent was removed from the filtrates, and the residue was taken up in water. The mixture was filtered through filter aid to remove any water insoluble material, and the aqueous solution was applied to a column of XAD-2. The column was eluted with further amounts of water. The aqueous eluents were freeze dried and submitted to the bioassay. They were inactive. After washing with a large volume of water, the column was eluted with methanol. The methanol eluents were concentrated to a small volume and allowed to stand several days. Several crops of luteolin-7-glucoside (Stafford, 1969) were deposited and were removed by filtration. The mother liquors, after removal of the bulk of the luteolin-7-glucoside by crystallization, were chromatographed on Sephadex LH-20 with methanol. The fractions were monitored by treatment of aliquots with 1:1 formic–hydrochloric acids and ferric chloride reagent. By this means, those fractions containing procyanidins could be located by the rose color formed. These fractions giving a positive color test were eluted early from LH-20. They were combined and the solvent removed. The late fractions from the column gave a positive ferric chloride test and, by paper chromatography in 15% acetic acid, were judged to contain further amounts of luteolin-7-glucoside. This was verified by isolation of luteolin-7-glucoside from these fractions.

Paper chromatography in the same solvent system for the fractions that gave the rose color and spraying with 1:1 formic–conc. HCl gave an elongated pink spot. The R_f of this material was much less than that obtained from 3′,4,4′,5,7-pentahydroxyflavan. The combined procyanidin fractions were examined for homogeneity by NMR. Although the spectrum was not interpretable, no signals for other known sorghum constituents were evident. The UV spectrum of the procyanidin in 1:1 formic acid–HCl was identical with that of 3′,4,4′,5,7-pentahydroxyflavan or 3,3′,4,4′,5,7-hexahydroxyflavan under the same conditions.

Isolation of Dhurrin. Dhurrin was prepared as previously described (Mao et al., 1965) from seedlings of high-cyanide sorghum (B Wheatland

type). The procedure was modified to include a step for removal of sugars by elution through XAD-2.

Isolation of p*-Hydroxybenzaldehyde and* p*-Hydroxybenzoic Acid.* Sorghum (IS 809) was extracted and the extracts worked up as described for the GBR line. Solvent was removed from the material which was retained on XAD. The brown gum remaining was taken up in warm wet tetrahydrofuran (THF). The THF solution was separated from some THF-insoluble material by filtration through celite. The bulk of the feeding-deterrent activity resided in the THF-soluble fraction. THF was removed, and the residue was again taken up in a small amount of methanol. The methanol solution deposited several crops of luteolin-7-glucoside upon standing. The NMR spectrum of the mother liquors indicated that they contained largely dhurrin and smaller amounts of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde. Chromatography of the methanol-soluble mother liquors on Sephadex LH-20 with methanol failed to concentrate the feeding deterrent activity in any single fraction. In a separate isolation run the THF-soluble material was chroatographed on silica gel with a THF-methanol gradient. Again biological activity was distributed through all of the fractions. *p*-Hydroxybenzoic acid was located in the fractions by monitoring with the NMR spectra and was isolated by crystallization from water. The IR spectrum was identical to that of an authentic sample. *p*-Hydroxybenzaldehyde was located in the fractions by monitoring with TLC (silica gel and a 1 : 1 chloroform–ethyl acetate solvent system) and spraying with 2,4-dinitrophenylhydrazine reagent. Those fractions showing a positive test were combined, the solvent removed, and the residue sublimed to give *p*-hydroxybenzaldehyde. The IR spectrum was identical with that of an authentic sample. In general, XAD-retained material, from which the bulk of the luteolin-7-glucoside had been removed by crystallization from methanol, could be analyzed by the NMR spectrum of the aromatic region. The spectra were surprisingly clean in this region, and an idea of the content could be obtained by observing the ratios between the aldehyde signal, the total aromatic signals (all AB doublets), and the benzylic resonance of the dhurrin (5.88 ppm in methanol-d_4).

RESULTS AND DISCUSSION

Bioassay. A behavorial bioassay was used as a guide in the isolation work to follow the feeding-deterrent activity of the extracts during the course of fractionation. The bioassay consisted of a synthetic diet to which substances to be tested would be added. After an appropriate time (24 hr), the percent of

aphids feeding was determined. The bioassay, when used in this fashion, suffers from two disadvantages: (1) it can only be used on water-soluble substances, and (2) it gives only an indication of feeding-deterrent properties, reflected perhaps in nonpreference in the plant. Resistance factors slowing the rate of reproduction or causing a decrease in weight gain of aphids will not be detected when the bioassay is used as described below.

In the initial phases of development of the bioassay, insect feeding on the diet was low and erratic. In order to improve the feeding levels, manipulation of diet constituents, including sugar levels, was undertaken with varying degrees of success. After the addition of a number of sodium and potassium salts, it was realized that there might be a pH factor in the degree of feeding. This was then explored by systematically adding a series of buffers to the standard Akey-Beck diet. As a result, the degree of feeding as a function of pH could be determined, and the results are illustrated in Figure 1. The maximum feeding occurred at pH 8.0. With proper care, about 90–95% of the aphids in a mixed population would readily feed within 24 hr on the Akey-Beck diet adjusted to pH 8.7. Sensitivity to dietary pH is not a new finding (Auclair, 1965; Srivastava and Auclair, 1971), although the greenbug seems to prefer an unusually high pH.

In an effort to dissolve normally water-insoluble materials in the diet for testing, the possibility of adding a nonionic detergent was explored. The addition of Tween-60 to the diet down to the 0.1% level resulted in such a large decrease in the number of aphids feeding that further work in this direction was given up.

Isolation. Since so little is known about the feeding habits of biotype C greenbugs, it appeared justified to use whole-plant extracts of sorghum as starting materials in the isolation procedure rather than specific plant parts.

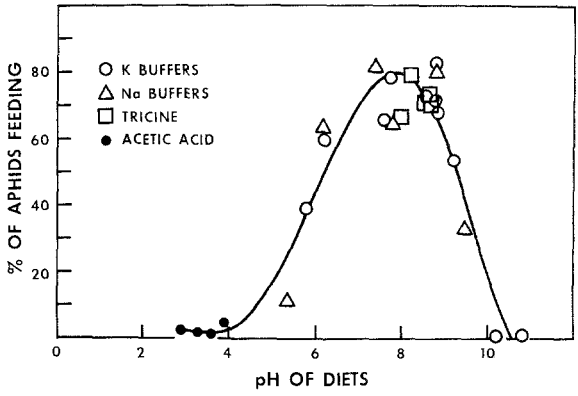

FIG. 1. Degree of feeding as a function of pH.

The bulk of the isolation work was carried out using a resistant breeding line of sorghum, IS 809 (Johnson et al., 1974), and a related commercially available greenbug-resistant line, G-449 GBR, the latter developed by Funk Seeds International.

As with most economically important crops, the major classes of chemical constituents in sorghum have been extensively studied. As a result, cyanohydrin glycosides, triterpenes, and a variety of phenolic substances including tannins are known to occur in sorghum. The concentrations of such compounds varies greatly among varieties and plant parts and is dependent upon plant age. The feeding-deterrent activity was confined to the polar extracts, although activity in the waxy fractions cannot be definitely excluded because of limitations of the bioassay. The active materials were soluble in acetone, methanol, and water.

To avoid hydrolysis of the cyanohydrin glucoside, dhurrin, known to be in sorghum, and any other glycosides, fresh plant material was crushed and, without drying, was promptly extracted with methanol or acetone. This procedure, designed to inactivate enzymes, was necessary since it is known that sorghum also contains a β-glucosidase which will hydrolyze dhurrin to yield free HCN and *p*-hydroxybenzaldehyde (Mao and Anderson, 1967, and references cited therein; Kojima et al., 1979). A key step in the isolation procedure was the adsorption of the extracts on XAD-2. Phenols and other aromatic substances are adsorbed on XAD while sugars and aliphatic compounds are not (Loomis et al., 1979). Aromatic compounds can be recovered from XAD by elution with methanol. When the aqueous sorghum extracts were filtered through a column of XAD, all of the feeding-deterrent activity was retained on the XAD and was recovered by elution with methanol. These results indicate that the feeding-deterrent materials were aromatic and very polar.

Chromatography of the methanol-soluble XAD-retained fraction on Sephadex LH-20 with methanol did not confine the feeding-deterrent activity to any particular fraction, suggesting that two or more biologically active components were present in the extracts. Similar results were obtained by chromatography of the active, tetrahydrofuran-soluble, XAD-retained materials on silica gel. Again, feeding-deterrent activity was distributed throughout all of the column fractions.

By working up the column fractions (see Methods and Materials), samples of *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, dhurrin, and luteolin-7-glucoside were isolated. At this juncture, it cannot be excluded that *p*-hydroxybenzaldehyde arises from partial hydrolysis of dhurrin during the isolation procedures.

The literature on tannins and possible tannin precursors in sorghum is extensive and conflicting (Yasumatsa et al., 1965; Stafford, 1965, 1967; Bate-

Smith, 1969; Bate-Smith and Rasper, 1969; Nip and Burns, 1971 and references cited therein; Fletcher et al., 1977; Haslam, 1977). The inconsistency of much of this work is likely due to a combination of differences in (1) the varieties of sorghum studied, (2) the degrees of maturity of the plant material studied, and (3) the plant parts studied. Nevertheless, there seems to be some general agreement that there are one or more substances present in sorghum which are likely tannin precursors and which upon treatment with strong mineral acid give a "blue rose" color with absorption maxima about 550 nm. Moreover, the spectral properties of this rose-colored material are similar to those obtained when 3′,4,4′,5,7-pentahydroxyflavan is treated with acid under the same conditions.

The material causing the rose color, procyanidin (Bate-Smith, 1969; Fletcher et al., 1977; Haslam, 1977), was retained on XAD. It was freed from other identifiable materials by chromatography on Sephadex LH-20 with methanol. The fractions giving the rose color did not contain flavonoids as judged by a weak ferric chloride test. The NMR spectra indicated the absence of dhurrin and related compounds. The material in these fractions had an ED_{50} of 0.08%, making them the most active feeding-deterrent materials so far isolated from sorghum.

Commercial tannic acid also showed very high activity (Table 1). Todd et al. (1971) also observed high biological activity of tannic acid.

Feeding Deterrence. Each of the substances isolated from the XAD-retained, feeding-deterrent fractions was tested at a series of concentrations in order to establish an ED_{50}. These data are summarized in Table 1 along with that for related substances, and results for dhurrin and *p*-hydroxybenzaldehyde are illustrated in Figures 2 and 3. An ED_{50} is defined as that concentration of material in the diet which caused a 50% reduction in feeding compared with the controls.

TABLE 1. FEEDING DETERRENCY OF SORGHUM AND RELATED CONSTITUENTS

Compound	ED_{50} (% of the diet)
Dhurrin	0.16
p-Hydroxybenzaldehyde	0.13
p-Hydroxybenzoic acid	0.36
Luteolin-7-glucoside	No activity at the 1% level
Amygdalin	0.5
Benzaldehyde	0.4
Chelidonic acid	0.15
Procyanidin	0.08
Benzyl alcohol	0.08
Tannic acid (commerical)	0.02

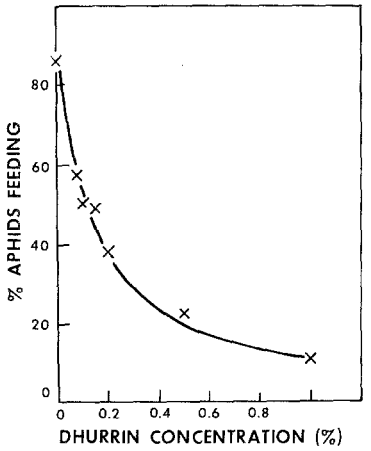

FIG. 2. Effect of dhurrin concentration on feeding.

Although chelidonic acid, a known sorghum constituent, was not isolated, it is available commercially and was screened for activity (Bough and Gander, 1972). However, since chelidonic acid is present in appreciable amounts only in sorghum seedings, it probably does not play an important role in aphid resistance.

Dhurrin levels are also known to decrease with increasing maturity (Hogg and Ablgren, 1943; Woodhead and Bernays, 1977). As a result, its deterrency role is likely to be important only in young plants. Moreover, there seems to be no general relationship between sorghum cyanide levels and greenbug resistance (Starks, personal communication).

Since dhurrin appeared to be somewhat unacceptable to greenbugs, several other related cyano derivatives were surveyed for their feeding-

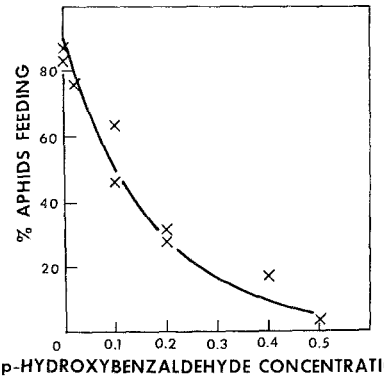

FIG. 3. Effect of *p*-hydroxybenzaldehyde concentration on feeding.

deterrent effects. When potassium cyanide was added to the diet at a molar equivalent to the ED_{50} of dhurrin (0.16%), the feeding rate dropped to 25%. When aphids feeding on the control diet were exposed to open diet containing dhurrin of KCN in the test chamber on the aphid side of the membrane, they all died. The greenbug thus shows a moderate tolerance to ingested cyanide compared to cyanide obtained through the respiratory system.

Relative to dhurrin, the aphids showed a high tolerance towards the commercially available cyanohydrin glycoside amygdalin (Table 1). This suggests that the aldehyde hydrolysis product of the cyanohydrin may play the major role in dhurrin toxicity or acceptability. This is supported by the fact that benzaldehyde, the aglycone of amygdalin, had an ED_{50} of only 0.4%, whereas *p*-hydroxybenzaldehyde, the aglycone of dhurrin, had an ED_{50} of 0.13. In spite of care to prevent dhurrin hydrolysis during the isolation work, it is likely that the *p*-hydroxybenzaldehyde is an artifact arising from partial hydrolysis of the dhurrin.

Dhurrin is localized in sorghum vacuoles (Saunders et al., 1977, 1978). If the greenbug biotype C is a phloem feeder, then the role of dhurrin as a feeding deterrent under field conditions may be minimal. Such is apparently the case for the dihydrochalcone, phlorizin, which acts as both a probing and ingestion deterrent for some aphids (*Myzus persicae*) in apples. *Aphis pomi* feeds on apples and only on the phloem. But since phlorizin does not occur in the phloem, it apparently does not play a role in plant resistance in this case (Montgomery and Arn, 1974).

Possible synergistic effects of the substances isolated from sorghum were explored by testing the six possible combinations of dhurrin, luteolin-7-glucoside, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoic acid combined as two component mixtures. In no case was the antifeeding activity significantly higher in the combinations than would be expected by the sum of the materials alone. Similar results have been obtained with sorghum constituents as feeding deterrents towards *Locusta* nymphs. Again, no synergistic effects were observed (Adams and Bernays, 1978).

A number of questions remain to be answered before sorghum selection for greenbug resistance by chemical analytical methods can be placed on a routine basis. These include: (1) Is greenbug biotype C a phloem feeder or does it feed the mesophyll parenchyma? (2) What is the distribution of deterrent compounds in the various plant parts, especially those parts on which the insect feeds? (3) How do the levels of deterrent compounds vary with age of the plant? (4) What compounds in sorghum affect greenbug growth and rate of reproduction?

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ABSTINONS
Male-Produced Deterrents of Mating in Flies

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Abstract—*Glossina morsitans morsitans* Westwood and *Musca domestica* Linne have an efficient strategy for preventing wasteful homosexual activity in males: hexane-soluble substances on the cuticle of the male flies terminate courting by other males on contact. The inhibitory activity of these materials is demonstrated by the abstention of males from mating with females treated with male extract.

Key Words—Abstinon, sex pheromone, *Glossina*, *Musca*, Diptera, Muscidae, homosexual mating inhibitor.

INTRODUCTION

Vision is often employed by diurnal insects when searching for a mate, but unlike scent or sound, visual perception is often nonspecific. Even though an insect may recognize another member of its species by sight, sexual recognition is often poor, and in many species of insects males court and attempt to copulate with other males. Such errors are rarely made by tsetse flies. Mature tsetse males are sexually aroused by the movement of other flies. They will initiate sexual behavior with males in the absence of females, and the level of arousal is increased if the flies are put into motion. The courting ceases, however, immediately when physical contact is made with another male, while contact with females results in rapid copulation (Langley et al., 1975). It has recently been shown that the female produces sex pheromone which stimulates mounting and copulation in the male. Small quantities of this attractant are also present in the male fly (Carlson et al., 1978); nonetheless, aroused tsetse males are inhibited upon touching males er-

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roneously being courted and immediately walk away from them. In the housefly, male recognition of females is very pronounced, yet a low frequency of homosexual courting attempts do occur (Rogoff, 1964).

We supposed that a chemical substance in the male cuticle might terminate male homosexual activity. If so, females which had been treated with this inhibitor might be rendered unattractive to males which touch them. Thus, an attempt was made to extract the substance using hexane washings of males and to test its inhibitory effect on courting behavior.

METHODS AND MATERIALS

Tsetse Flies (*Glossina morsitans morsitans* Westwood). Male extract was prepared by immersing week-old, virgin males in hexane (Merck A.R.) (10 flies/cc) for 2–6 hr. Dead females killed by freezing were tested for mating stimulatory activity. These same positive females were then treated with different doses of the male substance, and male response to the treated females was scored again. The extract was slowly applied with a hypodermic needle, with the female, mounted on the sharp end of an entomological pin, placed in a cool airstream in order to dry quickly without dripping of the fluid material. Treated flies were dried for ½ hr and placed in separate paperlined 2.5 × 7-cm glass test tubes containing one 7-day-old male. The scoring of male response was as in Carlson et al. (1978). Since male tsetse flies are inactive under such confined conditions, the tubes were tapped and moved until the male fly moved or made several contacts with the female. Behavior of the male was scored as follows: "nil" for no response; "one" for a short arrest of the male movement on the dead female; "two" for correct positioning of the body; "three" for the maximal response—attempt at copulation including flexing of genitalia.

Behavioral responses of male tsetse flies with cork decoys treated either with sex attractant alone or with sex attractant followed by male extract were tested as follows: Extract of sex attractant was obtained by submerging 3-week-old females in hexane (10 females/cc) for 6 hr, and applied to cork pieces, 2 × 3 × 10 mm, as described above. The responses of males to the treated decoy were scored as with the treated female. After positive responses to the decoys were observed, these same decoys were then treated with different doses of male extract, and the responses of male flies were tested to determine possible modification in their behavior. Normal sexual response of these male flies was verified after each experiment.

Houseflies (*Musca domestica* L.). As in the *Glossina* experiment, test objects were dead females. Scoring of male behavior was first done with untreated females, after which these female flies were treated with male extract and male mating response was scored again. The male extract was

prepared by immersing 7-day-old males in hexane (20 flies/ml) for 2 hr. Otherwise the experiments followed the general line of Rogoff et al. (1964). Sets of five virgin males more than 7 days old were placed in a filter-paper-lined 9-cm Petri dish with a dead fly (freshly killed by freezing) of the same batch. There is a difference in the procedure from that used for the tsetse fly because the housefly sex attractant is more volatile than that of the tsetse fly. Male flies introduced into the Petri dish are attracted to the female and there is no need to manipulate the dish to bring about contact between the male and female flies. The behavior of the males was observed for 1 hr and scored as follows: "one," abrupt male–female contact, with a short flutter of wings; "two," a longer flutter of wings; "three," attempts at copulation. Male-to-male approaches were scored together as homosexual mating attempts. Twelve to eighteen replicates were carried out for each treatment.

RESULTS AND DISCUSSION

All female controls treated with large quantities of the hexane solvent elicited the maximal response (stage 3) as did untreated females (Table 1). In the tsetse fly, application of as little as 0.1 male equivalents on the dead female produced a potent antiaphrodisiac effect; 80% of the male flies did not even score up to stage one with the treated females and no attempts at copulation with them were made. Increasing the amount of the extract to 0.5 male equivalents abolished male mating behavior entirely (Table 1).

In the housefly experiments, application of male extract on the dead female did not completely abolish mating response but it reduced the response to the low, normal level of homosexual mating activity (Table 2). The relative dose levels of male extract required to achieve this inhibition were higher than in the tsetse fly: 2.0 male equivalents reduced male response levels such that

TABLE 1. DECREASE IN ATTRACTION OF FRESHLY KILLED *G. morsitans* FEMALE FOLLOWING APPLICATION OF MALE FLY EXTRACT

Solvent quantity (cc)	Male extract (specimens equivalent)	Number of tests	Male reaction (%)			
			0	1	2	3
0.4	0	20				100
0.2	0	60				100
0.0	0	60				100
0.1	0.5	20	100			
0.05	0.25	100	96	4[a]		
0.02	0.1	20	80	20		

[a]Doubtful attempts.

TABLE 2. DECREASE IN ATTRACTION OF FRESHLY KILLED FEMALE *Musca* FOLLOWING APPLICATION OF MALE EXTRACT

Extract in male equivalent on dead female	Solvent quantity (ml)	Average[a] male sex activity per hr			Homosexual activity[b]	Total activity
		Stage 1	Stage 2	Stage 3		
0	0.1	0	2.13	10.2	0.3	12.26
2	0.1	0.6	0.06	0	0.1	0.76
0.5	0.025	1.2	1.06	0.8	1.0	4.06
0.25	0.05	1.266	1.533	1.8	1.13	5.72
Dead male	0				0.64	0.64

[a] More than 60 males/treatment.
[b] Occurring between the set of five males in each replicate.

very few of the tested flies reached stage 2 and none reached stage 3. Mating response levels with 2 male equivalents were reduced to ca. 0.5% of the pooled stage 2 and 3 levels of the controls.

To determine whether the male extract inhibits the courting response elicited by the female sex attractant, we used cork decoys treated with extracts of both sexes. Decoys treated with a dose of 2 female equivalents elicited a maximum score of three in over 70% of the males (Table 3). This response decreased to "nil" when an extract of 3 male equivalents was added to the decoy. Addition of smaller quantities gave a partial inhibition (Table 3). In the decoy series we used larger quantities of both male and female substances than in the experiments with a dead female, and the results were dose dependent and similar to the other series. The results with the decoys indicate that the male-produced material inhibits the behavior triggered by the sex pheromone.

Further evidence that the inhibition is carried out by means of sensory

TABLE 3. DECREASE IN ATTRACTION OF A 2-FEMALE-EQUIVALENT EXTRACT CORK DECOY FOLLOWING APPLICATION OF MALE *G. morsitans* EXTRACT

2-female-equivalent extract + different equivalents of male extract	Number of tests	Male reaction (%)			
		0	1	2	3
0	66	6	6	16.5	71.5
3	20	100			
2	25	88	12		
1.5	25	84	12	4	
1	25	52	36	12	
0.5	20	35	35	25	5
0.25	25	36	36	28	

cues results from our work on chemoreceptors of pheromones and antipheromones in flies (Schlein et al., 1981). In this work, coating of the tarsi resulted in a high percentage of homosexual activity both in tsetse flies and houseflies, apparently due to the sealing of the male substance receptors. This permitted the expression of the sex attractant present in male cuticular lipids in small quantities (Carlson et al., 1978). The homosexual behavior elicited by sealing the tarsal chemoreceptors is an indication that the abstention behavior of males upon touching other males is chemically mediated.

Mechanisms for inhibiting responses to sex attractants are found in nature. For example, in Lepidoptera they serve to block the response of closely related species which have components in common with the attractant of the female of the species. There they serve as a mechanism for reproductive isolation by preventing interspecific mating (Tumlinson et al., 1976).

In several species of muscid flies, cuticular lipids of newly emerged males and females were analyzed and found to be nearly identical. However, in older flies the chromatogram profiles were different not only quantitatively, but also qualitatively (Uebel et al., 1975, 1977, 1978; Harris et al., 1976). The possibility that some of the substances specific to the males serve as mating deterrents should be examined. Similarly, while there is no distinction between the stimulatory effect of cuticular lipids of the antennae of young male and female *Blatella germanica* in arousing sexual response, older males lose their potency for stimulation, apparently due to the accumulation of some inhibitory male chemical factor (Ishii, 1972). Often, in species where attempts to copulate between males occur, behavioral patterns have developed for ridding the attacked male of the assaulting male. For example, in locusts, an assaulting male is simply kicked off. Assaulted *Drosophila* males flick their wings in the same way as unreceptive females (Chapman, 1969). It is plausible that any behavioral mechanisms are more costly in time and energy than a chemical mechanism which terminates homosexual mating attempts at an initial stage.

Male-produced mating deterrents have been described in several cases as mechanisms for ensuring female monogamy. For example, males of the butterfly *Heliconius erato* produce pheromones which are transferred to the female genitals during mating and deter subsequent males from mating attempts (Weaver, 1978). However, the present finding is the first description of a male-produced substance which protects males from homosexual assault. Since we anticipate that this strategy is employed in additional species, we propose the term "abstinons" for male-produced substances which have antihomosexual activity.

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RECEPTORS OF SEX PHEROMONES AND ABSTINONS IN *Musca domestica* AND *Glossina morsitans*

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Abstract—Receptors arranged in two pairs on the inner and outer sides of the proximal end of the tibia of legs of *Musca domestica* L. and *Glossina morsitans morsitans* Westwood are described here for the first time. In the male fly, these receptors function in perception of sex pheromones, as demonstrated in experiments in which the sense organs were coated with paraffin. Similar techniques showed that sense organs for abstinon are located on the tarsi. Scanning electron microscopy and light microscopy show that the tibial sense organs of *Musca* may be sensilla of the coeloconic type.

Key Words—Sex pheromone receptors, coeloconic sensilla, abstinon receptors, Diptera, Muscidae, *Musca, Glossina.*

INTRODUCTION

The sex recognition system of *Musca domestica* L. and *Glossina morsitans morsitans* Westwood comprises both visual and chemical elements (Ragoff et al., 1964; Langley et al., 1975). In the arousal of sexual behavior in diurnal flies, the visual stimulus precedes or occurs concurrently with the chemical one. However, the visual cue is a generalized one as indicated by positive male fly reactions to different moving decoys. In contrast, a species-specific chemical cue is required for the progression of sexual behavior which is discontinued at an initial stage if the chemical stimulus is not encountered.

The chemical structure of female sex pheromones in the two species of flies and the pattern of sexual behavior induced by them were described in Ragoff et al. (1964), Carlson et al. (1971, 1978), and Langley et al. (1975). Wasteful homosexual behavior is prevented both in houseflies and tsetse flies by male-produced pheromone, termed abstinon by Schlein et al. (1981).

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The sense organs for sexual perception in flies have not yet been located except for the tarsal sense organs of *Stegomyia* mosquitoes which form part of their mechanisms for recognition of conspecific females (Nijhout and Craig, 1971). Carlson et al. (1978) suggested *Glossina* tarsal chemoreceptors (Lewis, 1954) as possible sex pheromone receptors.

The present study indicates that in *Musca domestica* and *Glossina morsitans* males, the receptors of sex pheromones and abstinons are located on the legs. Organs for perception of sex pheromones are located on the upper part of the tibia, and tarsal sensilla are sensitive to the abstinons. We describe here for the first time two pairs of sensilla, restricted to the upper tibia of dipteran species, which we believe are the sex pheromone receptors.

METHODS AND MATERIALS

Musca domestica L. Sexes were separated on the day of eclosion. The flies were fed on sucrose and kept at 24–26° C and 60–80% relative humidity.

The experiments followed the general line of Rogoff et al. (1964). Five mature virgin males more than 7 days old were placed in filter-paper-lined 9-cm Petri dishes with a dead fly (freshly killed by freezing) of the same brood. The behavior of the males was observed for 1 hr and scored as follows: "nil" for no contact; "stage 1," abrupt male–female contact, with a short flutter of wings; "stage 2," a longer flutter of wings; "stage 3," attempt at copulation. Homosexual mating attempts (male-to-male approaches) which occurred were also scored.

Glossina morsitans Westwood. Pupae were kept at 28° C and 70% relative humidity, and adult flies at 25° C and 70% relative humidity. The sexes were separated on the day of eclosion. The flies were allowed to engorge on guinea pigs. The experimental procedure and scoring were adapted from Carlson et al. (1978), which took into account the fact that tsetse flies are not very active. Mature treated or untreated virgin males older than 7 days were placed individually in glass tubes of 2.5 × 7 cm into which a freshly killed female was introduced. The tube was then tapped and moved until several male–female contacts were observed. The male behavior was scored as follows: "nil" for no response; "one" for a short arrest of the male movement on the dead female; "two" for correct positioning of the body; "three" for the maximal response—attempt at copulation including flexing of genitalia.

The sense organs of male flies were covered using a small drop of melted paraffin wax. The wax was applied with a fine pointed iron wire attached to the tip of a small soldering iron. The length of the wire was adjusted so that the temperature of the tip would be just above the paraffin melting point (56° C). The drop of melted paraffin formed a ring which covered both the outer and inner sensilla. Male flies were etherized and paraffin was applied to all six legs.

Areas covered in the two experimental groups (I and II) and the control group (III) were as follows: (I) sense organs on the upper part of the tibia and the adjacent area; (II) the tarsi; (III) the lower part of the tibia or the femur. There were two additional control groups: (IV) etherized but not treated; (V) not etherized, not treated. Treated flies were returned to cages overnight for recovery and untreated etherized flies were allowed at least 1 hr of recovery time before testing.

Permanent mounts of legs of different dipteran species, fixed in Bouin's fixative and stained with Mayer's hematoxylin, were examined under the compound microscope. Receptors (of *Musca* and *Glossina* males) were also examined under the Cambridge scanning electron microscope (SEM). The SEM material was washed in acetone and coated with gold vapor.

RESULTS

In both *Musca* and *Glossina*, similar paired sensilla located at the outer and inner sides of the proximal end of each tibia were observed in both sexes. The experiments which lead us to believe that these are the sex pheromone receptors of the flies are described below.

In Musca, the sensilla are shallow convex structures, found within an elliptical saucer-like depression ($6 \times 3\,\mu m$). There was an orifice on the upper surface of each sensillum (Figure 1a and b, outer sensilla; Figure 2, inner

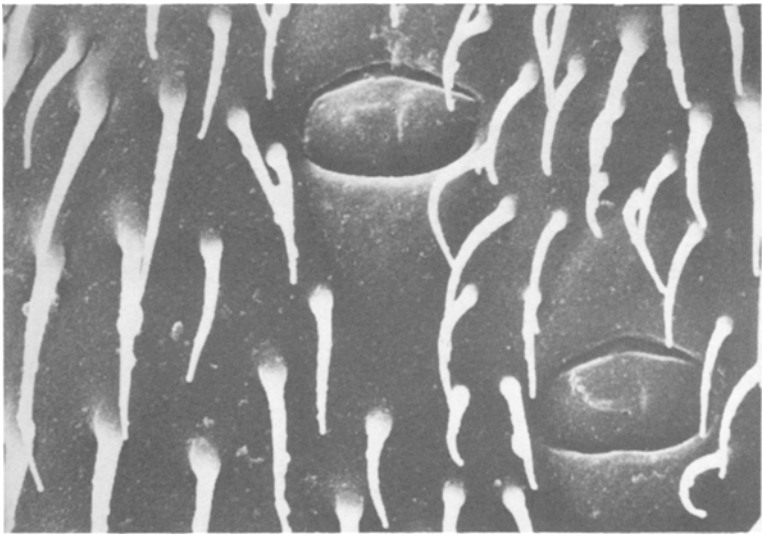

FIG. 1. Outer pair of tibial sensilla of *Musca domestica* (SEM). (a) 3100×; (b) upper sensillum of 1a, 9700×; (c) lower sensillum of 1a, 9700×.

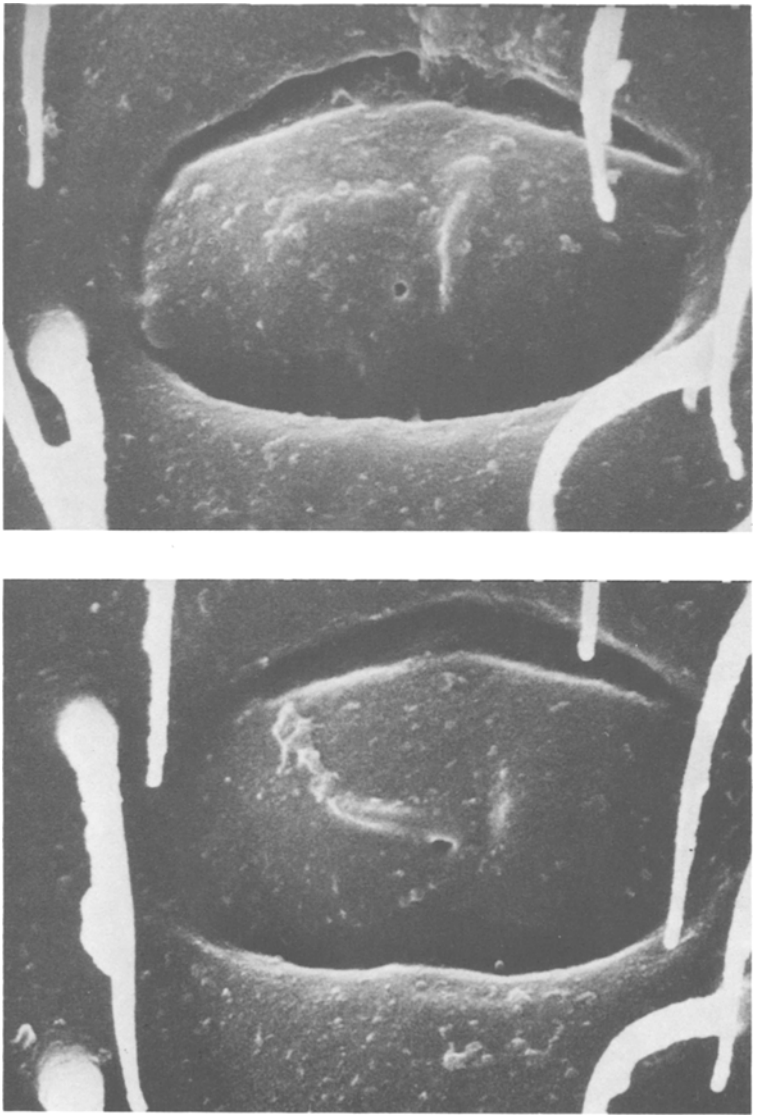

FIG. 1. (*cont.*)

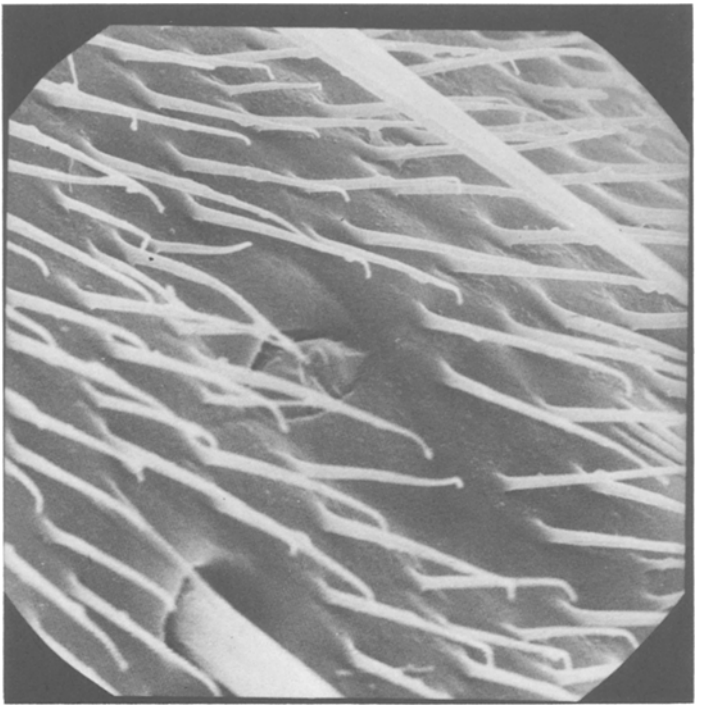

FIG. 2. Inner tibial sensilla of *M. domestica* (SEM, 2000×).

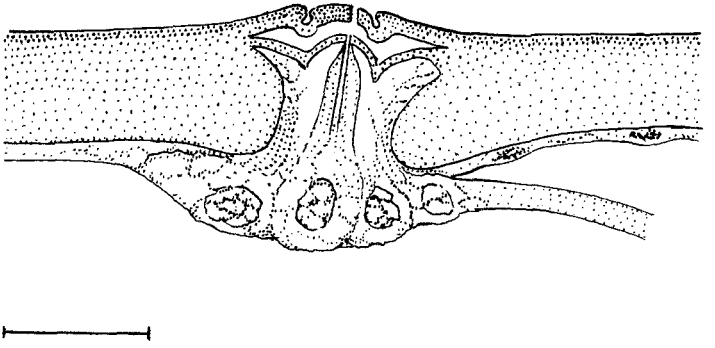

FIG. 3. Tibial sensillum of *M. domestica* as seen in light microscopy, schematic drawing.

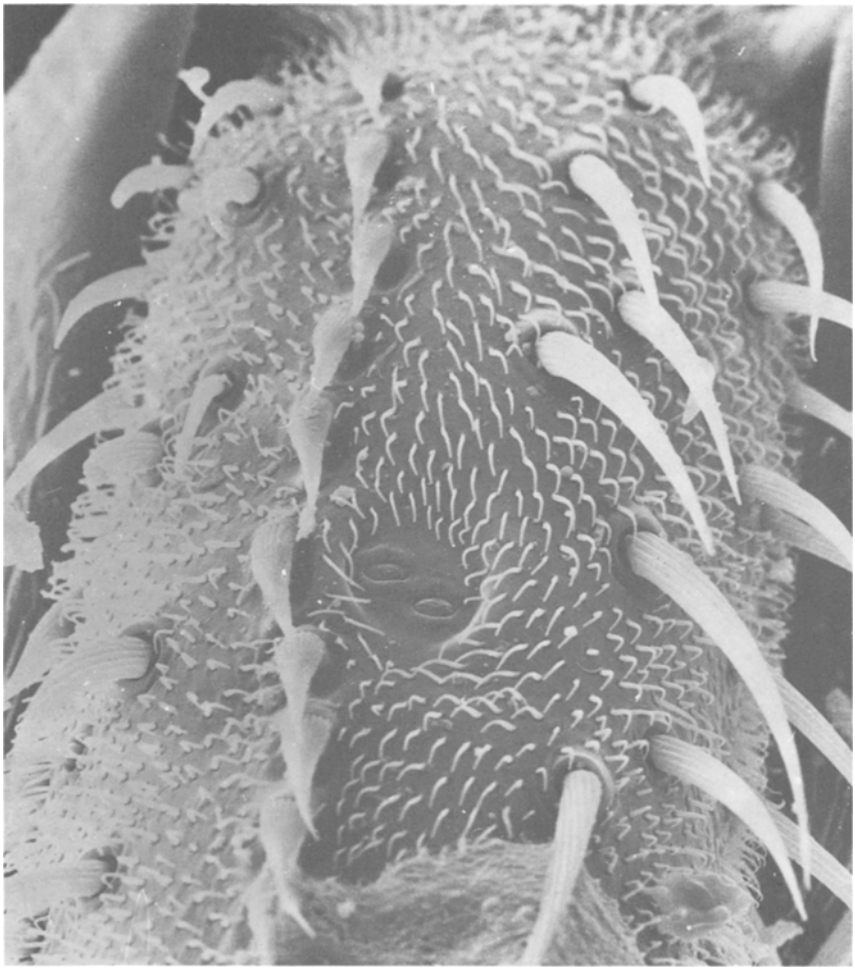

FIG. 4. Outer pair of tibial sensilla of *Glossina morsitans* (SEM). (a) 700×; (b) 3000×.

sensilla) leading into a lumen as observed in light microscopy sections (Figure 3). The floor of the lumen is a thick-walled peg with a single opening, giving the dendrites reaching the tip of the peg access to the exterior. Four cells were found at the base of the peg, of which two were the chemosensory neurons and two accessory cells. Morphologically, these sensilla may be classified as coeloconic chemoreceptive types.

In *Glossina*, the two outer sensilla were close together and found within a single, round, saucer-like depression (Figure 4a and b), while the inner (Figure 5) ones are within separate depressions. In light microscopy, the

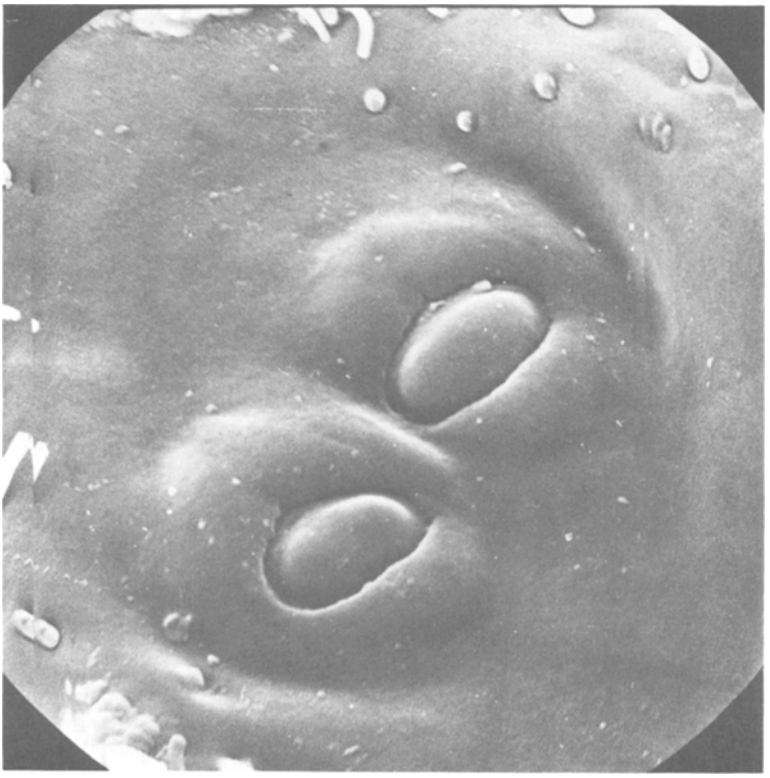

FIG. 4. (*cont.*)

sensilla resemble those of *Musca*; however, no orifice was observed when scanning the surface with the SEM, and due to the small size of the sensilla (3–4 μm diameter), transmission electron microscopy would be needed to determine the structural details.

In the seven additional dipteran species examined, representing a wide phylogenetic range [*Stomoxys calcitrans* L. (Muscidae), *Sarcophaga falculata* Pandele (Sarcophagidae), *Anopheles gambiae* Giles, *Culex pipiens* L., *Phlebotomus major* Annandale, *Phlebotomus papatasi* Scotoli (Psychodidae), and *Pseudolynchia canariensis* Macquart (Hippoboscidae)], all males and females had these tibial organs. This suggests that these tibial sense organs are general to Diptera.

Behavioral responses associated with these structures were studied by coating different sections of the leg in male flies. Coating the tibial receptors of *Musca* males reduced both the quantity and quality of male sexual behavior from an average of 11 strikes per male (control) to an average of 1.57 strikes

FIG. 5. Inner tibial sensillum of *G. morsitans*, 5400×.

(Table 1) and from 60% stage-3 strikes, involving manipulation of the male genitalia to strikes where sexual behavior involved, at most, clutching the female fly. In control flies where the distal part of the tibia or the femur was coated, only a slight reduction in sexual activity was observed (Table 1).

Coating of *Glossina* tibial receptors completely abolished mating behavior, while control flies in which the distal part of the tibia or the distal part of the femur was coated behaved normally (i.e., 100% stage-3 activity) (Table 2).

Coating of the tarsi of *Musca* males did not decrease the overall sexual activity, although not many flies reached the maximal stage-3 level. However, the treated males attempted to mate with each other; thus the level of homosexual activity increased eightfold (from 0.64 to 4.95 strikes/hr/male) (Table 1). In untreated *Musca* males it was not always clear whether the male-to-male behavior was courting or aggression. In contrast, the increase in homosexual relations in tarsi-coated flies comprises many attempts at copulation.

TABLE 1. AVERAGE SEXUAL RESPONSE PER HOUR OF *Musca domestica* L. MALES IN WHICH DIFFERENT LEG SENSE ORGANS WERE COATED

Group	Area coated	Sex object	Response level			Total male vs. female sexual activity	Homosexual[a] activity	Total sexual activity	Number of males
			1	2	3				
I	Tibial sense organs	Dead female	0.66	0.27		0.93	0.64	1.57	75
III	Control: distal tibia or femur	Dead female		2.63	5.03	7.66	1.07	8.73	70
II	Tarsi	Dead female	1.10	3.50	0.50	5.10	4.95	10.05	40
IV	Control: untreated	Dead female		3.50	6.74	10.24	0.80	11.04	35
V	Control: untreated	Dead male					0.64	0.64	30

[a]Any male-to-male approaches in the replicates.

TABLE 2. SEXUAL RESPONSE OF *Glossina morsitans* WESTWOOD MALES IN WHICH DIFFERENT LEG SENSE ORGANS WERE COATED.

Group	Area coated	Sex object	Response level 0	1	2	3	Number of males
I	Tibial sense organs	Dead female	22	2			24
III	Control: distal part of tibia	Dead female				22	22
II	Tarsi	Dead female	3	2	5	4	14
II	Tarsi	Dead male	7	2	5		14
V	Control: untreated	Dead male	20				20

Coating of the tarsi of *Glossina* males caused severe difficulty in locomotion; thus many males failed to mate. However, the males which attempted mating failed to discriminate between males and females (Table 2). About half the males with coated tarsi attempted to mate with males. This is of particular interest since untreated tsetse males show absolute abstention from homosexual activity.

The plausible inference from these observations is that the tibial receptors detect the presence of sex pheromones, while tarsal chemoreceptors are sensitive to abstinons (Schlein et al., 1981).

As noted, the sensilla are present in females as well as in males. However, in females, direct electrophysiological recording from the sensillum would be needed in order to determine whether a response to sex pheromone is present since the behavioral parameters which we used in the males do not apply to females.

DISCUSSION

While the olfactory receptors of the lepidopteran sex pheromones have been the subject of a great many studies, hardly any work has been carried out on dipteran sex-recognition pheromone receptors. The only previous insight into these sense organs was from a small experiment with males of the mosquito *Aedes albopictus* Skuse. Following inactivation of the two distal tarsal segments (Nijhout and Craig, 1971), heterospecific contacts with *A. aegypti* L. females were maintained which otherwise would have been terminated.

Male *Drosophila* flies will often court females of other species. *D. melanogaster* Meigen approaches and taps *D. simulans* females with its

foretarsi (Manning, 1966). This is the normal preliminary to courtship, and males certainly get specific contact-chemical information on the female's identity in this manner. The male may proceed with courting when the *D. simulans* is a newly emerged female (probably not yet covered with the specific pheromone). With mature *D. simulans* females, the male *D. melanogaster* will turn away (Manning, 1966).

In both the above instances, coating of the tarsi impaired the distinction between conspecific and heterospecific mating partners such that abnormal heterospecific pairings were attempted. In our experiments, however, coating of the tarsi (III) impaired the distinction between sexes within the same species. We believe that the same mechanism is operative in all three cases. In each, impaired perception of an inhibitory cue permitted continuation of mating with an inappropriate partner. In the first and second instances, mating was not inhibited by alien pheromones of the congeneric species. In the third instance, homosexual mating attempts were not inhibited by the abstinon of the other males.

The stimulatory effect of the sex pheromone is apparently perceived through the tibial sensilla and not through the tarsal receptors. This sense organ and its unique function appear to be widely distributed among Diptera. Thus, our observations shed some light on the nature of the interaction between the abstinons and the sex pheromones of the flies.

Pheromone perception has been found to be influenced by inhibitors in many species (Tumlinson et al., 1976). Often the chemical structure of the inhibitors is closely related to that of given pheromones. The inhibitors could be effective at the peripheral receptor or through integration in the central nervous system. Peripherally, inhibitors might block the pheromone receptor site. It is also possible that an inhibitor could stimulate different receptors from those activated by the pheromone. In that way, information from the pheromone and from the inhibitor would be transferred independently to the CNS. The inhibition would thus originate at the level of the brain. The receptors for abstinon and for the sex pheromone are located far apart and on different segments of the leg; thus it can be easily claimed that the inhibitory effect of abstinon works at the CNS level. The intensity of the information from the two receptors depends on the concentration of each stimulant. In cuticular lipids of the male tsetse there is a very low concentration of sex attractant (Carlson et al., 1978) and probably a high concentration of abstinon; therefore, homosexual advances between males are inhibited upon contact. However, when the abstinon receptors are blocked, the low concentration of sex attractant is enough to stimulate continuation of sexual behavior.

The outer tibial sensilla are situated on the laterally protruding "knee" joint, ensuring maximal opportunity to brush against neighboring flies. The

extremely low volatility of the *Glossina* sex pheromone presumably requires actual physical contact between receptor and pheromone in order to activate the receptor. When, during the courtship process, the male mounts the female, the outer tibial sensilla of the male are no longer in contact with her. However, in the mounting position the male's inner tibial sensilla take over, apparently ensuring continuous sensory input during the entire process of copulation.

The phenomenon of contact sex pheromones has been described in many Diptera. In several muscid species, the chemical structure of the pheromones has already been elucidated (Carlson et al., 1971, 1978; Uebel et al., 1975a,b, 1977, 1978). Similar behavioral experiments carried out with lower Diptera, *Culiseta* (Lang, 1977) and *Culicoides* (Linley and Carlson, 1978) also demonstrate the presence of contact sex pheromones.

Contact sex-recognition pheromones appear to be widespread in Diptera. The tibial sensilla described in this paper presumably play a similar role in all dipteran species using these pheromones.

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KAIROMONE RESPONSE IN *Thanasimus* PREDATORS TO PHEROMONE COMPONENTS OF *Ips typographus*

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Abstract—*Thanasimus formicarius* (L.) responds to racemic ipsdienol and ipsenol and less to (*S*)-*cis*-verbenol. All three are pheromone components in several bark beetles of the genus *Ips*. Synergistic effects appeared when the components were combined. Methylbutenol alone, the specific pheromone component of *Ips typographus*, elicited no response, but synergestic effects appeared when methylbutenol was combined with *cis*-verbenol and ipsdienol. The sympatric species *Thanasimus femoralis* (Zett.) responds to (*S*)-*cis*-verbenol, while ipsdienol and ipsenol synergize the response.

Key Words—Kairomone, pheromone, *Thanasimus*, *Ips typographus*, ipsdienol, ipsenol, *cis*-verbenol, methylbutenol, traps, attractant, behavior, Scolytidae, Cleridae.

INTRODUCTION

Pheromones produced by bark beetles are utilized by predacious clerids in order to find the habitat infested by their prey. The chemical messengers of bark beetles act as kairomones for the scolytid predators (Borden, 1977). The clerid species are usually predators on several species of bark beetles, often species of different genera. Most bark beetles have a multicomponent pheromone (Silverstein and Young, 1976), and some components are shared by several species. There is little information available on the mechanisms involved in clerid response to the habitat infested by their prey.

The first discovery of predators utilizing bark beetle pheromones was made by Wood et al. (1968) in California. *Enoclerus lecontei* (Walcott) was captured in field traps baited with *cis*-verbenol, ipsdienol, and ipsenol, the three pheromone components of *Ips paraconfusus* (Lanier). The clerid responded to ipsdienol and ipsenol but *cis*-verbenol failed to elicit a response

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from the predator. The ternary mixture was the most attractive. *E. lecontei* also responds to *Ips pini* (Say) (Lanier et al., 1972) which has ipsdienol as a pheromone component. *Thanasimus dubius* (F.) and *Thanasimus undatulus* Say are attracted to synthetic frontalin, which is part of the pheromone of several *Dendroctonus* species in North America (Vité and Williamson, 1970; Pitman, 1973; Kline et al., 1974; Dyer et al., 1975). Moser and Brown (1978) captured *T. dubius* in bucket traps baited with a mixture of frontalin and α-pinene, which are used as an attractant for *Dendroctonus frontalis* Zimm.

Thanasimus formicarius (L.) and *T. femoralis* (Zett.) (= *T. rufipes* Brahm) (Biström, 1977) are predators on several species of bark beetles in Europe (Saalas, 1917, p. 435; Gauss, 1954). Both clerids are attracted to the synthetic pheromone components of *Ips typographus* (L.) (Bakke and Kvamme, 1978). Three components are known for the aggregation pheromone of *I. typographus*, i.e., methylbutenol, (*S*)-*cis*-verbenol, and ipsdienol (Bakke et al., 1977; Krawielitzki et al., 1977). Ipsenol is also produced by the male beetle a few days after the excavation of the nuptial chamber (Bakke, 1976), but its behavioral role has not been clarified.

The aim of this paper is to elucidate the response of the two clerid species to the single components of the *I. typographus* pheromone and to their combinations.

METHODS AND MATERIALS

Field experiments were conducted in May and June 1979 in forests at Kongsberg, Lardal, and Eidskog in southern Norway and at Målselv in northern Norway.

The beetles were attracted to traps baited with the pheromone components. Traps were made of black, ridged, cylindrical drainpipes of polyethylene (12.5 × 150 cm) (Figure 1). Each trap had 310 holes (diameter 5 mm) drilled between the ridges. At one end, the pipe was covered with a lid, and at the other end, a funnel with a collecting bottle was mounted.

The traps were placed on a stick in a vertical position with the lower part of the pipe, about 1 m above ground. Distance between the traps was 8–10 m.

Pheromone components known from *I. typographus* were tested alone and in combinations. These were: racemic 2-methyl-6-methylene-2; 7-octadiene-4-ol (ipsdienol), purity 96%; racemic 2-methyl-6-methylene-7-octene-4-ol (ipsenol), purity 94%; (*S*)-*cis*-verbenol, 99% purity, 94% optical purity (all obtained from Borregaard Industries Limited, Sarpsborg, Norway); and 2-methyl-3-butene-2-ol (methylbutenol) (obtained from Aldrich-Europe, Beerse, Belgium).

The dispensers were placed inside the traps in the lower part of the drainpipe. They were made of a polyethylene bag (80 × 60 mm; thickness of polyethylene 0.05 mm) and an absorbant cellulose sheet (40 × 40 × 4 mm).

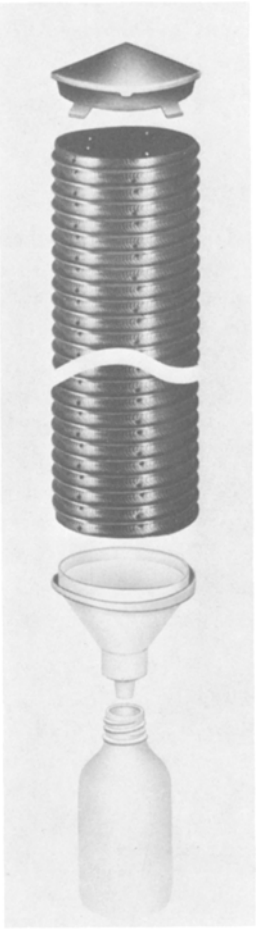

FIG. 1. The drainpipe trap model.

Fifty mg of the pheromone component was added to the cellulose, and it evaporated in small quantities through the polyethylene film. The rate of evaporation, which depends on temperature, was not measured. The temperature in the pipes was approximately 10°C above the air temperature on sunny days, when the beetles fly. The dispensers were still attractive after the termination of the experiments.

A total of 119 traps were used in the experiment. They were set up in the first week of May in southern Norway and in the first week of June in northern Norway. This was before the initial flight of *Thanasimus* spp. The traps were emptied three times during the 4-week experimental period.

In southern Norway we caught a total of 2645 specimens of *T. formicarius* and 158 specimens of *T. femoralis*. In northern Norway we trapped only one specimen (*T. femoralis*). Data from northern Norway are therefore omitted from the analysis.

RESULTS

T. formicarius responded clearly to ipsenol and ipsdienol when these two components were presented individually. (Significance probability ≈ 0.01 for Wilcoxon rank-sum test). A weaker response could be observed to *cis*-verbenol (Tables 1 and 2). Methylbutenol failed to elicit a response (Table 2).

When ipsenol or ipsdienol is combined with *cis*-verbenol, they elicited a response about two times the sum of the responses to the individual components. A synergistic effect could also be seen in the response to ipsdienol plus ipsenol and to the ternary mixture (Table 1). Chi-square analysis revealed that traps with *cis*-verbenol as part of the bait caught significantly more beetles than did traps without *cis*-verbenol ($\chi^2 = 238, 5\,df$). When methylbutenol was combined with *cis*-verbenol and/or ipsdienol, we noted a significant synergistic effect ($\chi^2 = 34, 5\,df$).

Only 6.3% of the predators trapped were *T. femoralis*. Despite the low

TABLE 1. *Thanasimus formicarius* AND *Thanasimus femoralis* TRAPPED IN RESPONSE TO VARIOUS PHEROMONE COMPONENTS OF *Ips* SPECIES (KONGSBERG, NORWAY, MAY 20 TO JUNE 4, 1979)

	T. formicarius						*T. femoralis*					
		Test number						Test number				
Test material	Mean	1	2	3	4	5	Mean	1	2	3	4	5
Ipsdienol	16.8	14	18	24	11	17	0.4	0	0	1	0	1
Ipsenol	16.4	20	2	35	11	14	0	0	0	0	0	0
cis-Verbenol	5.8	1	0	16	8	4	1.4	0	1	1	2	3
Ipsdienol + *cis*-verbenol	39.6	37	20	33	58	50	4.4	5	4	0	2	11
Ipsenol + *cis*-verbenol	38.8	21	32	79	22	40	3.2	0	1	0	3	12
Ipsdienol + ipsenol	44.0	73	41	60	13	33	0.8	0	0	1	1	2
Ipsdienol + ipsenol + *cis*-verbenol	103.2	125	198	66	60	67	9.2	4	5	3	3	31
Control	1.8	0	0	4	1	4	0.6	0	0	3	0	0

TABLE 2. *Thanasimus formicarius* AND *Thanasimus femoralis* TRAPPED IN RESPONSE TO VARIOUS PHEROMONE COMPONENTS OF *Ips typographus*[a]

	T. formicarius						*T. femoralis*					
		Test number						Test number				
Test material	Mean	1	2a	2b	3a	3b	Mean	1	2a	2b	3a	3b
Ipsdienol	18.6	15	12	3	28	35	0					
Ipsenol	15.8	19	2	1	15	38	0					
cis-Verbenol	8.8	14	1	4	12	13	1.6	0	0	0	1	7
Ipsdienol + *cis*-verbenol	56.4	45	19	5	77	136	3.0	5	3	0	2	5
Ipsenol + *cis*-verbenol	44.2	23	12	11	80	95	2.6	0	1	1	0	0
Ipsdienol + Ipsenol	20.2	8	24	6	26	37	0					
Methylbutenol	1.8	2	0	0	4	3	0					
Methylbutenol + *cis*-verbenol	16.4	12	0	2	38	30	1.6	0	1	2	0	5
Methylbutenol + ipsdienol	24.8	8	3	8	28	77	0.4	1	0	1	0	0
Methylbutenol + *cis*-verbenol + ipsdienol	43.4	10	12	5	103	87	2.0	1	0	1	6	2
Control	1.8	2	0	0	4	3	0.2	0	0	0	1	0

[a] Means of 5 tests from Lardal (1), Eidskog (2), and Kongsberg (3), May 15 to June 6, 1979.

number, it is possible to see some trends in their response to the pheromone components. Obviously ipsenol and ipsdienol alone or in combination had no attraction, whereas *cis*-verbenol alone appeared to elicit a response (Tables 1 and 2). Ipsenol and/or ipsdienol apparently had a synergistic effect when offered together with *cis*-verbenol. No synergistic effect could be seen when methylbutenol was combined with any of the other pheromone components (Table 2).

The number of beetles trapped varied between the geographical sites. Each trap group caught 71 *Thanasimus* beetles at Eidskog, 176 at Lardal, and 526 at Kongsberg. These differences explain the great variation in range seen in Table 2.

The results indicate a different response in *T. formicarius* and *T. femoralis* to the pheromone components of *Ips*. Ipsenol and ipsdienol are the main kairomonal components for *T. formicarius* and *cis*-verbenol for *T. femoralis*. The synergistic effect of other pheromone components could be observed in both species.

DISCUSSION

Ipsdienol and/or ipsenol, the pheromone components of *I. typographus*, which act as kairomones for *T. formicarius*, are produced by all European species of the genus *Ips* (Table 3). This explains the attraction of the clerid to habitats infested with all *Ips* species (Gauss, 1954).

The trap method may influence some of the results. Traps with methylbutenol as part of the bait always caught some *I. typographus*, while traps without this component caught none. Bark beetles crawling around in the bottle of the trap may release pheromone with their fecal material. The pheromone will evaporate from the bottle through the funnel and into the pipe, and mix with the components of the dispensers. The bait of the traps then could be supplied with the missing pheromone components necessary for the response of the predators.

trans-Verbenol, which is reported from many species of bark beetles (Vité et al., 1972; Francke and Heemann, 1976) has also been used as bait in similar traps (unpublished data). The response of *Thanasimus* spp. to *trans*-verbenol was significantly lower than to *cis*-verbenol. Because of the impurity in the test material (it contained about 10% *cis*-verbenol), the effect of *trans*-verbenol is doubtful.

Most of the pheromone components of bark beetles exist as enantiomers (Silverstein, 1977). The beetles often produce and respond only to one of the enantiomers (Wood et al., 1976; Vité et al., 1978), while the antipode may be inactive, or even strongly inhibitory to the response. The pheromone components used in this field test were all, except (*S*)-*cis*-verbenol, racemic mixtures. Only small amounts of enantiomers of bark beetle pheromones

TABLE 3. PHEROMONE COMPONENTS FROM EUROPEAN SPECIES OF *Ips*[a]

	Ipsdienol	Ipsenol	*cis*-Verbenol	Others	References
I. typographus	PR	P	PR	2-methyl-3-butene-2-ol, PR	Vité et al., 1972 Bakke et al., 1977
I. acuminatus	PR	PR	PR		Bakke, 1978
I. amitinus	P	P	P	*trans*-2-methyl-6-methylen-3,7-octadien-2-ol, "amitinol," P	Francke et al., 1980
I. cembrae	PR	PR		3-methyl-3-butene-1-ol, PR	Stoakley et al., 1978
I. duplicatus	PR				Bakke, 1975
I. sexdentatus	PR	P			Vité et al., 1974

[a] P = produced by the male beetle; R = positive response demonstrated to synthetic components in field tests.

have so far been available for field tests. As far as we know, there are no data available on the response of predators to synthetic enantiomers of bark beetle pheromones.

Predators of bark beetles also respond to volatile substances from host trees. Monoterpenes of conifers attracted *E. lecontei* in California (Rice, 1969) and *T. formicarius* in Czechoslovakia (Rudinsky et al., 1971). We have observed *Thanasimus* spp. in large numbers visiting pine and spruce logs in early spring several days before any bark beetle has started pheromone production. Obviously, the host substances are the olfaction stimuli, guiding the clerids to the logs. No pheromone components of *Ips* are found in *Tomicus piniperda* L. or *T. minor* (Hartig) (Francke and Heemann, 1976), two common bark beetles on pine in Europe and important prey for *T. formicarius* (Saalas, 1917). Either the volatiles from the trees are the only attractants for these bark beetles, or they produce pheromone components not yet identified, which also act as kairomones for these clerids.

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ON THE PERSISTENCE OF DISPARLURE IN THE HUMAN BODY[1,2]

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Abstract—Response of male gypsy moths from a sparse infestation in Italy to an observer apparently contaminated with disparlure at least two years previously is reported. Other examples of pheromone contamination of people or things are also recorded.

Key Words—Disparlure, *Lymantria dispar*, Lepidoptera, Lymantriidae, pheromone persistence.

For a number of years, many workers in North America who have used or tested disparlure (*cis*-7,8-epoxy-2-methyloctadecane), the synthetic pheromone of the gypsy moth (*Lymantria dispar* (L.) (Lepidoptera: Lymantriidae)) (Bierl et al., 1970), have observed that they, themselves, were often attractive to male moths. Most have assumed that disparlure had accidentally contaminated their clothing or perhaps bodies during routine handling. I report here a case of apparent persistence of bodily contamination for at least two years.

From 1971 through 1977, I have used racemic disparlure [never the (+) enantiomer] either as a bait in traps or in a variety of formulations for broadcast application in attempts to reduce mating success ("confusion" or "disruption" tests) in gypsy moth populations (see, for example, Cameron 1981a–c). In 1973, a microencapsulated formulation of the olefin precursor (2-methyl-*cis*-7-octadecene) was also tested (Cameron et al., 1975). Inevitably during these various tests, I was exposed to large quantities of disparlure and

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[2] The observations in Italy were made while the author was a Gastdozent at the Entomologisches Institute, Eidgenössische Technische Hochschule, Zürich, Switzerland.

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lesser quantities of the olefin, both in the vapor phase in treated field plots and through dermal contact with neat or formulated material. Since the end of the 1977 field season, however, I have not knowingly been in direct contact with disparlure.

On 4 August 1979, at approximately 1400 hr, I stopped with my family at a picnic area on the northeast edge of Lago di Mezzola about 1 km southeast of Novate Mezzola, Provincia Sondrio, Regione Lombardia, Italy. Within 2 min after I got out of the car, a male gypsy moth was fluttering about my exposed legs and exhibiting up-and-down searching behavior so often seen near a vertical silhouette (Richerson et al., 1976). Two more males appeared within the next 3 min, and another 2 males by the end of 30 min. At no time did any of the moths exhibit any apparent interest in other members of my family or other "vertical silhouettes" in the area.

All of the clothing, including footwear, that I was wearing was new within the previous year or less and could not have been exposed directly to disparlure. No other potential attractant, such as a commercial cosmetic or lotion, would appear to be involved, although no rigorous testing of such alternatives was conducted. Therefore, the circumstantial evidence is very strong that my body is contaminated with the pheromone and that the pheromone is extremely persistent. It would seem likely, also, that the quantities of lure required to exceed the behavioral threshold of a male moth ready to respond are exceedingly low—probably far lower than we have previously considered.

A search of the area surrounding the picnic ground revealed no obvious evidence of insect defoliation, no female gypsy moths and no egg masses, old or new. The predominant tree species was a *Populus* sp., possibly *deltoides*. At the time of these observations, the temperature was ca. 27–29° C, the sky was clear, and there was a light breeze blowing.

In light of this experience, other casual observations I have made in previous years assume increased significance.

1. Frequently during the evening hours in the summer of 1978 male gypsy moths would fly against the screened window of my home study in State College, Pennsylvania, if I was working at my desk with the window open. I assumed at the time, perhaps incorrectly, that they were responding to some contaminated object which provided a source of attraction in the room, even though I was never able to identify any likely source. Attraction to room lights is also a possibility which cannot be ruled out completely, although it is suspected not to be the major attractant in this case. Quite possibly I was the attractive source, one year after my last known direct contact with disparlure. The most likely source of the moths was a woodland ca. 200–300 m from the house; any population which may have existed was certainly sparse.

2. During late summer, 1976, about 4–5 weeks after I had been involved

in an aerial application of microencapsulated disparlure in a 47-hectare plot, I captured perhaps 30–40 male moths within a period of less than an hour as they fluttered about my legs or up my trousers while I attended an outdoor reception in State College. The house was located in a wooded area known to support a sparse population of the gypsy moth. Again, I was the only individual to whom the males responded.

3. In 1974, my young son assisted as part of the ground crew loading several formulations of disparlure for aerial application. Six to 7 weeks later, we were in Clearfield County, Pennsylvania, a county from which only very few individual gypsy moths had been reported up to that time, as it was perhaps 100 km west of the then known "general infestation" area in the state. Within minutes after we got out of our vehicle, a male moth was responding to my son's tennis shoes—the same shoes he had worn during ground-crew operations, but shoes that had been washed with detergent in a washing machine at least several times in the intervening weeks.

Similar personal or object contamination has been reported in the popular press for a scientist involved with studies of the pheromone of the Douglas-fir tussock moth (*Orgyia pseudotsugata* (McD.) (Lepidoptera: Lymantriidae)) (Anon., 1976). Ritter and Persoons (1976) noted the extreme potency and (until that time) activity for a least 2 years of subnanogram amounts of periplanone A and B, the sex pheromones of the American cockroach, *Periplaneta americana* (L.) (Orthoptera: Blattidae). Pink bollworm (*Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae)) female moths are reported to use their pheromone for scent marking (Colwell et al., 1978), although the implication in the paper is that this is a relatively transient phenomenon. Following removal of baited traps from the field during tests of the pheromone for the pea moth, *Cydia nigricana* F. (Lepidoptera: Olethreutidae), Wall et al. (1980) noted that insects responded to vegetation in an area within ca. 0.3 m downwind of where the trap had been located, or to other spots to which vegetation from the area downwind of the trap had been removed. The pheromone was adsorbed onto the vegetation, as determined by EAG and GC-MS evidence, which then remained attractive for at least 55 min until observations were terminated.

There is, therefore, at least scattered recognition of the possibility of stability and biological activity of insect pheromones under conditions other than normal insect use patterns. Indeed, it is at least some degree of demonstrated stability which underlies the applied use of synthetic pheromones. But there have also been unqualified statements such as those of Wood (1977) that insect behavior regulators (pheromones) ". . . are active in extremely small quantities, and are biodegradable," and Browne et al. (1979) that "Pheromones, like other natural product vapors in the environment, should eventually degrade," but in the same paper they note that frontalin and

exo-brevicomin appear quite stable in the presence of air and sunlight over a period of at least 3 weeks.

Occurrences of long-term synthetic pheromone stability, and contamination of persons as well as things, must raise a certain amount of caution among researchers and others using these materials. For example, is there any carry-over effect in a field plot, treated with a pheromone application one year, into tests in subsequent years (or particularly generations for multivoltine insects) conducted in the same plot? Charmillot (1980) has demonstrated reduction of codling moth [*Laspeyresia pomonella* (L.)] (Lepidoptera: Tortricidae) populations from 120.2 diapausing larvae per tree to 0.76, and fruit attack from >60% to 0.29%, in a small plot treated with codlemone (*E*-8,*E*-10-dodecadien-l-ol) evaporated from dispensers—but otherwise untreated to control codling moth—for three successive years. Long-term pheromone persistence in the field has not been demonstrated, but cannot be absolutely ruled out. Do observers who may be contaminated with a pheromone have any direct or indirect effect on observed behavior of insects, either in laboratory tests or in field trials? Are the standard "scrubbings" of wind tunnels or other special laboratory facilities, in which behavioral observations are increasingly made, inadequate to destroy all residual traces of pheromone that may have been introduced in previous tests?

If the answer to any of these or similar questions is "yes," researchers must recognize and evaluate, if possible, this additional confounding factor in their experimental designs. Perry et al. (1980) have suggested the use of modified Latin squares, to allow for the independent estimation of residual effects of previous treatments in field tests, as one possible way of overcoming this problem. If persistence in the human body does, indeed, occur, it could mean that one who has been exposed to quantities of a pheromone, such as during laboratory synthesis of neat material or in field trials especially involving broadcast applications, should seriously consider abandoning critical behavioral research with the same insects or others that use one or more of the pheromone components to which there has been such exposure. At a minimum, scientists should "bioassay" themselves from time to time.

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MACROCYCLIC LACTONES AND ISOPENTENYL ESTERS IN THE DUFOUR'S GLAND SECRETION OF HALICTINE BEES (Hymenoptera: Halictidae)

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Abstract—The volatile components of the Dufour's gland secretion of female halictid bees have been examined in 18 Nearctic species belonging to *Agapostemon*, *Augochlora*, *Augochlorella*, *Augochloropsis*, *Dialictus*, *Evylaeus*, *Halictus*, and *Lasioglossum*. Nine saturated and unsaturated macrocyclic lactones ranging from C_{18} to C_{26} have been identified. Four of these compounds, the saturated C_{26} and the unsaturated C_{20}, C_{22}, and C_{24} lactones, are new natural products reported for halictine bees. A series of eight esters containing branched C_5-alkenols and fatty acids has been identified in several species. The cell linings and pollen ball in *Augochlora pura pura* contain the same major lactones as the Dufour's gland. A discussion of the significance of the Dufour's gland secretion for apoid systematics and its function in the Halictidae is presented.

Key Words—Bees, exocrine products, mass spectrometry, *Agapostemon*, *Augochlora*, *Augochlorella*, *Augochloropsis*, *Dialictus*, *Evylaeus*, *Halictus*, *Lasioglossum*, macrocyclic lactones, isopentenyl esters, Dufour's gland.

INTRODUCTION

Halictid bees or "sweat bees" are a major group of Apoidea in North America. Over 500 species in 21 genera of these primitive bees are found in the United States (Hurd, 1979). Different halictids vary in their social behavior from solitary to eusocial. Although most species nest in the ground, a few nest in rotten logs. Halictids possess several exocrine glands whose secretions have

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important socioecological significance. One example is the Dufour's or alkaline gland, which is found in most female Hymenoptera, associated with the sting apparatus.

The Dufour's gland in halictids extends much of the length of the abdomen and varies considerably in size and shape (Lello, 1971). The fresh gland is white with a wrinkled surface. A short basal lobe gives the sac an asymmetrical, bilobed appearance. If the sac is ruptured under water, a colorless, viscous, immiscible fluid floats to the surface.

The chemistry of the Dufour's gland secretion has been the subject of several investigations. A number of North American (Hefetz et al., 1978; Bergström and Tengö, 1979) and European (Andersson et al., 1978; Bergström, 1974) species of Halictinae have been investigated. The secretions contain straight-chain hydrocarbons and macrocyclic lactones.

We have analyzed the Dufour's secretions of 18 species representing eight genera, *Agapostemon*, *Augochlora*, *Augochlorella*, *Augochloropsis*, *Dialictus*, *Evylaeus*, *Halictus*, and *Lasioglossum*. We report the identification of a series of eight isopentenyl esters and nine macrocyclic lactones, four of the latter and all of the esters being newly reported exocrine products for halictid bees. We have also analyzed the cell linings and pollen ball of *Augochlora pura pura*. A discussion of the function of the Dufour's gland secretions in these bees and their significance in apoid systematics is presented.

METHODS AND MATERIALS

During the summers of 1978 and 1979, bees were collected from flowers on the Howard University campus, College Park, Maryland, and on the Quantico Marine Training Base, Quantico, Virginia. *Augochlora pura pura* was collected in Iowa City, Iowa, near logs in which they were nesting. Individual specimens were placed in shell vials and stored in an ice chest prior to transport. In the laboratory they were maintained at 4° C until dissection, usually one or two days later. The Dufour's glands were excised under water and extracted with methylene chloride. Although some extracts contained as many as thirty glands, others contained as few as one.

Separate extracts were made of uncontaminated pollen balls and cell linings from *A. p. pura* nests in rotting logs. As much wood as possible was removed from the cell linings which were then washed in water before being extracted with methylene chloride.

Dufour's glands (10–20) of *Halictus rubicundus* and *H. ligatus* were extracted with 0.5 ml methylene chloride and each treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Applied Science Laboratories Inc., State College, Pennsylvania) according to the procedure of Langenbeck et al. (1975). The resulting solution was analyzed directly by GC-MS.

The extracts were analyzed on a Finnigan 3200 computerized gas chromatograph–mass spectrometer (GC-MS) utilizing a 2 m 3% OV-17 on Supelcoport 60/80 temperature programed from 60–300°C at 10°/min. Individual compounds were identified by comparison of their mass spectra and retention times either with those of standard compounds or with previously published data. Although Figures 1 and 2 are abbreviated for simplicity, each analysis was programed from 60–300°C, and the gas chromatograms exhibited no volatiles other than those shown. The isopentenyl esters used as standards were synthesized from the corresponding fatty acids and either 3-methyl-2-buten-1-ol or 3-methyl-3-buten-1-ol using *N,N'*-carbonyl diimidizole following the procedure of Staab (1962).

RESULTS

Dufour's glands were analyzed from 18 halictid species listed in Table 1. These glands contain saturated and monounsaturated lactones, together with isopentenyl fatty acid esters in some cases. The saturated C_{20} lactone is found in all species examined. The relative amounts of the other lactones vary from species to species, with the saturated compounds predominant. For example, a typical gas chromatogram of the Dufour's gland extract of *Augochloropsis metallica* is shown in Figure 1. The major component is the C_{18} lactone and the second largest component is an unsaturated C_{18} lactone, the position of the double bond being unknown. Smaller amounts of the C_{20}, C_{22}, C_{24}, and C_{26} lactones are also present (Table 1).

The major lactone in *Halictus ligatus* and *H. rubicundus* is the C_{22}, while the C_{20} and C_{24} are minor components and there are trace amounts of the C_{18} lactone. *Halictus parallelus*, on the other hand, contains equal amounts of the C_{20} and C_{22} lactones, a smaller amount of the C_{24}, as well as an unsaturated C_{22} lactone.

Augochlora pura pura contains approximately equal amounts of the C_{20} and C_{22} lactones. A C_{20} unsaturated lactone is also present along with two apparent terpenes with molecular weights of 380 and 408. *Lasioglossum fuscipenne* also contains these latter compounds in addition to the major components C_{18} and C_{20} lactones and smaller amounts of the C_{22} lactone and C_{20} and C_{22} unsaturated lactones. Figure 2 shows a typical gas chromatogram for *Agapostemon texanus* indicating the presence of these lactones plus compounds having molecular weights of 352, 380, and 408.

The lactones exhibit well-defined mass spectra which are useful in the identification of trace constituents. Thus, the C_{20} lactone exhibits a readily detectable molecular ion at m/z 310 (5%) with a loss of water to m/z 292 (5%). The next largest ion is a m/z 250 (M − 60) (CH_3CO_2H) with a smaller ion at m/z 264 (M − 46) followed by clusters of unsaturated ions at 125, 111, 97, 83,

TABLE 1. COMPOUNDS IDENTIFIED IN NORTH AMERICAN HALICTINE BEES.

Species	18-Octadecanolide	18-Octadecenolide	20-Eicosanolide	20-Eicosenolide	22-Docosanolide	22-Docosenolide	24-Tetracosanolide	24-Tetracosenolide	26-Hexacosanolide	3-Methyl-2-buten-1-yl hexadecanoate	3-Methyl-3-buten-1-yl hexadecanoate	3-Methyl-2-buten-1-yl octadecanoate	3-Methyl-3-buten-1-yl octadecanoate	3-Methyl-2-buten-1-yl eicosanoate	3-Methyl-3-buten-1-yl eicosanoate	3-Methyl-2-buten-1-yl docosanoate	3-Methyl-3-buten-1-yl docosanoate	Reference[b]
Augochlorini																		
Augochlora																		
A. pura pura			X[a]	X	XX									tr	tr	tr	tr	1
A. pura mosieri			X		XX		X							tr	tr	tr	tr	1
Augochlorella																		
A. striata			X	X	XX		X					tr	tr	tr	tr	tr	tr	1
Augochloropsis																		
A. metallica	XX	X	X	X	X	X	X		X									1
Halictini																		
Agapostemon																		
A. radiatus	XX		X		X		X					tr	tr					1
A. splendens	XX		XX		X													2
A. texanus	XX		X		X							tr	tr	tr	tr	tr	tr	1
Dialictus																		
D. coeruleus	X		X	X	X					tr	tr	tr	tr	tr	tr	tr	tr	1
D. cressonii	X	X	XX	X	X	X	X					tr	tr					1
D. laevissimus	X		X		X													2

D. lineatulus	X		X		X												2
D. nymphalis	X		X		X												2
D. pilosus	XX		X	X	X					tr	tr						1
D. rohweri	X	X	XX	X	X		X										1
	X		X		X		X										2
D. tamiamensis	X		X		X												2
D. versatus	X	X	XX		X					tr	tr	tr	tr				1
Evylaeus																	
E. quebecensis	X		X		X												2
E. truncatus	X		X	?	X		X										1
Lasioglossum																	
L. fuscipenne	XX		X	X	X	X				tr	tr	tr	tr	tr	tr		1
L. coriaceum	XX	X	X	X	X		?			tr	tr	tr	tr	tr	tr		1
L. leucozonium	X		X														2
Halictus																	
H. (Seladonia) confusus			XX	X	X					tr	tr	tr	tr	tr	tr		1
H. (H.) ligatus	X		X		XX		X										1
H. (H.) parallelus			XX		X	X	X	X									1
H. (H.) rubicundus	X		XX		XX		X										1
	X		X		X		X										3

[a] XX = major component, X = present, tr = trace.
[b] References (1) this study; (2) Hefetz et al., 1978; (3) Bergström and Tengö, 1979.

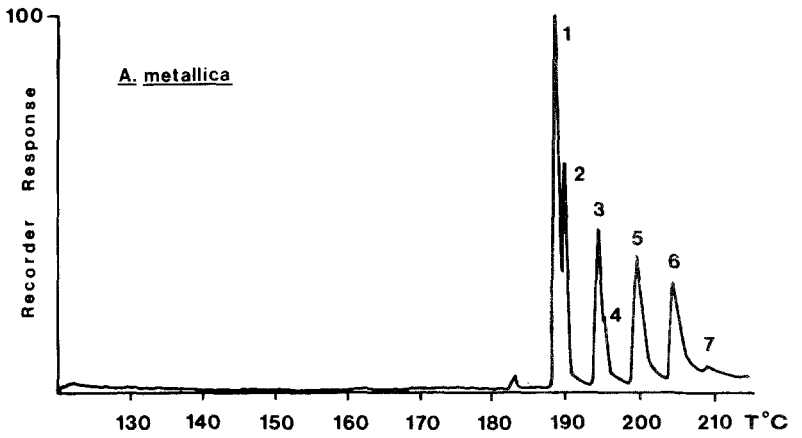

FIG. 1. Gas chromatogram of *Augochloropis metallica* Dufour's secretion. Peaks correspond to (1) 18-octadecanolide (mol wt 282), (2) 18-octadecenolide (mol wt 280), (3) 20-eicosanolide (mol wt 310), (4) 20-eicosenolide (mol wt 308), (5) 22-docosanolide (mol wt 338), (6) 24-tetracosanolide (mol wt 366), (7) 26-hexacosanolide (mol wt 394).

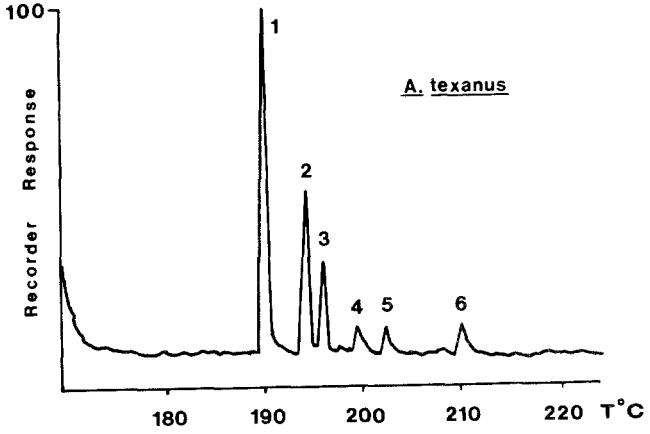

FIG. 2. Gas chromatogram of *Agapostemon texanus* Dufour's section. Peaks correspond to (1) 18-octadecanolide (mol wt 282), (2) 20-eicosanolide (mol wt 310), (3) mixture of 3-methyl-2-buten-1-yl and 3-methyl-3-buten-1-yl octadecanoate (mol wt 352), (4) 22-docosanolide (mol wt 338), (5) mixture of 3-methyl-2-buten-1-yl and 3-methyl-3-buten-1-yl eicosanoate (mol wt 380), (6) mixture of 3-methyl-2-buten-1-yl and 3-methyl-3-buten-1-yl docosanoate (mol wt 408).

69, 55 and 41. This type of fragmentation is seen in the other lactones as well, with the base peak appearing at either m/z 41 or m/z 55.

Also present in several of these halictid species (Table 1) are traces of a homologous series of apparently terpenoid esters with molecular weights of 324, 352, 380, and 408 (Table 2). Each of these exhibits a base peak at m/z 68 with an appreciable peak at 69. The molecular ion in each instance loses 85 amu, indicating a possible relationship between the base peak of 68 and this loss of 85. On the assumption that the fragment at M − 85 constitutes an acylium ion ($C_{15}H_{31}CO^+$, $C_{17}H_{35}CO^+$, $C_{19}H_{39}CO^+$, and $C_{21}H_{43}CO^+$) and that the 85 fragment consists of an isopentenyl group, a series of 3-methyl-2- (and 3-) buten-1-yl esters was prepared (Table 2). Comparison of the synthetic material with the natural product indicated that these halictids contain a mixture of 3-methyl-2-buten-1-yl and 3-methyl-3-buten-1-yl esters with the 3-isomer dominating in addition to the lactones. Although the isomeric esters co-eluted on a temperature programmed OV-17 column, the intensity of the 69 peak relative to the 68 peak differed markedly in the two isomers. The natural material exhibited a 69 peak whose intensity was significantly greater then the 69 peak of the 3-methyl-3-buten-1-yl but significantly less than the 69 peak of the 3-methyl-2-buten-1-yl isomer. Isothermal gas chromatography on 3% OV-17 allowed separation of the isomeric mixtures and verification that the original peak was a composite of the two isomeric esters, the relative amounts varying widely from species to species.

TABLE 2. HALICTID ISOPENTENYL ESTERS

Compound	Mol wt	m/z (relative intensity)
3-Methyl-2-buten-1-yl hexadecanoate	324	68(100), 69(88), 239(10), ×20→, 257(5), 281(2), 324(5)
3-Methyl-3-buten-1-yl hexadecanoate	324	68(100), 69(4), 239(10), ×20→, 257(5), 281(1), 324(2)
3-Methyl-2-buten-1-yl octadecanoate	352	68(100), 69(90), 267(10), ×20→, 285(5), 352(5)
3-Methyl-3-buten-1-yl octadecanoate	352	68(100), 69(40), 267(3), ×20→, 285(2), 352(2)
3-Methyl-2-buten-1-yl eicosanoate	380	68(100), 69(98), 295(4), ×20→, 311(2), 380(2)
3-Methyl-3-buten-1-yl eicosanoate	380	68(100), 69(47), 295(1), ×20→, 311(1), 380(1)
3-Methyl-2-buten-1-yl docosanoate	408	68(91), 69(100), ×20→, 323(3), 408(2)
3-Methyl-3-buten-1-yl docosanoate	408	68(100), 69(49), ×20→, 323(3), 408(2)

DISCUSSION

The Dufour's gland secretions of Hymenoptera have been studied extensively. In ants, the Dufour's gland may contain as many as 50 compounds representing many different chemical classes (Bergstrom and Lofqvist, 1971). Although the Dufour's glands of several bee families, including the Halictidae, are larger in relation to the abdomens than those of ants, the bee glands contain fewer compounds. Bees in different families contain different classes of compounds. The Dufour's gland of *Xylocopa virginica* (Anthophoridae) contains long-chained hydrocarbons (Vinson et al., 1978), while other anthophorids produce acetates (*Melissodes*, Batra and Hefetz, 1979) or triglycerides (*Anthophora*, Norden et al., 1980). Species of *Andrena* (Andreninae) produce farnesyl hexanoate and geranyl octanoate and a variety of other esters (Bergström and Tengö, 1974; Tengö and Bergström, 1975, 1978). Octadecyl butanoate and other esters are found in the Dufour's secretions of species of *Melitta* (Melittidae) (Tengö and Bergström, 1976).

Dufour's gland secretions of species of *Colletes* and *Hylaeus* (Colletidae) and eight genera of Halictidae (Table 1) are dominated by long-chain hydrocarbons and macrocyclic lactones (Andersson et al., 1966; Bergström, 1974; Bergström and Tengö, 1979; Duffield et al., 1980; Hefetz et al., 1978, 1979). These lactones include a homologous series of saturated compounds ranging from C_{16} to C_{24}. Our results expand this list to include the C_{26} compound and unsaturated C_{20}, C_{22}, and C_{24} lactones. The 16-hexadecanolide has been found only in European species of colletids and halictids and not in the 25 native Nearctic species analyzed to date. In *Colletes cunicularis* (Colletidae) it is a minor component, whereas in *Evylaeus calceatus* (Halictidae) it equals the amount of the C_{18} and the C_{20} lactones.

Bergström and Tengö (1979) state that both macrocyclic lactones and their corresponding ω-hydroxy acids were found in fresh Dufour's secretions of *C. cunicularius* and *E. calceatus* when examined by direct-inlet mass spectrometry (Bergstrom, 1974). However, there is no mention of these hydroxy acids in this earlier paper. Attempted silylation of these presumed hydroxy acids in extracts of two species (*H. ligatus* and *H. rubicundus*) and subsequent analysis by GC-MS gave only the C_{20}, C_{22}, and C_{24} lactones and none of the corresponding silylated hydroxy acids. Careful analysis of the mass spectra of each of these lactone gas chromatography peaks gave no indication that any open-chain ω-hydroxy acids are present in the Dufour's gland secretion.

Systematic Implications. The natural product chemistry of bees is a relatively unexplored field of chemical ecology. Among the short-tongued, more primitive bees (Figure 3), Dufour's gland secretions have been analyzed for representatives of only five of the 16 subfamilies. Macrocyclic lactones

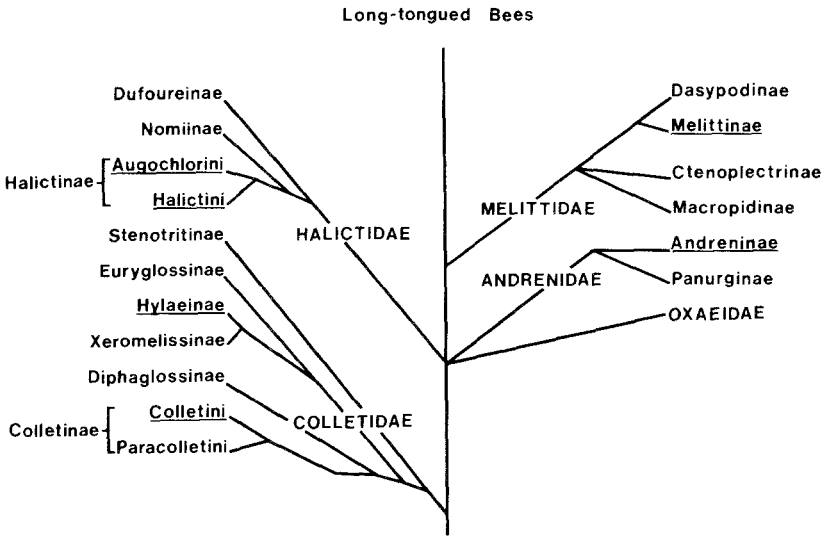

FIG. 3. Cladogram of families of short-tongued bees (adapted from Michener, 1974). Underlined taxa: Dufour's gland secretion chemically analyzed in at least one species.

occur in all Halictidae and Colletidae so far examined, while esters characterize all Andreninae and Melittinae. We therefore suggest as a preliminary hypothesis that the stem species that gave rise to the Halictidae and Colletidae was different from the stem species of the latter two subfamilies. Chemical analysis of representatives of the remaining subfamilies could help clarify their systematic relationships. We especially urge examination of the Paracolletini (often considered to be the most primitive of bees), the Stenotritinae, the Oxaeidae, and the other melittid subfamilies (particularly the Ctenoplectrinae).

Within the Halictinae, many of the lactones are common to numerous species, and there is no evidence of unique, species-specific blends. The higher taxa (Figure 4) are also not chemically unique, although trends are apparent that may have phylogenetic implications (Table 1). In the tribe Augochlorini, the closely related genera *Augochlora* and *Augochlorella* have nearly identical compounds, with C_{22} lactone a major component and C_{18} lactones absent, while the distinctive genus *Augochloropsis* has C_{18} lactone as a major component, lacks isopentenyl esters, and is the only taxon with a C_{26} lactone. *Agapostemon* is distantly related to the other Halictini but is not distinctly separated from them chemically. Species of *Agapostemon* all have C_{18} lactone as a major component and lack unsaturated lactones. The genera of the *Lasioglossum* genus group (*Dialictus*, *Evylaeus*, and *Lasioglossum*) are not separated chemically from *Halictus*, and *Dialictus* and *Evylaeus*, often considered to form one genus, are not different from *Lasioglossum* s.str.

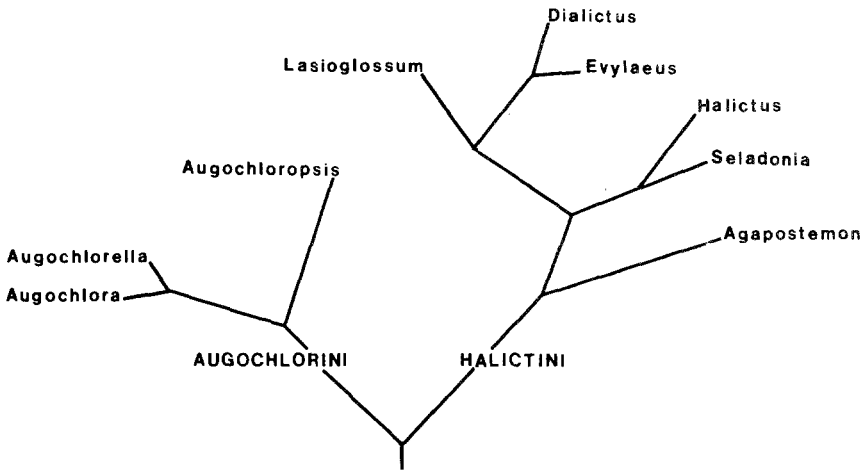

FIG. 4. Cladogram of the genera of Halictinae that have had Dufour's gland secretions analyzed (adapted from Eickwort 1969a,b, unpublished).

Within *Halictus*, the representative of the subgenus *Seladonia* differs from representatives of *Halictus* s.s. in lacking C_{24} lactones and possessing three sets of isopentenyl esters.

Functional Implications. The function of the Dufour's gland secretion in Hymenoptera varies among taxa. It functions in alarm, defense, trail laying in ants (Blum and Hermann, 1978); host marking and discrimination in some parasitic wasps (Vinson and Guillot, 1972); and as a source of the sex pheromone in others (Weseloh, 1976).

All halictid bees line the walls of their nest cells with a wax-like, waterproof coating (Michener, 1974). Claude-Joseph (1926), Malyshev (1935), Batra (1964, 1968), and Roberts (1969) observed halictines applying this lining with their glossae and legs and hypothesized a salivary gland origin. However, other lines of evidence suggest that the Dufour's glands secrete the cell lining. The Dufour's glands are best developed in those bees that are actively constructing cells (Batra, 1966; May, 1974 and references therein). Species of *Sphecodes*, which are cleptoparasites and do not construct or provision their own cells, have atrophied Dufour's glands (Hefetz et al., 1978). Batra (1968) observed that *Dialictus versatus* opened its sting chamber and deposited a chemical while it was lining a cell. May (1974) found many similarities in the thin-layer chromatograms of Dufour's gland contents and cell linings of *Augochlora p. pura*. She hypothesized that bees use their glossae to spread the Dufour's gland secretion about the cell. The data we present here are the first conclusive direct evidence that Dufour's gland secretions are used

to line halictine nest cells. Similar evidence has been presented by Hefetz et al. (1979) for colletid cell linings and by Norden et al. (1980) for anthophorid cell linings. May's (1974) data can be interpreted in light of our results; she found homologs with molecular weights of 310, 338, and 366 in cell linings and Dufour's gland contents; her mass spectra indicate macrocyclic lactones, and although she did not identify her compounds, they correspond to the C_{20}, C_{22}, and C_{24} lactones reported here.

Cell linings function to preserve homeostatic moisture conditions in the cells, which are essential for larval survival (May, 1972). They may also help prevent contamination by pathogens and adsorb odors that act as cues to prevent digging bees from breaking into completed cells (May, 1972, 1973).

Our data (Table 3) show clearly that the cell lining, pollen ball, and Dufour's gland of *A. p. pura* share the same C_{20} and C_{22} lactones. The Dufour's secretion is apparently multifunctional, serving as a cell wall lining and as a nutritional source for the larvae, as has been shown for the triglyceride secretion of *Anthophora abrupta* (Anthophoridae) (Norden et al., 1980). We postulate that in the Colletidae the Dufour's gland secretion may also be incorporated in the liquid food provisions in the cells. We are presently investigating this possibility.

The homologous series of 3-methyl-2-buten-1-yl and 3-methyl-3-buten-1-yl esters in the Dufour's gland secretion of species of Halictinae represent a new insect natural product, as semiterpenoid isopentenyl esters have not previously been demonstrated. However, a semiterpenoid alcohol (2-methyl-3-buten-2-ol) has been found to play an important role in bark beetle aggregation (Bakke et al., 1977).

TABLE 3. COMPOUNDS IDENTIFIED IN DUFOUR'S GLAND, CELL LINING, AND POLLEN BALL OF *Augochlora pura pura*

Source	Methyl *n*-Hexadecanoate	Ethyl *n*-Hexadecanoate	*n*-Hexadecanoic acid	20-Eicosanolide	20-Eicosenolide	22-Docosanolide
Dufour's gland	–[a]	–	–	XX	X	XX
Pollen ball	X	X	X	X	–	X
Cell lining	X	X	X	X	–	X

[a] XX = major compound, X = present, – = absent.

NOTE: After submission of this paper the authors received a manuscript submitted by James Cane to the *Journal of Chemical Ecology*. It was demonstrated that the Dufour's secretion and cell linings of *Lasioglossum albipes* share some of the same compounds.

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EFFECTS OF DECOMPOSING RICE STRAW ON GROWTH OF AND NITROGEN FIXATION BY *Rhizobium*

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Abstract—Five phenolic compounds produced by decomposing rice straw and sterile extracts of decomposing rice straw in soil were very inhibitory to growth of three strains of *Rhizobium*. The effects were additive and in several instances synergistic. The phenolic compounds also reduced nodule numbers and hemoglobin content of the nodules in two bean (*Phaseolus vulgaris*) varieties. Extracts of decomposing rice straw in soil (same concentration as in the soil) significantly reduced N_2 fixation (acetylene reduction) in Bush Black Seeded beans. This may explain in part the great reduction in soybean yields in Taiwan following rice crops when the rice stubble is left in the field.

Key Words—*Rhizobium*, N_2 fixation, legumes, phenolic compounds, allelopathy, rice, acetylene reduction, decomposing crop residues.

INTRODUCTION

Stevenson (1967) pointed out that the soils of rice paddies in Japan and India have been found to contain high enough concentrations of aliphatic acids to inhibit growth of rice. He pointed out additionally that anaerobic conditions are favorable to the microbial synthesis of organic acids. Chandromohan et al. (1973) isolated vanillic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and three unidentified phenolic acids from rice field soil at Annamalainagar, South India. They found that cinnamic acid (a related compound) was inhibitory to the growth of rice seedlings even at a 10^{-4} M concentration.

The unharvested parts of rice plants are customarily mixed with the soil through plowing or other mechanical manipulation, because this has been thought to be beneficial. It has been commonly observed, however, that

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productivity of the second rice crop in a paddy is less than that of the first crop. Chou and Lin (1976) did a comprehensive study, therefore, on the effects of decomposing rice residues in soil on the growth of rice plants. They found that aqueous extracts of decomposing rice residues in soil inhibited radicle growth of rice (*Oryza sativa* L.) and lettuce (*Lactuca sativa* L.) seedlings and growth of rice plants. Maximum toxicity occurred in the first month of decomposition and declined thereafter. Extracts of the soil in rice fields were also inhibitory to rice and lettuce, and the toxicity was persistent for 4 months. Five phytotoxins, *p*-hydroxybenzoic, *p*-coumaric, vanillic, ferulic, and *o*-hydroxyphenylacetic acids, were identified from decomposing rice residues under waterlogged conditions; several unknowns were also isolated. Three of the phytotoxins identified by Chou and Lin (1976) were the same as three identified by Chandramohan et al. (1973).

The results of Chou and Lin (1976) were strongly supported by subsequent research by Chou et al. (1977). The same phytotoxins were identified in paddy soil and amounts were higher in paddies in which rice stubble was left than in paddies from which the rice stubble was removed. They found that the quantities of leachable nitrate (NO_3^-) and ammonium-nitrogen (NH_4^+) were lower also in paddies where rice stubble was left than in paddies where the stubble was removed. No research was done, however, on effects of decaying rice straw on nitrogen fixation.

In the southern part of Taiwan, a crop of rice is often followed immediately by a legume crop, commonly various types of beans (*Phaseolus spp.*, *Vigna spp.*, etc.) or soybeans [*Glycine max* (L.) Merr.]. The AVRDC Soybean Report for 1976 (Asian Vegetable Research and Development Center, 1978) cited considerable data indicating that soybean yields in southern Taiwan were increased by several hundred kilograms per hectare when the rice straw was burned before planting the soybeans rather than allowing the straw to remain in the field and decompose. No explanation was given for this result, but it is certainly possible that phytotoxins produced by the decomposing rice straw inhibited nitrogen fixation by *Rhizobium* in the nodules of the soybean plants, or inhibited growth and yield of the soybean plants directly. Therefore, the goal of this project was to determine the effects of decomposing rice straw on growth of *Rhizobium* and on nodulation, hemoglobin formation, and nitrogen fixation in legumes.

METHODS AND MATERIALS

Effects on Rhizobial Growth of Known Phytotoxins from Decomposing Rice Straw. The bacteria chosen for tests included *Rhizobium leguminosarum* Frank emend. Baldwin and Fred, American Type Culture (ATC) strain 10314; *R.* sp, ATC strain 10703; and *R. japonicum*, Taiwan strain WSM

isolated from *Glycine max* nodules. A yeast extract–mannitol medium (Society of American Bacteriologists, 1957, p. 113) was used for all strains, and the same medium was used for liquid inocula and solid plates. Fifteen grams of bacto-agar were added per liter if a solid medium was desired. All stock cultures and test plates were kept at 30°C, which is near optimum for these organisms.

The five phenolic acids identified by Chou and Lin (1976) in decomposing rice residues were dissolved in methanol to make three concentrations, 10^{-4} M, 5×10^{-4} M, and 10^{-3} M. The compounds were *p*-coumaric, ferulic, *p*-hydroxybenzoic, *o*-hydroxyphenylacetic, and vanillic acids. The phenolic solutions were tested for antibacterial activity by the diffusion technique on solid media against seeded cultures of the selected strains of nitrogen-fixing bacteria. Filter paper disks saturated with a given phenolic solution were used for the tests. Approximately 0.1 ml of solution was required to saturate a disk. When methanol was used as the solvent, it was allowed to evaporate before placing the disk on the seeded Petri plate. Control disks were saturated with methanol and thereafter treated the same as test disks.

Two tenths of a milliliter of a 24-hr liquid culture of *Rhizobium* was used in seeding the plates, and the test and control disks were added immediately. The plates were routinely examined and zones of inhibition measured 3 days after the disks were applied.

Additive and Synergistic Effects of Known Phytotoxins on Growth. The following methanolic solutions of the five known phenolics in decomposing rice straw were made: separate 10^{-3} M solutions of each of the phenolics; a solution containing *p*-coumaric and vanillic acids, each in a 10^{-3} M concentration; a solution containing *p*-hydroxybenzoic and *o*-hydroxyphenylacetic acids, each in a 10^{-3} M concentration; a solution containing ferulic and vanillic acids, each in a 10^{-3} M concentration; and a solution containing all five phenolics, each in a 10^{-3} M concentration. Each of the nine solutions was tested against all test strains of *Rhizobium* as described previously.

Effects of Extracts of Decaying Rice Straw on Growth of Rhizobium. Rice straw consisting of a mixture of several cultivated varieties and hybrids was chopped into pieces 2–3 cm long and incorporated into soil in ratios of 0, 25, and 100 g/3 kg of air-dry soil. Each mixture was placed in a large container and 2 liters of distilled water were added. The containers with the mixtures were placed in a greenhouse room, and the straw was allowed to decompose for 2 weeks. Other containers were set up similarly, and the straw was allowed to decompose for 4 weeks.

At the end of the decomposition period, an aqueous extract was obtained by squeezing the residue–soil in several layers of cheesecloth and centrifuging for 20 min at 16,000 *g*. The extract was concentrated to one fifth of the original

volume (Chou et al., 1977) by flash evaporation and then was filter sterilized with 0.45 μm Millipore filter pads. The sterile extracts were bioassayed against the same strains of *Rhizobium*.

Effects of Known Phenolics on Nodulation and Hemoglobin Formation in Bean Plants. Kidney bean seeds (*Phaseolus vulgaris* L., var. Four Season) were inoculated with the appropriate Nitragin inoculum and five seeds were planted per pot (26 cm, top diameter) in Vermiculite which was previously leached thoroughly with tap water followed by distilled water. Immediately after planting, 200 ml of Evan's nutrient solution (Moore, 1974), containing one twentieth of the nitrogen recommended for a complete solution, were added to each pot. The pH of the nutrient solution was set at 6.0.

After 2 weeks, the plants were thinned to one plant per pot, and the Vermiculite was leached thoroughly with tap water followed by distilled water. One day after thinning and leaching, 200 ml of a 10^{-3} M solution of a known phenolic in Evan's minus-nitrogen solution were added to each of five pots and 200 ml of a 10^{-4} M solution was added to each of five pots. This was repeated for all five phenolics making a total of fifty test pots. Each of five pots was watered with 200 ml of Evan's minus-N solution only as controls.

Twelve days later the pots were watered again with 200 ml each of the appropriate phenolic in Evan's minus-N solution or with just the minus-N solution in the controls. Between treatments and after the second treatment, the pots were watered when necessary with the minus-N solution alternated with distilled water.

Forty-five days after planting, the roots of each plant were carefully washed free of Vermiculite and the number of effective nodules (wrinkled, pink in color) was counted. The nodules were collected, combined from all plants of a given treatment, and frozen until the amount of hemoglobin (heme) could be determined. The hemoglobin was extracted with pyridine containing a small amount of sodium hydrosulfite, and the heme content was determined quantitatively according to the method of Virtanen et al. (1947) as an indication of the relative amount of hemoglobin present.

This experiment was repeated with some modifications, using another variety of bean grown in Taiwan, Bush Black Seeded, Accession No. 361. Only the 10^{-3} M concentration of each phenolic was used, *o*-hydroxyphenylacetic acid was omitted, and a solution containing a 10^{-3} M concentration of each of the four other phenolics was tested instead. The phenolic solutions were added immediately after planting this time, and again two weeks later after thinning to one plant per pot. The plants were harvested 31–33 days after planting for nodule counts and hemoglobin determinations.

Effects of Decomposing Rice Straw on Acetylene Reduction (N_2 Fixation) by Rhizobium *in Bean Nodules.* Bush Black Seeded beans were inoculated with the appropriate Nitragin inoculum, and four seeds were

planted per pot in Vermiculite which was previously leached thoroughly with tap water followed by distilled water. As soon as the hypocotyl hooks appeared above the surface, 200 ml of a 10^{-3} M concentration of a known phytotoxin in Evan's nutrient solution, containing one twentieth of the nitrogen (N/20) in a complete solution, were added to each of four pots, and 200 ml of a 10^{-4} M solution were added to each of four pots. This was repeated for all five known phenolic phytotoxins from decaying rice straw. Four control pots were set up for each phenolic and each of these received 200 ml of the N/20 solution.

Extracts of rice straw decomposed for 4 weeks in soil were made as described previously, except the volumes were not reduced and they were not filter sterilized after centrifugation. Ratios of straw to soil were the same as before: 0 g, 25 g, and 100 g/3 kg soil. Two hundred milliliters of a given extract were added to each of four pots. The pots watered with the extract of soil without straw served as the controls. Two days later, each pot which received one of the extracts was given 200 ml of the N/20 nutrient solution to supply necessary minerals.

Two weeks after planting, the plants were thinned to one per pot, and the pots were leached thoroughly with tap water followed by distilled water. Two days later, each pot was watered with 200 ml of the appropriate phenolic made up in Evan's minus-nitrogen solution or with Evan's minus-N solution only (controls). Those previously watered with extracts of decomposing rice straw were watered similarly again. Two days later these were watered with 200 ml each of the minus-N solution to furnish other necessary minerals. Between treatments and after the second treatment, all pots were watered when necessary with the minus-N solution alternated with distilled water.

Thirty-two to 34 days after planting, the root system of each plant was carefully washed free of Vermiculite and transferred to a 500-ml bottle with a serum cap for determination of the nitrogen fixation rate by the acetylene reduction method (Huang, 1978). Acetylene was injected into each bottle to give an acetylene partial pressure of 0.1 atmosphere. Samples of the the gas phase were withdrawn after 1 hr, and the acetylene and ethylene were separated and quantitated by means of a gas chromatograph equipped with a glass column 0.8 m long and 3 mm ID packed with Porapak R, and a hydrogen ionization detector. The oven temperature was 65° C and the carrier gas was N_2 flowing at 30 ml/min. The acetylene reduction rate was calculated on a per plant basis.

RESULTS AND DISCUSSION

Effects on Growth of Rhizobium of Phenolics Known to be Produced by Decaying Rice Straw. All phenolics tested inhibited growth of all test strains

TABLE 1. EFFECTS OF KNOWN PHENOLICS ON GROWTH OF NITROGEN-FIXING BACTERIA[a]

Test organism[b]	*p*-Coumaric acid			Ferulic acid			*p*Hydroxybenzoic acid			*o*-Hydroxyphenylacetic acid			Vanillic acid		
	10^{-3} M	5×10^{-4} M	10^{-4} M	10^{-3} M	5×10^{-4} M	10^{-4} M	10^{-3} M	5×10^{-4} M	10^{-4} M	10^{-3} M	5×10^{-4} M	10^{-4} M	10^{-3} M	5×10^{-4} M	10^{-4} M
R 10703	1.2[c]	0.5	0.2	0.2	0.0	2.0	0.5	0.8	0.5	1.0	0.2	0.8	2.0	2.0	1.5
R 10314	3.2	4.0	1.8	2.5	2.0	2.0	4.2	1.8	1.8	1.2	0.5	0.5	1.5	1.0	1.0
R WSM	3.0	2.8	1.8	1.2	0.8	1.2	0.8	0.5	0.6	1.2	1.0	0.5	0.9	0.8	0.8

[a] Controls had no inhibition.
[b] Symbols: R, *Rhizobium*; numbers or letters are strain designations (see text).
[c] Each figure is mean radius (mm) of inhibited zone of two trials.

of *Rhizobium* (Table 1). In most tests, the amount of growth inhibition increased with an increase in concentration of the phenolic acids. In a few instances, growth inhibition was about the same in all concentrations of a given phenolic acid. The relative inhibitory activity of a given phenolic varied considerably, depending on the test organism. For example, *p*-coumaric acid was considerably more inhibitory to *Rhizobium* strains 10314 and WSM than to strain 10703.

Rhizobium strain 10314 was very strongly inhibited by both *p*-coumaric and *p*-hydroxybenzoic acids, and *Rhizobium* strain WSM was very strongly inhibited by *p*-coumaric acid. It is noteworthy, therefore, that Chou et al. (1977) found *p*-coumaric and vanillic acids to be present in greatest concentrations in rice paddy soil. Vanillic acid was the most inhibitory compound tested again *Rhizobium* strain 10703.

Chou and Lin (1976) found that the concentrations of the phenolic compounds in decomposing rice straw in soil were often high. For example, the concentration of *o*-hydroxyphenylacetic acid was 10^{-2} M in the first week of the decomposition period. Thus the concentrations used in the present tests were realistic ones. Chou and Lin reported that a 10^{-4} M concentration (15.2 ppm) of *o*-hydroxyphenylacetic acid suppressed radicle growth of lettuce and rice seedlings significantly and a 50-ppm concentration completely inhibited radicle growth of rice.

In most tests with *Rhizobium* strains, there was an additive effect from combined phenolics over the effects of the individual phenolics (Table 2). Moreover, in most tests of combined phenolics against *Rhizobium* strains 10314 and 10703, there were pronounced synergistic effects in addition to the additive effects. The occurrence of synergistic action is important because all five compounds generally occur together in paddy soil. It appears, therefore,

TABLE 2. ADDITIVE AND SYNERGISTIC EFFECTS OF KNOWN PHENOLICS ON NITROGEN-FIXING BACTERIA[a]

Test organism[b]	C[c]	F	H	P	V	A	CV	HP	FV
R 10314	0.5[d]	0.2	0.2	0.0	0.5	2.2[e]	3.0[e]	1.5[e]	3.5[e]
R 10703	1.0	1.0	0.0	0.2	0.0	1.8	2.2[e]	1.8[e]	1.8[e]
R WSM	0.2	1.0	0.0	0.0	0.0	0.5	0.2	1.0[e]	1.0

[a]Concentration of each phenolic was 10^{-3} M in every test. Controls had no inhibition.
[b]Symbols for organisms: see Table 1.
[c]Symbols for phenolics: C, *p*-coumaric acid; F, ferulic acid; H, *p*-hydroxybenzoic acid; P, *o*-hydroxyphenylacetic acid; V, vanillic acid; A, all previous five; CV, *p*-coumaric and vanillic acids; HP, *p*-hydroxybenzoic and *o*-hydroxyphenylacetic acids; FV, ferulic and vanillic acids.
[d]Each figure is mean radius (mm) of inhibited zone of two trials.
[e]Synergistic effect.

that some of the nitrogen-fixing bacteria could be inhibited considerably even when the phenolics are present in relatively low concentrations.

Effects of Extracts of Decaying Rice Straw in Soil on Growth of Rhizobia. The extract of the 100-g concentration (rice straw) was inhibitory to all test organisms except *Rhizobium* strain 10703 (Table 3). The control extract (soil only) was slightly inhibitory to strain 10314, but the amount of inhibition was less than that resulting from extracts of soil with added rice straw. The amount of inhibition resulting from 25 g of straw was generally intermediate between that of the control and that of the 100-g concentration. The extracts of straw decomposed for 2 weeks were more inhibitory to *Rhizobium* than 4-week extracts. The amounts of inhibition compared favorably with amounts resulting from combinations of the known phenolics. These results demonstrate conclusively that there are compounds in decomposing rice straw in soil which are inhibitory to *Rhizobium*.

Effects of Known Phenolics on Nodulation and Hemoglobin Formation in Bean Plants. Each concentration of each phenolic, except the 10^{-4} M concentration of *o*-phydroxyphenylacetic acid, appeared to cause at least a slight reduction in mean nodule number in Four Season beans (Table 4). Plants treated with a 10^{-3} M concentration of each phenolic consistently had lower mean nodule numbers than those treated with a 10^{-4} concentration of the same phenolic. The consistent results suggest that the reductions in mean numbers were biologically significant. Only a few reductions were statistically significant, however. This probably resulted from the small number of plants in each test set and the control.

The mean heme content per plant was reduced considerably by each concentration of each phenolic acid (Table 4). Again, there was a consistently greater reduction of the heme content by the 10^{-3} M concentration of each

TABLE 3. EFFECTS OF STERILE EXTRACTS OF DECOMPOSING RICE STRAW IN SOIL ON GROWTH OF NITROGEN-FIXING BACTERIA

Test organism[a]	Ratio of rice straw to soil					
	Decomposed for 2 weeks			Decomposed for 4 weeks		
	Control, 0 g/3 kg	25 g/3 kg	100 g/3 kg	Control, 0 g/3 kg	25 g/3 kg	100 g/3 kg
R WSM	0.0[b]	0.1	0.4	0.0	0.1	0.2[c]
R 10314	0.5	0.9	2.8[c]	0.1	0.1	0.4[c]
R 10703	0.0	0.0	0.0	0.0	0.0	0.0

[a] See Table 1 for symbols of organisms.
[b] Each figure is mean radius (mm) of zone of inhibition of two trials.
[c] Significantly different from control at 0.10 level or better.

TABLE 4. EFFECTS OF PHENOLICS ON NODULE NUMBERS AND HEME CONTENT OF FOUR SEASON BEANS[a]

Treatment and concentration		Mean nodule number ± SE	Mean heme/plant (μg)	Reduction in heme (%)
p-Coumaric acid	10^{-4} M	137.2 ± 6.8	92.7	38.5
	10^{-3} M(3)	73.0 ± 10.1 [b, c]	78.5	47.9
Ferulic acid	10^{-4} M	132.6 ± 12.8	137.3	8.9
	10^{-3} M	126.2 ± 15.4	114.2	24.2
p-Hydroxybenzoic acid	10^{-4} M	124.8 ± 13.7	111.0	26.3
	10^{-3} M	118.4 ± 13.6[d]	103.7	31.2
o-Hydroxyphenylacetic acid	10^{-4} M(4)	160.8 ± 8.0	103.4	31.4
	10^{-3} M	147.0 ± 14.2	83.5	44.6
Vanillic acid	10^{-4} M	145.0 ± 8.3	142.7	5.3
	10^{-3} M	128.6 ± 12.3	127.7	15.3
Controls (4)		151.8 ± 5.5	150.7	

[a]Five plants in each set except where indicated differently in parentheses after description of treatment.
[b]Significantly different from control mean at 0.05 level or better ($DF = 5$).
[c]Significantly different from 10^{-4} at 0.05 level or better ($DF = 6$).
[d]Significantly different from control at 0.10 level ($DF = 7$).

phenolic than by the 10^{-4} M concentration. The percent reduction in heme varied from a low of 5.3% to a high of 38.5% in the 10^{-4} M concentration of the five phenolics, and from a low of 15.3% to a high of 47.9% in the 10^{-3} M concentrations. *p*-Coumaric acid, one of the two compounds with the highest concentrations in rice paddy soils (Chou et al., 1977), reduced both the mean nodule number and heme content per plant the most. Vanillic acid had the least effect on heme content at both concentrations.

TABLE 5. EFFECTS ON NODULE NUMBERS AND HEME CONTENT OF BUSH BLACK SEEDED BEANS OF KNOWN PHENOLICS[a]

Treatment	Mean nodule number ±SE	Heme/plant (μg) *per*	Reduction in heme (%)
p-Coumaric acid	106.1 ± 11.4[b]	211.2	15.2
Ferulic acid	87.4 ± 7.1[b]	160.0	35.7
p-Hydroxybenzoic acid	97.5 ± 3.8[b]	200.0	19.6
Vanillic acid	112.0 ± 7.1[b]	198.0	20.4
All of above	68.7 ± 6.9[b]	160.0	35.7
Control	153.9 ± 10.9	248.9	

[a]Concentration of each phenolic in each test 10^{-3} M.
[b]Significantly different from control mean at 0.01 level or better. $DF = 17$.

Hardy et al. (1971) reported that the amount of nitrogen fixed is positively correlated with the hemoglobin content of the nodules. It is probable, therefore, that most test plants in this experiment fixed less nitrogen than the controls due to their lowered nodule numbers and reduced hemoglobin content.

In a related experiment with Bush Black Seeded beans, each phenolic markedly reduced the mean nodule number, and the reduction was highly significant statistically in every test (Table 5). There was a definite additive effect also because the solution containing all four phenolics caused the greatest reduction in nodule number. Ten plants were used in each test set and the controls in this experiment.

All phenolic solutions also reduced the mean hemoglobin (heme) content per plant, with the percent reduction ranging from 19.6 to 35.7% (Table 5). Ferulic acid and the combined phenolics solution each reduced the heme content by 35.7%, and these solutions were also most effective in reducing nodule numbers.

Effects of Known Phenolics and of Extracts of Decomposing Rice Straw

TABLE 6. EFFECTS ON N_2-FIXATION (ACETYLENE REDUCTION) BY BUSH BLACK SEEDED BEANS OF KNOWN PHENOLICS AND OF EXTRACTS OF DECAYING RICE STRAW IN SOIL (MEAN ± SE) ($DF = 6$)

Treatment		C_2H_4 (μmol/plant/hr)
p-Coumaric acid	Control	5.64 ± 0.50
	10^{-4} M	7.75 ± 0.32[a]
	10^{-3} M	5.08 ± 0.72[b]
Ferulic acid	Control	7.30 ± 0.82
	10^{-4} M	7.09 ± 0.62
	10^{-3} M	5.65 ± 1.29
p-Hydroxybenzoic acid	Control	3.84 ± 0.50
	10^{-4} M	6.10 ± 0.29[a]
	10^{-3} M	4.06 ± 0.58[b]
o-Hydroxyphenylacetic acid	Control	6.38 ± 0.80
	10^{-4} M	5.13 ± 0.69
	10^{-3} M	4.53 ± 0.80
Vanillic acid	Control	2.22 ± 0.39
	10^{-4} M	3.45 ± 0.59[a]
	10^{-3} M	2.57 ± 0.85
Extract of decaying rice straw in soil	0 g/3 kg soil (control)	8.56 ± 1.64
	25 g/3 kg soil	3.71 ± 0.77[a]
	100 g/3 kg soil	2.45 ± 0.35[a]

[a]Significantly different from respective control at 0.05 level or better.
[b]Significantly different from 10^{-4} M at 0.05 level or better.

on Acetylene Reduction (N_2 Fixation) by Rhizobium *in Bean Nodules.* Ferulic and *o*-hydroxyphenylacetic acids appeared to reduce the rate of N_2 fixation (acetylene reduction) in nodules of Bush Black Seeded beans because the rate decreased consistently with increasing concentrations of these compounds (Table 6). The differences from the controls were not statistically significant, however. The 10^{-4} M concentrations of *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids significantly stimulated N_2 fixation, whereas the 10^{-3} M concentrations had no effects. These results were surprising in view of the previous experiment indicating that all the phenolics tested significantly reduced nodule numbers and hemoglobin content of the nodules in Bush Black Seeded beans. The difference was probably due to the more advanced stage of development of the plants at harvest in the present experiment. Technical difficulties with the gas chromatograph forced us to postpone harvest for an extra week, and the bean plants were fruiting heavily. Some of the nodules appeared to be decaying and the hemoglobin contents appeared lower in many of the older nodules. Thus, there was much variability in acetylene reduction rates.

The extracts of decaying rice straw in soil (both concentrations) significantly inhibited N_2 fixation by Bush Black Seeded beans (Table 6). Thus the evidence is very strong that decomposing rice straw in paddies is inhibitory to N_2 fixation by legumes.

The results of our research suggest that this inhibition of N_2 fixation by decaying straw may be responsible, at least in part, for the large decreases in yields of legumes following rice crops, when the rice straw is left on the land. This hypothesis needs to be tested by measuring N_2 fixation by several legumes under field conditions, with and without residues of rice straw. The possibility of a direct inhibition of legume plant growth by decaying rice straw needs to be tested also.

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THE SEX PHEROMONE GLANDS OF *Dermacentor variabilis* (SAY) AND *Dermacentor andersoni* STILES
Sex Pheromone Stored in Neutral Lipid[1]

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Abstract—Evidence is presented indicating the presence of the tick sex pheromone, 2,6-dichlorophenol in lipid droplets in the foveal glands of *Dermacentor variabilis* (Say) and *Dermacentor andersoni* Stiles. The pheromone appears to be dissolved in the lipid droplets. The droplets consist of neutral lipids, mostly triacylglycerides and cholesterol esters. The esterified fatty acid profiles of foveal gland triacylgylcerides are different from those of other tissues examined or reported for other ticks. The percentage of shorter chain, mostly saturated fatty acids were decreased, while the longer chain polyunsaturated fatty acids were increased. The biological advantages of lipid solution for storage, translocation, and release of the sex pheromone from the female tick are discussed.

Key Words—*Dermacentor variabilis*, *Dermacentor andersoni*, 2,6-dichlorophenol, sex pheromone, neutral lipid, sex pheromone glands, ticks.

INTRODUCTION

The role of phenolic compounds as sex pheromones in metastriate ticks has been reported by several workers (Berger et al., 1971; Berger, 1972;

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Sonenshine et al., 1974, 1976; Chow et al., 1975; Wood et al., 1975). Little is known about the production, storage, and release of these compounds or the physiological functions that regulate these events. The sex pheromone, 2,6-dichlorophenol, has been associated with the foveal glands in *Dermacentor variabilis* (Say) and *Dermacentor andersoni* Stiles (Sonenshine et al., 1977). The source of other phenolic pheromones in ticks is unknown.

Ultramicroscopic study of the foveal glands reveals abundant accumulation of lipid or lipid-like droplets when the tissues fixed in glutaraldehyde are stained with lead citrate and with osmium tetroxide (Vernick et al., 1978). In contrast, these droplets are almost all lost when the foveal glands are dehydrated and cleared in xylene prior to embedding in paraffin. The possibility that storage and translocation of pheromone is associated with these lipid materials is suggested by these findings. Storage in liquid lipids would tend to minimize cytotoxic effects of the phenols, facilitate translocation, and provide a slow, constant release from the tick's body surface.

This paper reports new evidence associating the sex pheromone, 2,6-dichlorophenol, with neutral lipids in the foveal glands of *D. andersoni* and *D. variabilis*. The lipid composition of these glands is described. The possible role of neutral lipids in sex pheromone activity is reviewed.

METHODS AND MATERIALS

The Rocky Mountain wood tick, *Dermacentor andersoni*, was colonized with specimens obtained from the U.S. Public Health Service, Rocky Mountain Laboratory, Hamilton, Montana. The American dog tick, *D. variabilis*, was colonized with specimens collected near Montepelier and Ashland, in central Virginia. Ticks were reared and stored for experiments as described in previous reports (Sonenshine et al., 1977).

Histochemistry. Foveal glands were excised from unfed or partially fed females of both species. Tissues removed for frozen sections were fixed in cold 10% buffered neutral formalin, embedded in Lipshaw M-1 embedding matrix (Lipshaw Manufacturing Co., Detroit, Michigan), frozen with Cryokwik (IEC, Needham Heights, Massachusetts), and sectioned at 10 μm in an International Equipment Co. Cryostat (Needham Heights, Massachusetts) held at $-20°$ C. The frozen sections were mounted on subbed slides. Histochemical tests were done with oil red "O" (ORO) and Sudan black B (SBB) according to the methods described by Pearse (1968) to reveal neutral lipids (NL). Tissues removed for paraffin sections were also fixed in cold 10% buffered neutral formalin, dehydrated, cleared in xylene, and embedded after three changes of parrafin (mp, 56° C). The following histochemical tests were done, in accordance with methods described by Pearse (1968): Periodic acid

Schiff (PAS) for polysaccharides, with controls predigested with 1% diastase prior to treatment; mercuric bromphenol blue for basic protein; toluidine blue for degrees of metachromasia; Millon's reagent for tyrosine-containing proteins, and dimethylaminobenzaldehyde (DMAB) nitrite for tryptophan-containing proteins. Salivary glands from the same specimens were used as controls. Sudan black B was used on tissues fixed according to the McMannus technique for 5 weeks.

Chlorine Analysis. Excised foveal glands of unfed and partially fed ticks of both species were prepared for ultramicroscopic examination as described by Vernick et al. (1978); cacodylate rather than phosphate was used as a buffer, and agar embedding was omitted. Sections were cut on a Porter-Blum MT-2 ultramicrotome. Some sections were cut at 0.2μm, mounted on support grids, and the unstained specimens were examined with a JOEL Co. JSM U-3 scanning electron microscope at the Department of Electron Microscopy, University of South Carolina, Columbia, South Carolina. Analysis of the chlorine content of different secretory droplets, intervening tissue, and adjacent epon was made (1) for intervals of 1000 sec with an EDAX Energy Dispersive X-ray analyzer, model 505, and Teletype Interface, model 604, and repeated (2) for intervals of 250 sec with an EDAX model 9100 analyzer. Other sections, cut at 0.1μm and mounted on ordinary grids were examined with a Hitachi model 500 transmission electron microscope at the Bureau of Biologics, Food and Drug Administration, DHEW, Bethesda, Maryland. Analysis of the chlorine content of secretory granules and adjacent tissue was made with a Kevex-7000 Micro-X Elemental Analyzer.

Electrophoresis. Polyacrylamide gels were prepared with four concentrations, 3.5%, 4.8%, 7.0%, and 12.0%, to form a step gradient. Twenty-five feeding *D. variabilis* females were inoculated with 0.05–0.1 μCi of ^{36}Cl as NaCl (3 mCi/g specific activity, Amersham Searle, Des Plaines, Illinois). The foveal glands were excised, homogenized with a tissue grinder, and disrupted further with ultrasonic sound (Branson Sonic Power Co., Danbury, Connecticut); the mixture was concentrated and aliquots (20 μl) were deposited on the top of each gel. Electrophoresis was done in a BioRad model 150 A gel electrophoresis cell with a BioRad model 400 (Richmond, California) power supply at 3 mA/gel. Gels were stained with Coomassie brilliant blue (CBB) for proteins, ORO for neutral lipids, and assayed for radioactivity with a Beckman model LS 250 liquid scintillation counter (Fullerton, California). Other gels were sectioned without staining and assayed for 2,6-dichlorophenol by GC at S.U.N.Y., Syracuse, New York.

Lipid Analysis. Young, unfed virgin females (1–2 weeks post emergence) were fixed with cold buffered 10% neutral formalin; 46 *D. variabilis* and 35 *D. andersoni* females were used. The foveal glands were excised, freed of all cuticle and adjacent soft tissue, and placed in a mixture of chloroform–

methanol (2:1, v/v) (Gurr and James, 1971). Other extracts were made of midgut hemolymph, salivary glands, and cuticle. The extracts were washed 2×, cleansed of extraneous nonlipid material, and concentrated (300 μl); aliquots were spotted onto TLC plates. To detect neutral lipids, TLC was done on precleaned Baker Flex IB_2 plates coated with 250-μm silica gel G (J.T. Baker Chemical Co., Phillipsburg, New Jersey). The plates were developed in petroleum ether–diethyl ether–acetic acid (80:20:1) (Mangold, 1965) and stained with 10% phosphomolybdic acid (PMA) in ethanol or charred with 50% H_2SO_4. Identification of lipid classes was aided by comparison with authentic standards (Supelco, Inc., Bellefonte, Pennsylvania) as well as staining and comparison of R_f values as described by Kates (1972). Similar techniques were used to detect phospholipids on Whatman K5WF plates (Whatman, Inc., Clifton, New Jersey). The solvent system was chloroform–methanol–water (65:25:4) and identification was aided by comparison with authentic standards (Analabs, Inc., Vineland, New Jersey). To quantify the lipid classes in the foveal gland extract, the chromatograms with known internal standards were scanned with a Kontes system II recording densitometer (Kontes, Inc., Vineland, New Jersey). Replicates were examined in the UV range.

To trace the pheromone in the different lipids, 191 partially engorged *D. variabilis* females were inoculated with approximately 1–2 μCi of [^{36}Cl]NaCl prior to excision of the foveal glands; small segments of cuticle, ca. 1.0 cm^2, epidermis, and muscle fragments were also included. Alloscutal segments of similar composition, but without foveal glands, were excised for comparison. Lipids were extracted and identified as described above. Samples of the identified lipids were assayed by liquid scintillation spectrometry and exposure to X-ray film (Kodak type NS-2T). Other samples were coinjected on the GC (Varian model 2740, FID mode) with 5 μg of unlabeled authentic 2,6-dichlorophenol, and the 2,6-dichlorophenol fraction was collected. Details of column packing and other parameters were described previously (Sonenshine et al., 1977).

The esterified fatty acid (ESA) composition of the foveal gland, midgut, and integumental lipids was determined by GC. Triacylglycerides (TAG) and phospholipids were extracted from thin-layer silica gel plates with chloroform–methanol–ethyl ether (1:1:1, v/v) and chloroform–methanol–acetic acid–water (50:39:1:10, v/v), respectively. Each sample was dried at 45°C under nitrogen, redissolved in benzene, and transmethylated with sodium methoxide in methanol (Applied Sciences). The resultant fatty acid methyl esters were extracted with ethyl ether, dried, and redissolved in heptane. GC was done on a Packard 427 GC (Downer's Grove, Illinois) using 10% SP 2330 on a 100–120 Chromasorb WAW glass column (Supelco) programed from 150° to 230°C at 8°/min. A minigrator (Spectra-Physics, Santa Clara, California) was utilized for electronic integration of peak areas. Peaks were

identified by comparison of retention times with those of standard fatty acid methyl esters.

Biosynthesis of lipids by tick tissue samples containing foveal glands was tested by incubating the tissue samples with [U-^{14}C]glucose (140 mCi/mmol specific activity) and [U-^{14}C]glycerol (140 mCi/mmol specific activity) (Amersham Searle, Des Plaines, Illinois). Lipids were extracted and separated on TLC plates as described above. The presence of labeled lipids was detected by exposing X-ray film to the TLC plates; differences in synthesis of the different compounds were determined by radioassay of each lipid class.

RESULTS

Histology and Histochemistry. Frozen sections of the foveal glands stained with SBB and ORO revealed positive staining reactions, suggesting accumulation of neutral lipids (Figure 1A and B). In contrast, formalin-fixed tissues dehydrated in ethanol, cleared in xylene, and embedded in paraffin revealed highly vacuolated lobes with the cytoplasm of the cells represented by only a fine matrix of protein strands; the SBB test was negative (Figure 1C). However, when these tissues were fixed by the McMannus technique prior to dehydration, the cell contents were retained and stained positive with SBB (Figure 1D). Polysaccharides, perhaps including glycogen, were demonstrated by the strongly positive PAS reaction observed with both frozen sections and paraffin sections; the reaction was negative in controls predigested with diastase. Basic protein was revealed by the positive bromphenol blue reaction. The Millon and DMAB nitrite tests for tyrosine and tryptophan, respectively, were negative.

Chlorine Analysis. High concentrations of chlorine were found only in secretory droplets. Little chlorine was found in the droplet-free areas of the tissue and, with the exception of one sample, virtually none was found in the epon in which the tissue was embedded (Table 1). Much greater accumulations of chlorine were found in *D. andersoni* than *D. variabilis* granules.

Electrophoresis. Polyacrylamide gel electrophoresis of aliquots of a homogenate of 156 *D. variabilis* foveal glands revealed 30 ng of 2,6-dichlorophenol, all at the top of the gel. Radioassay of fractions of a similar gel prepared with glands (122 pairs) from ticks inoculated with ^{36}Cl (as [^{36}Cl]NaCl) revealed radiochlorine activity distributed more or less uniformly in trace amounts, with very small peaks only at the origin and the dye front. No significant accumulations of radioactive chlorine were found in any of the protein bands. Similar results were obtained with *D. andersoni* foveal glands (50 pairs). None of the gels stained with ORO.

Foveal Gland Lipids and Fatty Acids. Table 2 summarizes the major lipid classes of the foveal glands. A total of 1.377 μg of lipids and free fatty

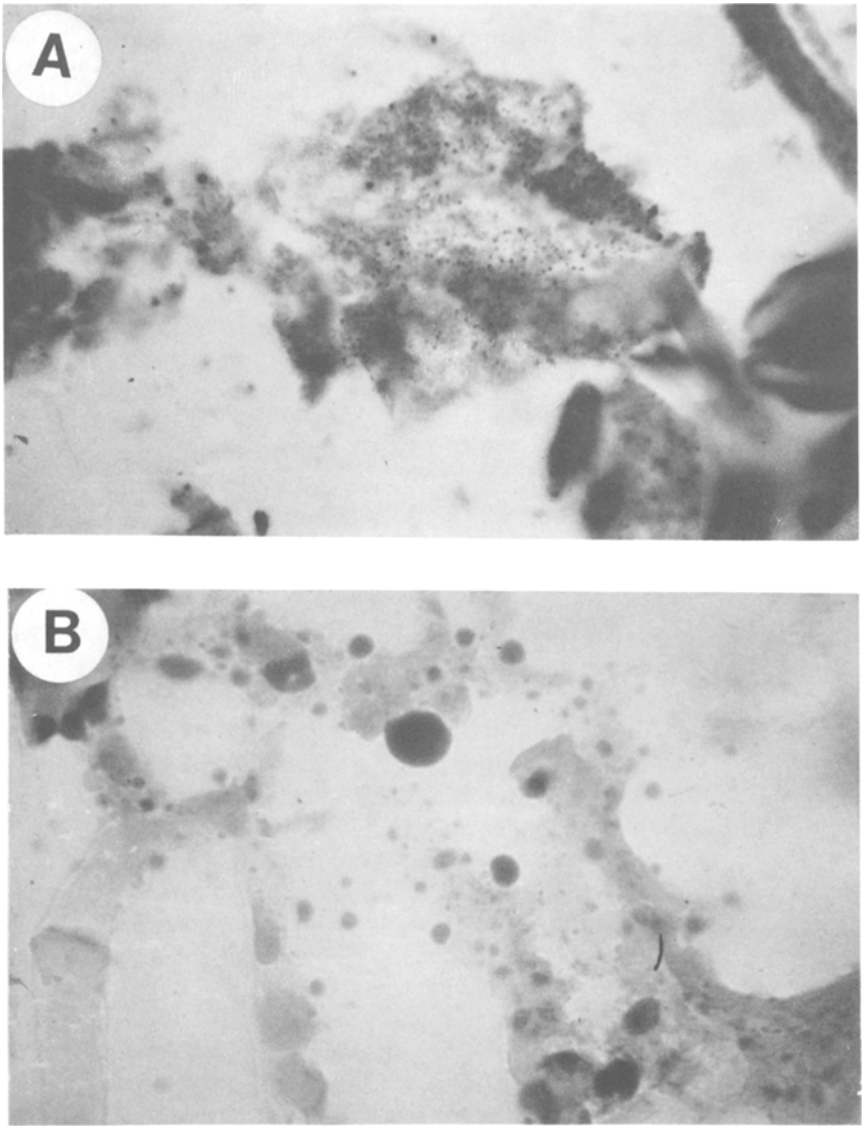
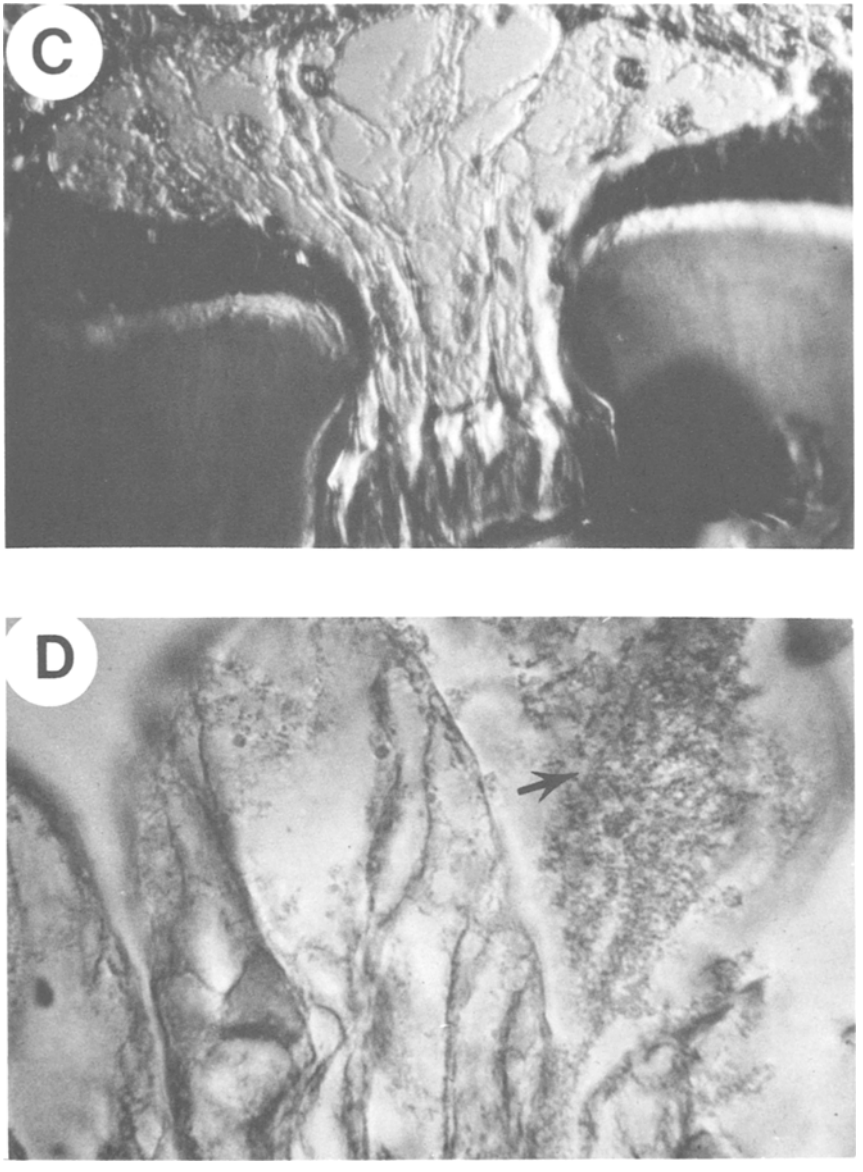


FIG. 1. Histochemical studies of foveal gland lipids in *D. variabilis* and *D. andersoni*. (A) Black-staining granules found in frozen sections of extirpated *D. variabilis foveal* glands stained with Sudan black B; 1500×. (B) Red-staining granular clusters found in frozen sections of extirpated *D. variabilis* foveal glands stained with oil red 0; 1500×. (C) Highly vacuolated lobes shown in a cross-section of *D. andersoni* foveal gland,



fovea dorsale, and adjacent cuticle; formalin fixation, routine alcohol dehydration, paraffin sections; 400×. (D) Dark-staining granular mass (arrow) dislodged from adjacent lobes of extirpated *D. variabilis* foveal glands. Tissues fixed according to McMannus (Pearse, 1968) to preserve lipids; paraffin sections; 1000×.

TABLE 1. CHLORINE CONTENT OF SECRETORY GRANULES OF FOVEAL GLAND SECTIONS VIEWED WITH ELECTRON MICROSCOPE

A. Thick sections (0.2 μm) viewed with the SEM[a] (*D. variabilis* and *D. andersoni*)

	Chlorine-to-osmium ratio		
Sample No.	*D. variabilis*	*D. andersoni*	Epon only
1	0.233:1.0	38.90:1.0	0.0:1.0
2	0.408:1.0	17.85:1.0	0.01:1.0

B. Thick sections (0.2 μm) viewed with the SEM[a] (*D. variabilis* only)

	Chlorine only (photons/sec), foveal glands		
Sample No.	Secretory granule	Nongranular tissue	Epon only
1	2.614	0.810	0.000
2	3.148	0.312	0.000
3	12.269	0.000	
4	2.676		
Mean ± SD	5.177 ± 4.100	0.374 ± 0.334	0.000

C. Thin section (0.1 μm) viewed with the TEM[b]

	Chlorine only (photons/sec), foveal glands		
Sample No.	Secretory granule	Nongranular tissue	Epon only
1	12.135		1.064
2	15.691		
3	15.921		
Mean ± SD	14.582 ± 2.098		

[a] Unstained sections mounted on support grids, carbon coated, viewed with a JOEL Co. scanning electron microscope.
[b] Unstained thin sections, not mounted on support grids, viewed with a Hitachi transmission electron microscope.

acids were found per foveal gland in *D. variabilis*. The major lipid class was neutral lipid, 0.793 μg/gland. Triacylglycerides, and cholesterol esters were present in the greatest amounts, although small quantities of cholesterol were also detected. Free fatty acids were also abundant, but only small amounts of phospholipids were present. Somewhat smaller quantities of lipids and free fatty acid, 0.311 μg lipid/gland, were extracted from the foveal glands of *D. andersoni*. Again, the same three neutral lipids were present, with cholesterol relatively more abundant than in the extract from the *D. variabilis* glands.

Free fatty acids were much less abundant in the *D. andersoni* gland extract, and phospholipids were absent.

Fatty acid profiles of the foveal glands and other tick organs are illustrated in Figure 2. The fatty acid composition of the TAG from the midgut of *D. variabilis* showed large amounts of oleate, the predominant fatty acid, accounting for 71.3% of the total. Palmitate, stearate, linoleate, palmitoleate, and, to a lesser extent, arachidonate, were the most common fatty acids present (Figure 2A). Similar fatty acid profiles were found in the TAG from dorsal integument and midgut phospholipid (not figured) from these ticks, and in the midgut lipids of *D. andersoni*. In contrast, the TAG extracted from foveal glands of *D. variabilis* showed a distinctly different fatty acid composition (Figure 2B). Oleate, although still the most abundant fatty acid, was only 19.4% of the total. Increases were found in linolenic and arachidonic acids, as well as several even longer chain polyunsaturated fatty acids. Three unidentified peaks, including one between palmitoleate and stearate (X), were from 7 to 10% each. A similar fatty acid profile was found in the TAG extracted from the foveal glands of *D. andersoni* (not figured). However, in contrast with *D. variabilis*, palmitate was somewhat more abundant (21.5%). The TAG composition of both foveal glands and other body tissues in the two ticks was highly unsaturated and, presumably, liquid at ambient temperatures.

Evidence of ^{36}Cl labeling was found in the neutral lipid fraction, but not the phospholipid fraction of the crude foveal gland extract of radiochlorine-treated ticks. Thin-layer chromatograms revealed an unknown compound, R_f 0.50, which fluoresced when excited by UV; when X-ray film was exposed to the TLC plates (72 hr), a weak positive reaction was found at this same

TABLE 2. LIPIDS AND FATTY ACIDS OF FOVEAL GLANDS OF TWO SPECIES OF HARD TICKS

Compound	*D. variabilis* Amount/gland (μg)	*D. variabilis* %	*D. andersoni* Amount/gland (μg)	*D. andersoni* %
Free fatty acid	0.485[a]	35.2	0.053[a]	17.0
Triacyglycerides	0.428[b]	31.1	0.110[b]	35.4
Cholesterol esters	0.235[b]	17.1	0.070[b]	22.5
Cholesterol	0.130[a]	9.5	0.078[a]	25.1
Phospholipids	0.099	7.2		
Totals	1.377		0.311	

[a] Mean of two samples.
[b] Mean of three samples.

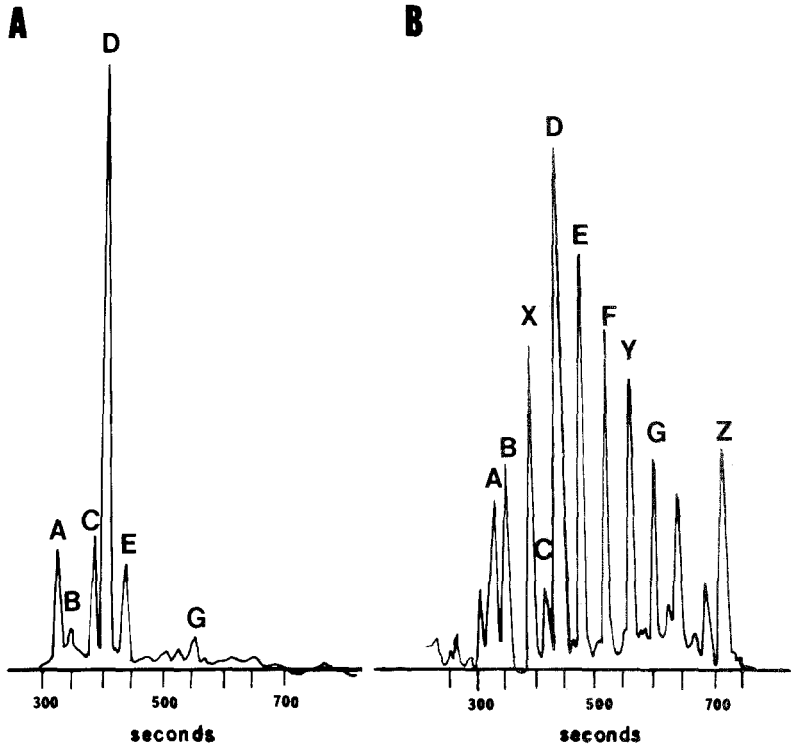


FIG. 2. Gas-liquid chromatograms of the fatty acid (as methyl ester) composition of a triacylglyceride extract from *D. variabilis* foveal gland and midgut tissues. Part A = midgut; part B = foveal gland. A = palmitate; B = palmitoleate; C = stearate; D = oleate; E = linoleate; F = linolenate; G = arachidonate; X, Y, Z, = unknowns.

location. This and adjacent neutral lipids (triacylglycerides and alkyl diglycerides) were extracted from the plate and coinjected with authentic, unlabeled 2,6-dichlorophenol into the GLC. No radiochlorine was found when the GC-reisolated 2,6-dichlorophenol was assayed by liquid scintillation spectrometry. When foveal glands from partially fed *D. variabilis* were incubated with [^{36}Cl]NaCl, the same UV-positive compound described above was found to have incorporated radiochlorine (62 cpm above background). No other compounds isolated from the glands had ^{36}Cl, and no ^{36}Cl was found in the extract except at the origin of the chromatograms. No UV-positive lipids or ^{36}Cl incorporation were found in any of the lipid fractions from integumental segments without foveal glands.

Synthesis of Lipids by Foveal Glands and Epidermis. When *D. andersoni* foveal glands, adjacent epidermis, and muscle fragments were incubated in the presence of [^{14}C]glucose, ^{14}C was found in the lipid fraction. The dominant

neutral lipids were triacylglycerides (31.5% of the ^{14}C incorporated); small quanties of cholesterol ester (2.3%) were also labeled. Most of the ^{14}C in the lipid fraction from this crude extract was used for the synthesis of phospholipid (66.2%), especially phosphatidylinositol (48.8%) and phosphatidylethanolamine (11.9%). When these tissues were incubated in the presence of [^{14}C]glycerol, most of the glycerol which was incorporated in tissue lipids was used for phospholipid synthesis (85.9%); triacylglycerides were the only major neutral lipids to have been synthesized with labeled glycerol. Epidermal tissues from the same ticks utilized the labeled substrates for similar lipid synthesis, although with slightly more triacylglycerides, trace amounts of cholesterol ester, and slightly less phospholipid.

DISCUSSION

The results confirm the presence of abundant neutral lipid droplets in the foveal glands, mostly triacylglycerides and cholesterol esters. Sex pheromone, 2,6-dichlorophenol, appears to be dissolved in the neutral lipid droplets. No evidence of chemical bonding to any specific neutral lipid or lipoprotein was found, and the pheromone did not cochromatograph with any of the neutral lipid classes.

The neutral lipids found in the foveal glands of *D. variabilis* and *D. andersoni* are characteristic of those found in ixodid ticks generally. Chow et al. (1972) found triacylglycerides and cholesterol to be the dominant neutral lipids in *Boophilus microplus* (Canestrini). The esterfied fatty acid profiles in this study in midgut and integumental tissues are also similar to the fatty acid profiles of whole ticks, including *B. microplus*, *Hyalomma dromedarii* Koch, *H. excavatum* Koch, and *D. andersoni* (Chow et al., 1972; Maroun and Kamel, 1973). However, oleate and palmitate were much more abundant in midgut and integumental tissues than in the whole tick extracts described by these other workers. The foveal gland esterified fatty acid profiles were very different from either the midgut and integumental tissues of the same ticks or other, whole ticks. Oleate and other common fatty acids were decreased (except palmitate) in the foveal gland, while the longer chain polyunsaturated fatty acids, such as linolenic, arachidonic, and the unknown fatty acids X and Z were increased. The unique composition of the foveal gland fatty acids is suggestive of a specialized storage and/or secretory function.

Storage of pheromone in neutral lipid droplets composed primarily of triacylglycerides provides a convenient means of masking this potentially toxic compound and translocating it to the surface in the oily droplets. Replenishment of the neutral lipid secretions appears to be within the tick's synthetic capabilities. Release of the pheromone from the oily liquid spread

on the body surface would be gradual, providing long-lasting attraction to mate-seeking males. The use of neutral lipids to store and release pheromone may also have practical value in developing pheromone-containing formulations for control of ticks. Triacylglyerides have been used as "keepers" for sustained release of the gypsy moth pheromone, disparlure (Beroza et al., 1971).

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AN OLFACTOMETER FOR BARK BEETLE PARASITES[1]

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Abstract—An "H"-type olfactometer was designed and built to test the response of bark beetle parasites to various beetle and tree host odors. The design has several advantages over other types of olfactometers. Strong air currents are not utilized, parasites have free movement in the test chamber, and a concentration gradient of test compound is maintained. Parasites tested in the olfactometer demonstrated strong positive responses to air drawn over logs infested with bark beetle larvae and varied responses to tree host odors.

Key Words—Parasites, bioassay, *Dendroctonus frontalis*, Coleoptera, Scolytidae, olfactometer, bark beetles.

INTRODUCTION

The hymenopterous parasites of the southern pine beetle, *Dendroctonus frontalis* Zimmermann (SPB), engraver beetles, *Ips* spp.; and the eastern juniper bark beetle, *Phloesinus dentatus* (Say), show preferences for the hosts on which they developed even though many are common on two or more of these beetle hosts (Kudon and Berisford, 1980). However, the stimuli to which the parasites respond when searching for a host have not been determined. Camors and Payne (1969) indicated that only α-pinene of the more common SPB pheromone components contributed to attraction of the parasite *Heydenia unica* C&D. However, most parasites are attracted to the SPB when beetle broods are primarily 3rd and 4th instar larvae (Berisford et al.,

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unpublished data) and are not attracted to α-pinene. Since α-pinene is probably always present at infested and uninfested pine trees at varying levels, it appears that α-pinene may not play a primary role as a chemical attractant for many SPB parasites. Dixon and Payne (1980) found evidence that some bark beetle pheromones may play a role in host location by some parasites.

In an effort to determine the nature of attractants for bark beetle parasites and to gain added information on host perference of parasites, a specialized olfactometer was designed. Olfactometers of the "Y" design (Barrows, 1907; McIndoo, 1926) have several drawbacks when utilized in this type of study. They are not easily adapted to comparison tests of two different chemical stimuli, and for correct operation a relatively strong airflow is needed through the testing chamber. Experiments with olfactometers involving a strong airflow showed that many of the small hymenopterous parasites of bark beetles brace and remain motionless. In addition, Y olfactometers have two separate testing chambers traversed by the test specimens (the two branches of the Y), both of which must be maintained at exact airflow, humidity, light level, etc. McIndoo (1926) also noted that test insects had difficulty orienting to the direction of the odors at the junction of the branches. Possibly insects that enter a branch of the Y in exploratory behavior may become trapped yet appear to be giving a perferential response. Other types of olfactometers (Wieting and Hoskins, 1939; Folsom, 1931; Chamberlain, 1956) all have various drawbacks for use with hymenopterous parasites. For example most olfactometers have strong air currents and few allow adequate determination of the concentration of chemicals. We describe herein an "H" olfactometer designed to be used specifically for bark beetle parasite-attractant and host-perference studies.

METHODS AND MATERIALS

The olfactometer is illustrated in Figure 1. The air stream enters at point labeled IN. An entry flow meter is calibrated in standard cubic inches per minute (SCIM) with the air flow adjusted to 885 SCIM (14,500 cc/min). The first filter (1) contains activated charcoal to remove impurities, odors, etc., and the second filter is desiccating calcium chloride. Two motorized syringes (3) are inserted in the tygon tubing of the appropriate channels at a point immediately behind the filter Y tube. In the side view of the motorized syringe (8), the drive gear, motor gear, and cutoff alarm switch are visible.

The large drive gear is intermediate between the motor gear and a geared track. There is a direct link between the plunger of the syringe and the geared track. The motor operates on 28-V AC pulses of variable duration and interval supplied through a relay actuated by a LM 555[2] linear integrated

[2]Part number of National Semiconductor Incorporated. Available under similar part number from a variety of sources.

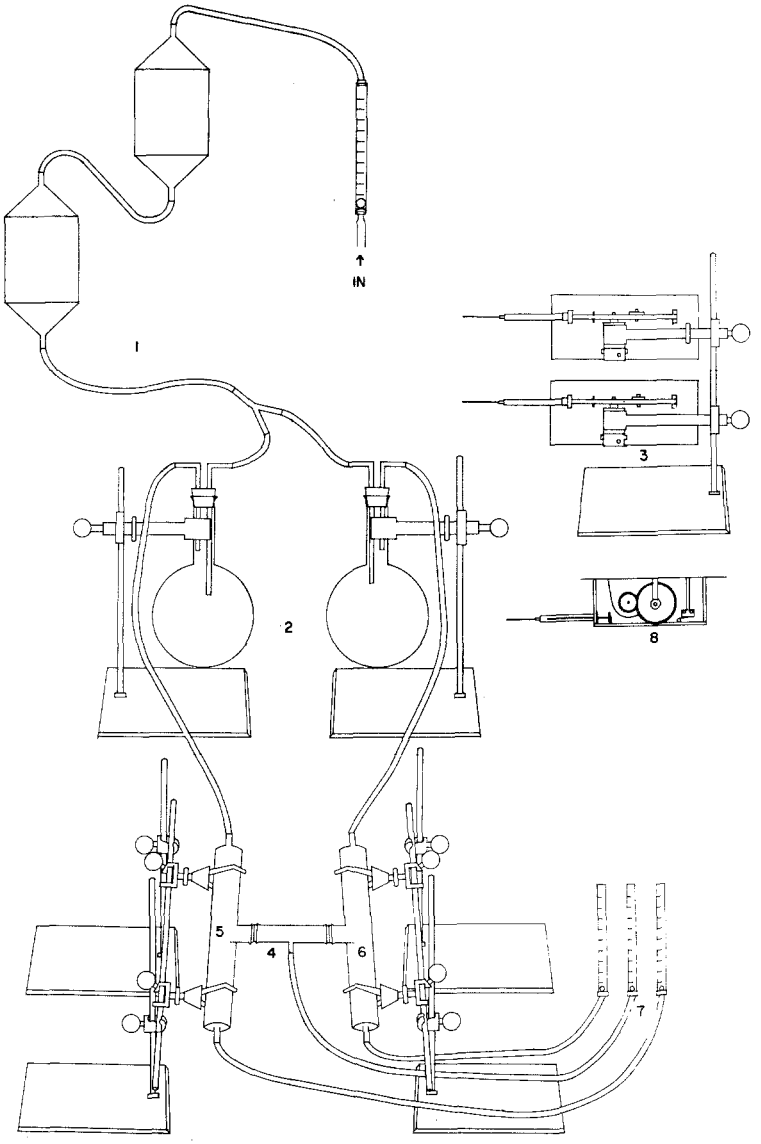

FIG. 1. Diagram of H olfactometer: (1) air line filters; (2) pulse reservoir flasks; (4,5,6) H olfactometer proper; (7) flow tubes; (3,8) motorized syringes.

circuit timing device. Variable pulse duration and interval enables a wider range of elution rates than an analog system. A cutoff system activates an alarm while simultaneously blocking power from the motors when the syringe is empty.

Since each motorized syringe is activated by pulses, reservoir flasks (2) serve to smooth out the flow of test volatiles in the air stream. The right channel (6) and left channel (5) plus the bioassay chamber (4) compose the olfactometer proper. The insect is confined to the bioassay chamber by Saran® screening located at the junctions of the bioassay chamber and the left and right channels. Exit flow meters (7) ensure airflow balance between right and left channels and enable determination of airflow through the bioassay chamber. Temperature is monitored at both the air input and the exit.

The bioassay chamber and left and right channels are constructed of glass tubing with an inside diameter of 2.5 cm. An extension of the rear wall of the bioassay chamber into each side channel deflects part of the air current into the bioassay chamber. By varying the duration and interval of the pulse operating the motorized syringe, the effective concentration of test compounds can be altered. Compounds can also be tested by direct egress into the air system. Air drawn over logs infested with bark beetles was directed into the channels of the olfactometer for testing against bark beetle parasites.

Various preliminary tests were performed to determine the efficacy of the olfactometer. Smoke was injected into the apparatus by syringe and observed with the aid of a laser beam (coherent light source) directed through the bioassay chamber. Titanium chloride smoke was also introduced into the main air stream. Smoke tests confirmed the existence of two gradients in the bioassay chamber. A test compound introduced into the right channel forms a gradient extending from the right channel to the central exhaust of the bioassay chamber. The second gradient extends from the exhaust to the left channel. Air samples containing α-pinene were removed while the apparatus was operating and were analyzed by gas–liquid chromatography. Analyses of air extracts showed an α-pinene concentration ratio of 10:1:0.5 for the right channel–middle exhaust–left channel. This ratio denotes the two gradients in the bioassay chamber as described above. Gas chromatograms of clean air directed through the apparatus before and 10 min after a trial run failed to show any significant level (6 ng/μl) of contaminant, including plasticizer from tygon tubing.

The reactions of the insects tested were recorded by visual observation. Four known bark beetle parasites, *Coeloides pissodis* (Ashmead) (Braconidae), *Heydenia unica* Cook & Davis, (Pteromalidae), *Dendrosoter sulcatus* (Musebeck) (Braconidae), and *Roptrocerus xylophagorum* (Ratzeburg) (Torymidae), were tested in the device. The bioassay chamber was divided longitudinally into five sections termed far right, near right, middle,

TABLE 1. PERCENTAGE OF TIME SPENT BY SPB PARASITES IN BIOASSAY CHAMBER SECTORS OF H OLFACTOMETER[a]

Parasite	No. specimens	Compounds			% Time (avg)				
		No. runs	R	L	FR	NR	M	NL	FL
Coeloides	15	30	α-Pinene	—	10	20	25	35	10
Roptrocerus	10	20	α-Pinene	—	10	20	30	25	15
Heydenia	10	20	α-Pinene	—	20	20	15	25	20
Coeloides	10	20	Cedarwood oil	—	20	30	20	10	20
Roptrocerus	9	18	Cedarwood oil	—	15	30	30	10	15
Heydenia	10	20	Cedarwood oil	—	15	35	20	15	15
Coeloides	12	24	Frontalure	—	15	20	25	15	25
Roptrocerus	10	20	Frontalure	—	25	15	15	25	25
Heydenia	10	20	Frontalure	—	15	20	25	25	15
Coeloides	7	14	Air over infested SPB logs	—	40	30	15	5	10
Roptrocerus	5	10	Air over infested SPB logs	—	50	15	15	10	10
Heydenia	8	12	Air over infested SPB logs	—	45	30	10	10	5
Dendrosoter	18	36	Air over infested SPB logs	—	55	25	10	5	5

[a]R, right; L, left; FR, far right; NR, near right; M, middle; NL, near left; FL, far left. All data treated as if test compounds were introduced into the right channel.

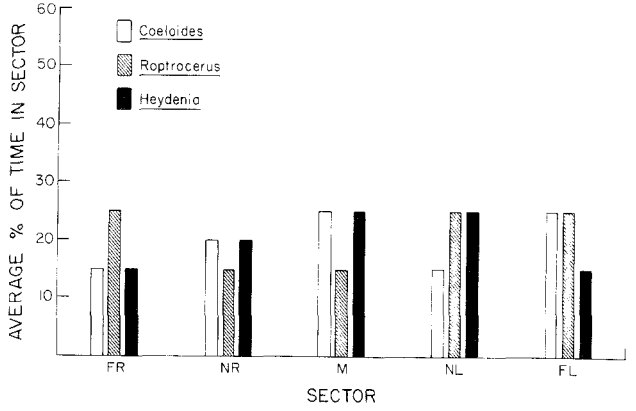

FIG 2. Percentage of time spent by SPB parasites in different sectors of the bioassay chamber of the H olfactometer when exposed to α-pinene. FR, far right; NR, near right; M, middle; NL, near left; FL, far left (see text).

near left, far left. The time each parasite spent in each sector was noted. A Brush Operations Monitor® was utilized to record the time the insect spent in each sector and to record the duration and occurrence of the pulse operating the syringe. Each insect was tested twice for 5 min. The second run was performed with the test compounds in opposite channels from the first run. Between each run clean air was forced through the chambers for 10 min.

Preliminary data have been gathered utilizing the olfactometer with SPB parasites. Tests compared three compounds: α-pinene, (99%, Aldrich Chemical Co.), cedar wood oil (Fisher Scientific Co.), and frontalure. Frontalure is a 1:2 mixture of α-pinene and frontalin (100%, Chemsamp Co.). These compounds were selected for testing since they are volatiles present in relatively large quantities at bark beetle-infested trees. Tests were all performed at various concentrations (except for α-pinene) but due to the uniformity of responses, concentrations were not included in the table. Tests utilizing α-pinene were all performed at an initial injection rate of 0.5 μl/15 sec. Using the 10:1:0.5 ratio obtained with α-pinene and the GLC gives a concentration/15 sec of 0.43 μl:0.04 μl:0.02 μl. Air drawn over infested SPB bolts that were attractive to parasites in the field was also tested. The parasites utilized were all reared from SPB, *P. dentatus* or *Ips* spp.

RESULTS AND DISCUSSION

Results of the bioassays are shown in Table 1. A total of 134 specimens were run in 264 separate trials. In Figures 2–5, all compounds were treated as having been run in the right channel of the olfactometer with the percentage of

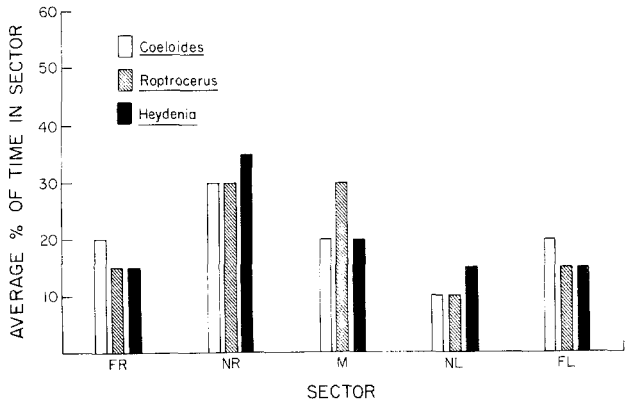

FIG. 3. Percentage of time spent by SPB parasites in bioassay chamber sectors of an H olfactometer when exposed to cedarwood oil. FR, far right; NR, near right; M, middle; NL, near left; FL, far left (see text).

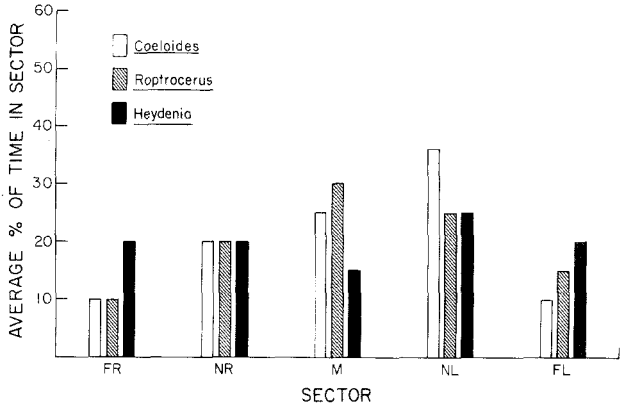

FIG. 4. Percentage of time spent by SPB parasites in bioassay chamber sectors of an H olfactometer when exposed to frontalure. FR, far right; NR, near right; M, middle; NL, near left; FL, far left (see text).

time spent in each sector of the bioassay chamber by the parasite given in regards to the right channel. This was done for simplification of presentation.

The percentage of time spent in each sector given in the table is an average for all the runs performed on that species of parasite. The data for α-pinene were variable from test to test (Figure 2).

The tests which used air drawn over SPB-infested log bolts effectively demonstrated chemotaxis as a possible mechanism of attraction to SPB by the parasites (Figure 5). Parasites reared from SPB showed a marked attraction to the side of the bioassay chamber supplied with air from the infested logs.

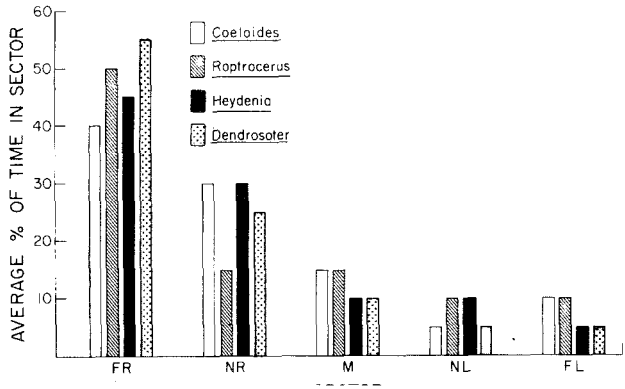

FIG 5. Percentage of time spent by SPB parasites in bioassay chamber sectors of an H olfactometer when exposed to air drawn over infested logs. FR, far right; NR, near right; M, middle; NL, near left; FL, far left (see text).

The "H" olfactometer is a useful tool to help elucidate olfaction as a possible mechanism by which bark beetle parasites locate their hosts. It has the following advantages for this type of study: (1) strong air currents in the test chamber are not necessary for proper operation; (2) a concentration gradient of the tested compound can be maintained; (3) the tested specimens have free movement through the various areas of the test chamber and concentration gradients; (4) the concentration gradient is easily variable; (5) two compounds can be tested at once; and (6) the test chamber is easily removed for cleaning.

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CHEMICALLY MEDIATED INTERACTIONS AMONG JUVENILE MOSSES AS POSSIBLE DETERMINANTS OF THEIR COMMUNITY STRUCTURE

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Abstract—A field study of the distribution patterns of six closely related species of mosses along a complex altitudinal gradient found evidence suggesting differential aggressiveness among juveniles rather than evidence of differential competitive abilities among adults of the species. Work by Bopp (1963, 1968) on the moss phytohormone, factor H, suggests a means by which such interactions might be mediated. An experimental system is proposed through which the effect of patterns of early moss development upon the determination of moss community structure may be assessed.

Key Words—Juvenile interactions, community structure, mosses, Polytrichaccae phytohormones.

INTRODUCTION

Most studies of the distribution patterns of plants in nature have utilized the locations of adult plants within the complex biotope for defining species patterns of distribution (Whittaker, 1967, 1977; Whittaker et al., 1973). While the majority of these studies make clear the differential distribution of species within the biotope (e.g., Werner and Platt, 1976; Parrish and Bazzaz, 1976; Bratton, 1976; Vitt and Slack, 1975; Glime, 1970), in contrast to animal studies (see Hutchinson, 1978, for review), they provide little evidence for the role of exploitation competition in regulating their patterns of distribution.

With the exception of Harper et al. (1965), Ross and Harper (1972), Forcier (1975), and Werner (1976), little attention has been paid to the importance of juvenile interactions in the determination of community structure. Such interactions are expected to be of particular significance in the

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interactions of lower plants and other organisms which are sessile throughout their life and whose juvenile and adult forms have radically different environmental needs and modes of interaction.

A recent study of the differential patterns of distribution of six closely related species of mosses in the family Polytrichaceae, along a complex altitudinal gradient, provided evidence for the differential distribution of the six species along a number of physical habitat parameter axes (Watson, 1980a,c). Little evidence, however, could be found to support a hypothesis of regulation of community structure based upon competition for limiting resources (or habitats) (e.g., Pianka, 1974). Another mechanism by which community structure may be regulated was suggested by the data.

METHODS AND MATERIALS

The distribution patterns of six closely related moss species were studied along a 1600-m altitudinal gradient along the northeast face of Mount Washington, New Hampshire. The species were *Polytrichum commune*, *P. juniperinum*, *P. piliferum*, *Polytrichastrum formosum*, *Pa. pallidisetum*, and *Pa. alpinum*. Nine sites were established at regular intervals along the gradient. A total of 189 subsamples (100 cm^2), chosen at random within each site, were examined to determine species number, species importance, and frequency of reproductive stems. The density of moss stems varied from an average of 400 stems/subsample in the tundra at the top of the mountain to 50 stems/subsample in the forested regions of the mountain. Details of the sampling protocol and analysis may be found in Watson (1979, 1980b).

RESULTS

Each species was found growing throughout the greater part of the altitudinal gradient, although the individual species displayed differences in their precise patterns of distribution (Watson, 1979, 1980a,b). *P. commune* was found in 39 of the subsamples, *P. juniperinum* 45, *P. piliferum* 15, *Pa. formosum* 56, *Pa. pallidisetum* 39, and *Pa. alpinum* 80. Twenty-nine percent of the subsample quadrats contained stems of two or more of the subject species growing together. These subsamples were termed multispecific subsamples (or mixtures), while subsamples which contained stems of just one of the subject mosses were termed monospecific (or pure) subsamples. The frequency at which stems of a particular species were found growing with stems of one or more of the other five closely related species was computed as:

$$\frac{\text{Number of mixed subsamples containing species X}}{\text{Total number of subsamples (i.e., pure + mixed) containing species X}}$$

The frequency of subsamples which contained both male and female stems of a particular species was computed as:

$$\frac{\text{Number of subsamples containing both } \sigma \text{ and } \varphi \text{ stems of species X}}{\text{Total number of subsamples containing species X (as above)}}$$

A positive relationship was observed between the frequency of subsamples containing a given species plus one or more closely related species and the frequency of samples of that same species which contained both male and female stems (Figure 1). The curve saturates quickly for frequency of bisexual samples because these species persist primarily as vegetative specimens and rarely express sexuality. The wide range among species in the proportion of samples in which each was found mixed with one or more of the other five species might simply reflect the habitat tolerances of adults of the individual species. This hypothesis, however, was not supported; for while *P. piliferum*, for example, was narrowly distributed along the transect, *Pa. alpinum* was among the most widely distributed of the species studied (Watson, 1979).

A second hypothesis is that the observed pattern might result from differences among species in the magnitude of spore production, viability, or physiological tolerance. If this were true, a positive relationship would be predicted between the frequency of occurrence of a given species on the mountain and the frequency of occurrence in mixture of that same species. Instead, a negative correlation was observed between the two ($r \approx 0.82$, $P < 0.05$), suggesting that an alternative mechanism must be acting to produce the observed patterns of distribution.

In these dioicous species, both multispecific and bisexual populations must result from the successful establishment of more than one spore within a small area (100 cm^2). This suggests a third hypothesis to explain the observed patterns of distribution—that species differ in their abilities to exclude coestablishing individuals, that is, they exhibit differential aggressiveness.

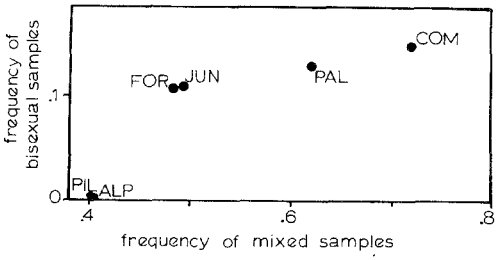

FIG. 1. Relationship between the frequency of samples of a given species, which contain one or more of the five other closely related species (mixed samples) and the frequency of samples of the same species which contain both ♂ and ♀ stems of that species (bisexual samples).

DISCUSSION

The means by which differences in aggressive potential among these moss species may be produced is unclear. Earlier analyses employing demographic methods (Watson, 1979) and formal niche theory (Watson, 1980a,b,c) failed to find evidence of competitive interactions among adults. The data presented above, however, subsequently caused us to focus upon the juvenile stages of moss development.

In 1959 Bopp reported that colonies of the moss *Funaria hygrometrica*, when initiated from individual spores sown at least 1 cm apart on sterile agar, failed to intersect despite the potential of isolated clones to achieve growth of several centimeters in diameter during the same time period. In contrast, clones initiated from individual spores separated by only 3 mm coalesced to form a coordinated developmental unit which behaved as though it were a single protonema. Similarly, colonies which were started from droplet inocula containing many spores behaved as though they were a single clone. Bopp proposed that the maintenance of a clear zone between neighboring colonies or clones did not involve competition for nutrients (see also Klein, 1967), but rather involved the production of a diffusable substance at a particular stage in development which secondarily inhibited lateral growth of both the protonema which synthesized it and neighboring receptive protonemata (Bopp, 1959, 1963). The substance was designated factor H and described as a natural plant product which behaves as a hormone (Klein, 1967). Recent work indicates that factor H may be a complex of substances, at least one of which, when applied to receptive protonemata, appears involved in the inhibition of lateral spread (Bopp and Knoop, 1974). Factor H is also involved in the induction of the normal developmental transition from two-dimensional protonemal growth to three-dimensional bud production and formation of leafy shoots (Bopp and Knoop, 1974). Low concentrations of a number of applied cytokinins mimic the inductive effect of factor H (Bopp, 1968; Spiess et al., 1971, 1972, 1973; Beutelmann and Bauer, 1977; Menon and Lal, 1974).

It is postulated that determination of whether or not two colonies or clones merge or remain distinct depends upon the developmental stage at which they meet. Thus, spatial patterning should reflect, in part, the outcome of interactions occurring among individuals at different points along a time-dependent developmental axis. The nature of the outcome may be expected to influence the ultimate structure of the adult community. A Petri dish system was developed to explore this prediction.

Because of ease in handling and the ability to compare our results with those of Bopp and his coworkers, we chose to use the same moss as they had, *Funaria hygrometrica*. While *F. hygrometrica* differs from the polytrichaceous moss studied above in the details of its developmental pattern, the

response of polytrichaceous moss to exogenously applied growth hormones is similar (Nehlsen, 1979 and personal communication).

Petri Dish System for Examining the Developmental Basis of Juvenile Aggressiveness in Moss

Methods. Moss was grown in sterile, 100-mm Petri dishes, on cellophane disks placed over nutrient agar (Hutner, 1953). Use of cellophane disks allowed for manipulation of the spatial array as an intact unit, as well as providing a means for altering the complexity of the growth medium without interfering with the monitoring of the development of spatial pattern.

The inoculum consisted of droplets containing spores from a single capsule suspended in nutrient medium (10–25 spores per droplet). Droplets were inoculated onto the plates at the points indicated in Figure 2, permitting observation of the interactive behavior of moss colonies which were initiated simultaneously, but which differed in developmental stage at the time of interaction. Spores were obtained from moss capsules which were surface sterilized using a modification of the method of Basile (1972) prior to suspension of the spores in nutrient medium. Moss capsules were collected on the grounds of the Missouri Botanical Garden, St. Louis, Missouri, in September 1975 and stored in vials, at room temperature, until use.

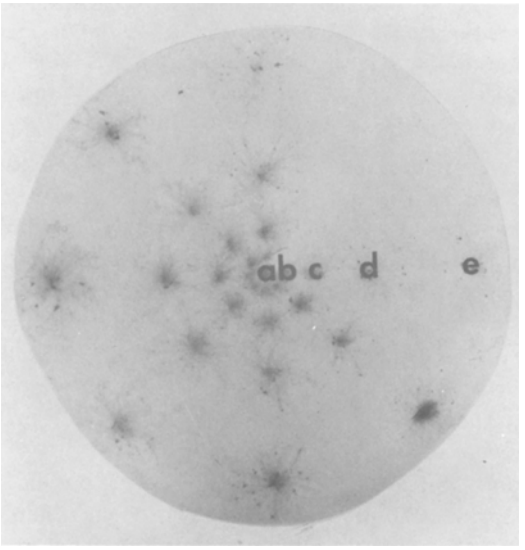

FIG. 2. Light photograph of a 34-day-old culture of *Funaria hygrometrica*. Distance of the rings (lettered a–e) are given from the center of the plate marked by the inoculum at point a; ring b, 3 mm; ring c, 8 mm; ring d, 18 mm; ring e, 38 mm.

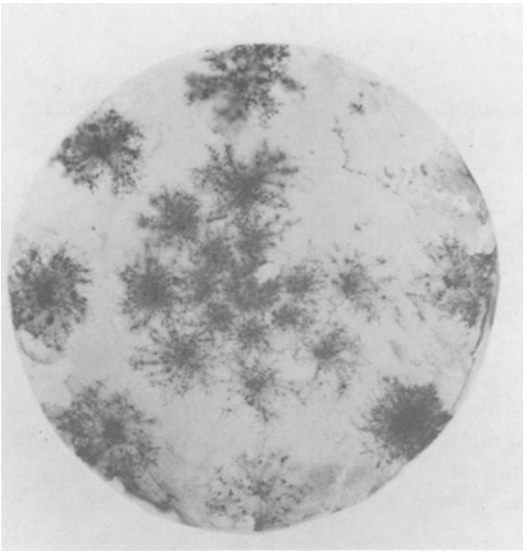

FIG. 3. A 48-hr autoradiographic exposure of the same plate presented in Figure 2, labeled with [^{14}C]TdR. The culture was labeled for 48 hr with [^{14}C]thymidine ([^{14}C]TdR) by transferring the cellophane disk into a Petri dish containing 2 ml [^{14}C]TdR, 2 μCi/ml (55.5 mCi/mM, New England Nuclear No. NEC-468) for 48 hr. Uptake was stopped by transfer of the disk onto 0.2 N NaOH agar plates overnight. The plates were washed free of label by serial passage of the cellophane onto agar plates containing cold TdR, 100 μg/ml. Efficiency of incorporation into the insoluble fraction ranged from 74 to 99%.

The plates were incubated in a growth chamber where they received 16 hr of light at 21°C and 8 hr of dark at 15°C. The substrate was not changed for the entire length of the experiment. Active growth was maintained throughout the observation period first as lateral growth of the filamentous protonemata (Figures 2 and 3), followed by aerial growth of the leafy shoots (Figure 4). Plants gave no indication of exhaustion of food reserves during the course of the experiment in that growth (but not lateral spread) proceeded continuously throughout. During the course of the study, 20–30 plates grown as described, were observed. The photographs presented here are representative of the patterns which were observed.

The spread of protonemal filaments (i.e., the growth of juveniles) could be determined by direct observation (Figure 1) and more detailed microscopic examination. The overall pattern of "community" structure, however, could be more readily visualized by pulse labeling the arrays with [^{14}C]thymidine, from which autoradiographs were prepared.

Results. The use of the format illustrated in Figure 2 ensured that colonies towards the periphery of the plate met at later developmental stages. Under the specified culture conditions colonies in rings a and b tended to meet

and merge early in development. Bud production was observed by day 13. Retardation of lateral growth, however, was not evident until day 20. Colonies in rings c, d, and e approached at later stages of development. The clear zones produced between colonies, in culture, were maintained for periods exceeding $2\frac{1}{2}$ months.

At 34 days, growth had resulted almost exclusively in the production of spreading protonemal filaments (Figures 2 and 3); buds and leafy shoots were evident, but not well developed. At 64 days, colony diameters had not increased significantly (Figure 4), indicating that lateral spread had ceased. Leafy shoot formation, however, was pronounced, evidence of the switch from an essentially two-dimensional to an essentially three-dimensional growth form.

Colonies in ring e were considered as internal noncompetitive controls due to the large area for unrestricted expansion available to them. In most instances these colonies exhibited far greater lateral spread than did any of the other colonies growing in the more internal rings.

Colonies grown alone in 100-mm Petri dishes obtained diameters equivalent to those achieved by the coalescence of all colonies in rings a–c in the experimental system, indicating that in a system in which growth is unrestricted by neighbors, large individual colony sizes may be achieved.

Discussion. Examination of the interactive behavior of colonies (Figures 2 and 3) reveals significant differences along the time-developmental axis.

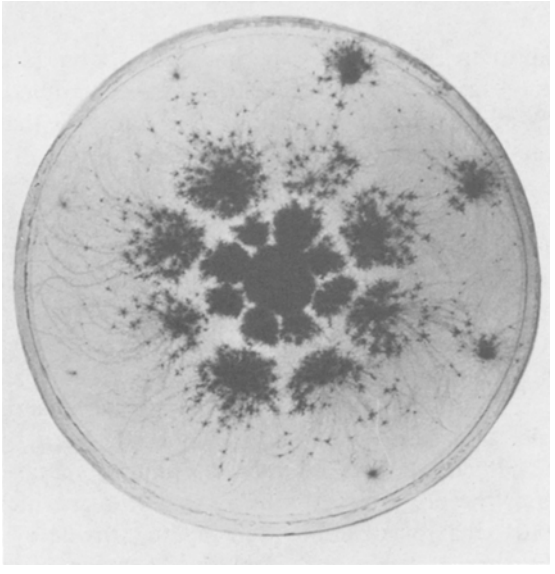

FIG. 4. Light photograph of a 65-day-old culture of *Funaria hygrometrica* initiated and cultured as described in Figure 2.

Colonies in rings a and b tend to meet and merge early in development. In contrast, colonies in rings c and d retain their individual identities. These colonies do not meet until later in development due to the greater distances between them. By this time, it is postulated, sufficient quantities of growth-inhibiting substance(s) have been produced to retard further lateral spread of protonemal filaments. The accumulation of the active substance(s) appears to be gradual. This is suggested in the observation that bud production is observed by day 13, while retardation of lateral growth is not seen until day 20. The lag in growth inhibition is likely due to insufficient accumulation of the active substances early in the time sequence.

The importance of endogenous plant substances in the regulation of plant density, and diversity, depends upon maintenance in the medium of a gradient of these substances which is of sufficient intensity to affect the growth of other plants (of either the same or of different species) within the environment. The establishment of such a chemical gradient within the culture medium is suggested by the asymmetric pattern of bud formation observed in ring d of a 65-day-old culture of *F. hygrometrica* (Figure 4) initiated as previously described. The asymmetric pattern of development suggests a decreasing concentration gradient of growth factor from the center of the plate, where moss biomass is high, to the periphery of the plate, where biomass is low and colonies are more spread out.

CONCLUSIONS

While admittedly most of the experimental work on the growth of moss in axenic culture has dealt with the biology of *F. hygrometrica*, studies by Nehlsen (1979 and personal communication) indicate that *Polytrichum juniperinum*, at least, may be expected to respond in similar ways to the presence of growth substances in its environment. These observations suggest that chemical factors may share similarities among moss taxa which would permit intraclonal, intraspecific, and interspecific regulation of community structure through the action of morphogenic substances held in common among them. Differences among individuals (taxa) in arrival time and susceptibility to leaked chemicals would appear likely to effect the outcome of competitive interactions among them. These observations suggest that the outcome of competition among juveniles may have a critical time component based upon priority of access to a particular habitat site.

If the critical competitive interactions among species occur during juvenile stages of the life cycle, it is likely that studies which focus on the distribution and behavior of adults will fail to provide evidence of such interactions. Further studies of the interactive behaviors of juveniles will be essential to the development of a comprehensive understanding of the

determinants of community structure both of plants and other sessile organisms.

The petri dish system described here may be easily modified to accept substrates of increasing complexity so as to produce experimental systems which bear closer analogy to natural systems.

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RUTTING BEHAVIOR AND ANDROGEN VARIATION IN REINDEER (*Rangifer tarandus* L.)

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Abstract—The rutting behavior of captive reindeer was studied during three rutting seasons. Blood samples from three males were taken for androgen analysis before, during, and after the rut. The amount of androgen increased at the end of August to a peak during late September–early October and decreased thereafter. Flehmen, urinating on the hindlegs, and two different low-stretch displays emerged during the prerut period. The preorbital gland enlarged and secretion occurred at the rim of the pocket. A conspicuous breath odor appeared very abruptly during the most intense period of rutting. A few days later a strong odor also appeared in the urine. The urination behavior and the two odors were confined to a brief period during the rut, while other behavior persisted afterwards.

Key Words—Androgen, breath odor, Cervidae, flehmen, preorbital gland, reindeer, rutting behavior, urine odor.

INTRODUCTION

Wild ungulate breeding and male sex drive are usually restricted to a clearly defined annual rutting period, a condition which is less pronounced in domesticated ungulates (Fraser, 1968). Photoperiod controls the activity of the gonads via the output of gonadotropins from the pituitary. Release of androgen from the testes is essential for the rutting activities of male red deer since castration abolishes all of these activities (Lincoln, 1972). Androgen levels in red deer (Lincoln, 1970), roe deer (Short and Mann, 1967), white-tailed deer (McMillan et al., 1974, Mirarchi et al., 1978), and in black-tailed deer (West and Nordan, 1976) show pronounced annual variations. Except for the long-day breeder roe deer, androgen output characteristically

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increases at the end of summer, a period recognized as the prerut. Peak levels coincide with rut and are then followed by a decrease. Reindeer and caribou show the same pattern but, compared with the above-mentioned species, the rut appears to be shorter (Whitehead and McEwan, 1973).

The increase in androgen concentration coincides with certain morphological, physiological, and behavioral changes. In the reindeer bull, signs of approaching rut include the growth of the mane, neck muscles, and scrotum; velvet is shed from the antlers; and the bulls become more aggressive. A characteristic of ungulates is the emergence of specific behaviors which are not normally performed outside the rutting season. These are of both sexual and aggressive nature. Several studies on reindeer and caribou have described social and sexual behavior (Pruitt, 1960; Espmark, 1964; Bergerud, 1975; Lent, 1965; Müller-Schwarze et al., 1978). During our previous observations on captive reindeer, it was observed that most behavioral changes appeared within a range of a few days in consecutive seasons.

This study describes the temporal relationship between specific rutting behaviors, olfactory cues, and variation in the concentration of androgen. Moreover it was our intention to describe the development of behavioral patterns and their role in social interactions.

METHODS AND MATERIALS

General Methods. Reindeer of the forest type, *Rangifer tarandus L.*, were studied in a 100 × 100-m pen during the rutting seasons of 1976–1978. The group consisted throughout of three adult males, three females, and their calves. Commercial food and water were liberally provided. The social structure of the forest reindeer during rut is quite closed, with a harem bull defending his group of females against subordinate bulls. Thus only one male would freely interact with the females. In order to permit subordinate males to also interact with females, each of these males were separated from the group for 15 min, together with a female and her calf, in an adjacent pen, where detailed observations were performed. After 15 min of observation ($N = 54$), they were reunited with the group. The existence, onset, duration, and termination of rutting behavior were noted.

Determination of social rank was based upon observations of agonistic encounters. As the reindeer were used to humans, close observation was possible without disturbing them.

From spring 1978 onwards, monthly blood samples were taken from the adult males. During the rutting period, blood samples were taken more frequently. The reindeer were caught by lasso, and 5–10 ml of blood were taken for androgen analysis. Plasma testosterone concentration was measured by radioimmunoassay (Damber and Jansson, 1978).

Behaviors Studied. An aroused bull rapidly tramps with his hindlegs while urinating. The tramping continues until the hindlegs are near the forelegs. He often shifts the hindlegs and urinates small quantities of strong smelling urine on the anterior metatarsi. Espmark (1964) termed this display "tramping and urinating" (t-u).

Flehmen or lip curl is a common habit in ungulates (Estes, 1972) and has previously been described on several occasions for *Rangifer*. The reindeer bull

FIG. 1. A reindeer bull urinating on his crossed hindlegs, as part of tramping and urinating behavior.

licks the urine from the ground or directly from the female as she urinates. After licking it, he curls his upper lip thereby wrinkling his nose. The head is raised and slightly stretched forward.

The reindeer bull, in common with other ungulates, performs low-stretch displays (Lent, 1975). One display involves rapid panting (Bergerud, 1975) when the bull approaches a conspecific in a low-stretch position. A functionally different behavior, tongue-flicking (t-fl), consists of a similar low-stretch approach, but at the end of the approach the bull rapidly flicks his tongue in and out striking it against his palate. This behavior is accompanied by rapid sniffing sounds.

RESULTS

Behavioral Patterns and Sequences. Urination and t-u are, during the rutting season, associated with aggressive acts (Figures 1 and 2). During the 1976 rut, t-u was the prerogative of the dominant bull, and observations in later years confirmed the association between high social rank and this type of behavior. In the dominant male, t-u was often preceded by an aggressive act and followed by the neutral acts of walking or standing. Subordinate males were often subjected to aggression, and urination often occurred as a response to this (Figure 2). Calves and females were, in this context, socially inert groups.

The two types of low-stretch displays are very similar regarding the overall locomotory pattern. Also the typical withdrawal of the muzzle is a common feature, although it was more pronounced in actual t-fl. Panting often progressed into the t-fl sequence, although the reverse situation was not observed. Panting was often performed at a distance and in association with

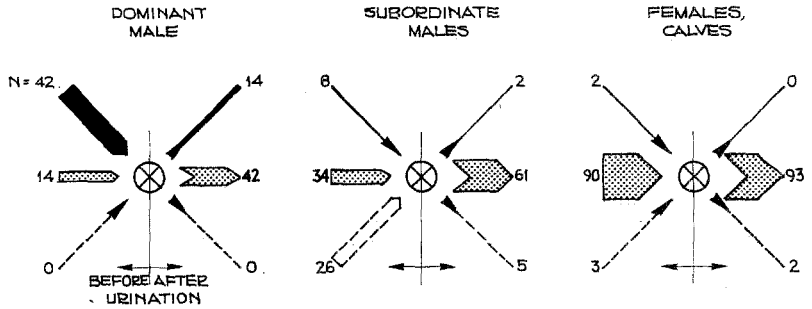

FIG. 2. Behavioral sequences immediately preceding and following urination in male reindeer. Upper solid arrows indicate that the male performs an aggressive act and the lower dotted arrows indicate that the male is being subjected to an aggressive act. Horizontal line indicates walking or standing.

TABLE 1. OBSERVATIONS OF DIFFERENT LOW-STRETCH DISPLAYS AND THEIR DIRECTIONS

	Directed towards						
Behaviour	Distant Animals	Calf	Female at Distant	Posterior part	Head and neck	Anogenital area	Σ
Panting	31	5	51				87
Panting–tongue-flicking	3	12	26	17	3	8	69
Tongue-flicking	2	58	60	36	9	26	191

situations such as chasing or herding. T-fl was performed near the female and was often directed towards her rear, sometimes terminating in a sniffing or licking of her anogenital area (Table 1). The preorbital gland widened during panting, while the nonaggressive nature of t-fl is suggested by the more-or-less closed preorbital pocket and the often averted antlers (Figure 3). After a low-stretch display, the bull undertook a brief but distinct sniffing of the

FIG. 3. A reindeer bull performing tongue-flicking, a low-stretch approach directed towards females. Note the closed preorbital pocket.

TABLE 2. BEHAVIOR IMMEDIATELY PRECEDING AND FOLLOWING PANTING, PANTING–TONGUE-FLICKING, AND TONGUE-FLICKING[a]

Behavior	Panting		Panting–tongue-flicking		Tongue-flicking	
Chasing, herding	6	10	5	4	2	4
Panting, tongue flicking	3	3	5	7	7	3
Sniffing the ground	2	21	6	52	8	48
Standing, walking	69	32	93	36	104	59
Tramping and urinating	—	9	2	12	1	8

[a] Data are derived from 15-min observations ($N = 54$) of a male together with one female and her calf in a separate pen.

ground. Neutral acts, such as walking or standing, preceded the displays (Table 2). This sniffing of the ground was also frequent during threat displays such as t-u or after flehmen. Flehmen (Figure 4) was usually elicited by female urine although other stimuli occasionally elicited it. The nature of the source is important in determining the behavioral response. The intensity of the behavior, as measured from licking the urine to the distinct masticatory

FIG. 4. A reindeer bull performing flehmen.

TABLE 3. DURATION OF FLEHMEN IN REINDEER ELICITED BY DIFFERENT SOURCES AND AT DIFFERENT TIMES

	N	Duration of flehmen (sec)
Female urine		
September 22–27	19	27.1 ± 1.4
October 3–7	21	33.2 ± 2.0
Calf urine	5	16.0 ± 2.4
Other objects	30	20.0 ± 1.5

movements terminating it, varies depending on what elicits it (Table 3). Its duration is significantly longer after sniffing fresh female urine than urine from calves ($P < 0.01$, Mann-Whitney U test), or undefined objects such as feces or old urine spots ($P = 0.01$). It was also shorter before copulation was observed (September 22–27) than in the following period (October 3–7), ($P < 0.05$).

Olfactory Signals and Skin Glands. The breath of the reindeer is normally odorless to a human being; a slight change of odor can be detected during the prerut, coincides with the appearance of the flehmen behavior, and may result from licking female urine. A more drastic change, readily discerned from one day to the next, occurred in late September. This odor onset was very abrupt, while the decrease was less pronounced. The odor can be detected in both the breath and in the saliva.

A few days later, a corresponding change in the odor of the urine appeared. This change was very abrupt and in the case of male A in September 1977 it occurred between 10 AM and 2 PM, but, as with the breath odor, the decrease was less distinct. The sharp odor was spread to the frontal parts of the hindlegs by the frequent t-u behavior.

The preorbital pocket was widened during all aggressive acts, and a white area was exposed at the anterior of the eye. This was seen in all seasons and in both sexes, although most frequently during the rut. An external proliferation of the preorbital gland of the males was seen in early September. The hairs of the anterior rim of the pocket were covered with a greasy secretion. In October, after the rut, the size of the gland decreased, but fat deposits remained for some time (Figure 5). Males M and G had more developed glands than male Q.

Development of Behavioral Patterns and Olfactory Signals. The appearance of behaviors and olfactory signals followed a similar temporal pattern both between individuals and between the two rutting periods studied (Figure 5). The first behavioral changes noted were the increase in different agonistic patterns that are not specific to the rutting period, followed by the

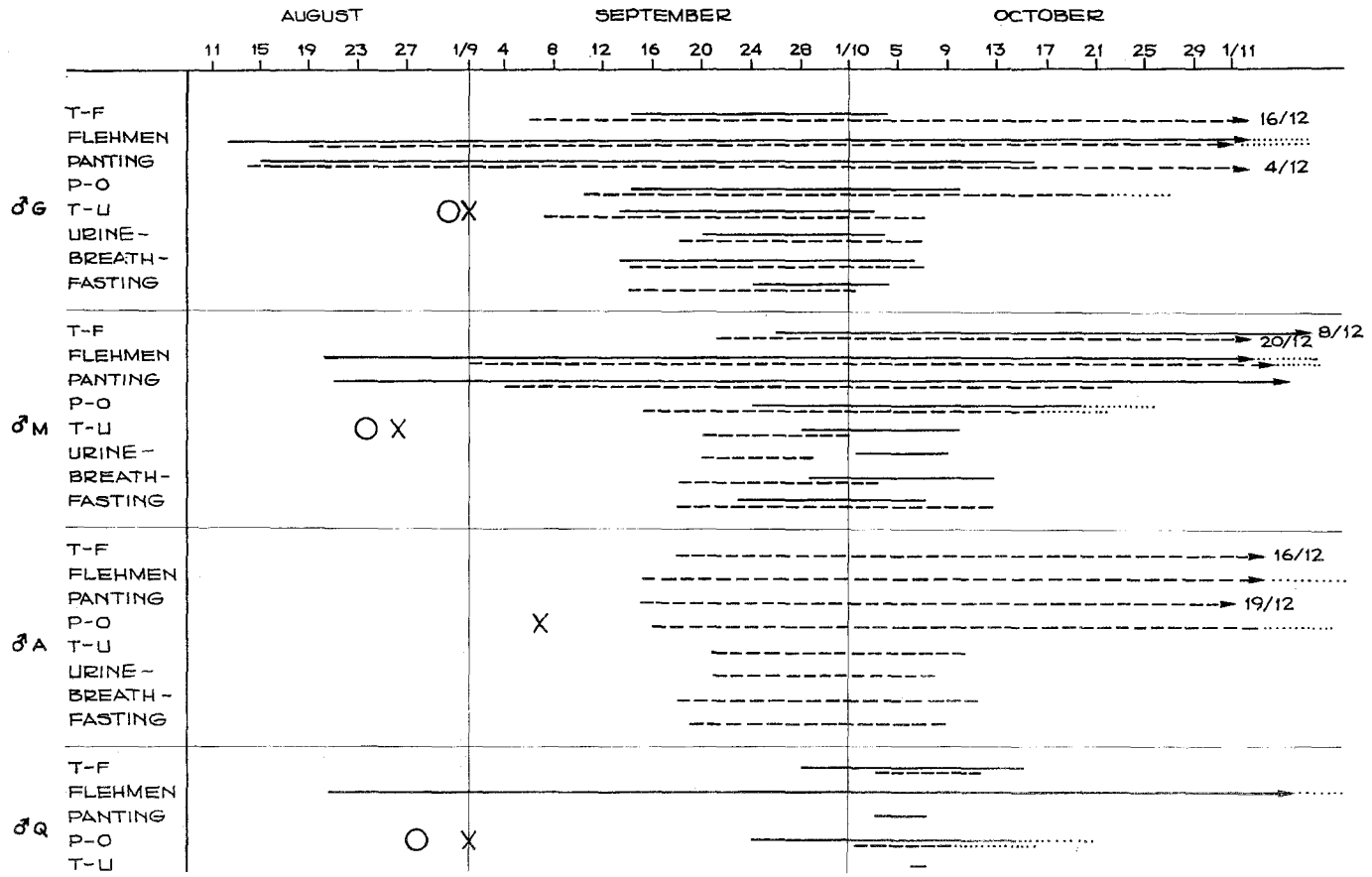

FIG. 5. The occurrence of different rutting behaviors in male reindeer: tongue-flicking (T-F), flehmen, secretion on the rim of the preorbital pocket (P-O), panting, tramping and urinating (T-U), urine odor, breath odor, and fasting. Solid line 1978, broken in 1977. O and X are the dates when velvet was shed in 1977 and 1978, respectively.

development of the agonistic low-stretch vocalizing behavior (panting). Panting was performed by the older males long after the rutting season was over. Low-stretch tongue-flicking usually appeared several weeks after panting appeared. Tramping and urinating were observed for a short period during the most intense rut. The young bull Q was only once seen to perform a complete t-u (in October 1978).

The first sign of sexual interest was an increased frequency of sniffing and licking of the female anogenital area in early August. During summer, flehmen was absent and was first observed in late August. It was most frequent in September and was performed continuously throughout the winter. As a rule, the odor in the breath appeared earlier than that in the urine (3.8 ± 0.9 days, $X \pm$ SE, $N = 5$) and remained longer ($\bar{X} = 2.4 \pm 0.9$ days, $N = 5$). When approaching rut, food intake was progressively reduced and finally stopped. The fasting period (Figure 4), defined as the period the bulls were not observed to eat commercial food, mainly coincided with the periods when the characteristic urine and breath odors occurred.

Social Structure and Dominance. The male hierarchy in 1977 was constantly G > M > A > Q. Male A was slaughtered in July 1978. During the following rut male G dominated the other males up to October. He then showed signs of having been injured and became lower in rank. Copulations were observed on September 27, 28, and 29, 1977. Copulations were not observed during the following rut, but examination of the external genitalia indicated that all females had been bred before September 30.

Androgen Variation. The output of androgen during spring and early summer was low and constantly about 0.5–1.0 ng/ml (Figure 6). The increase in androgen output started in all three mature males at the beginning of August and attained peak levels in late September. Androgen levels declined after the rut and, shortly before shedding of antlers (♂ M December 13–16; ♂ Q February 13–20), output had reached its basal level. The peak in male M occurred somewhat later compared with males G and Q and was also considerably higher, although the androgen maxima for males G and Q could have exceeded those of the two latest September samples. Data from November and beyond were inadequate but suggest that a small second peak occurred in mid-November.

Effect of Androgen Treatment. In February 1977, 1 g testosterone propionate (in oil) was implanted in male Q. No behavioral response was noted. Male Q was castrated February 13, 1979. Testosterone enantate was injected intramuscularly to male Q in quantities of 250, 500, and 500 mg on March 13, 19, and 27, respectively. Although antlers were shed in February, which normally leads to a subordinate position, he was very aggressive compared with the other reindeer and with his own behavior the preceding winter. At the beginning of April, he was seen tramping and urinating on his

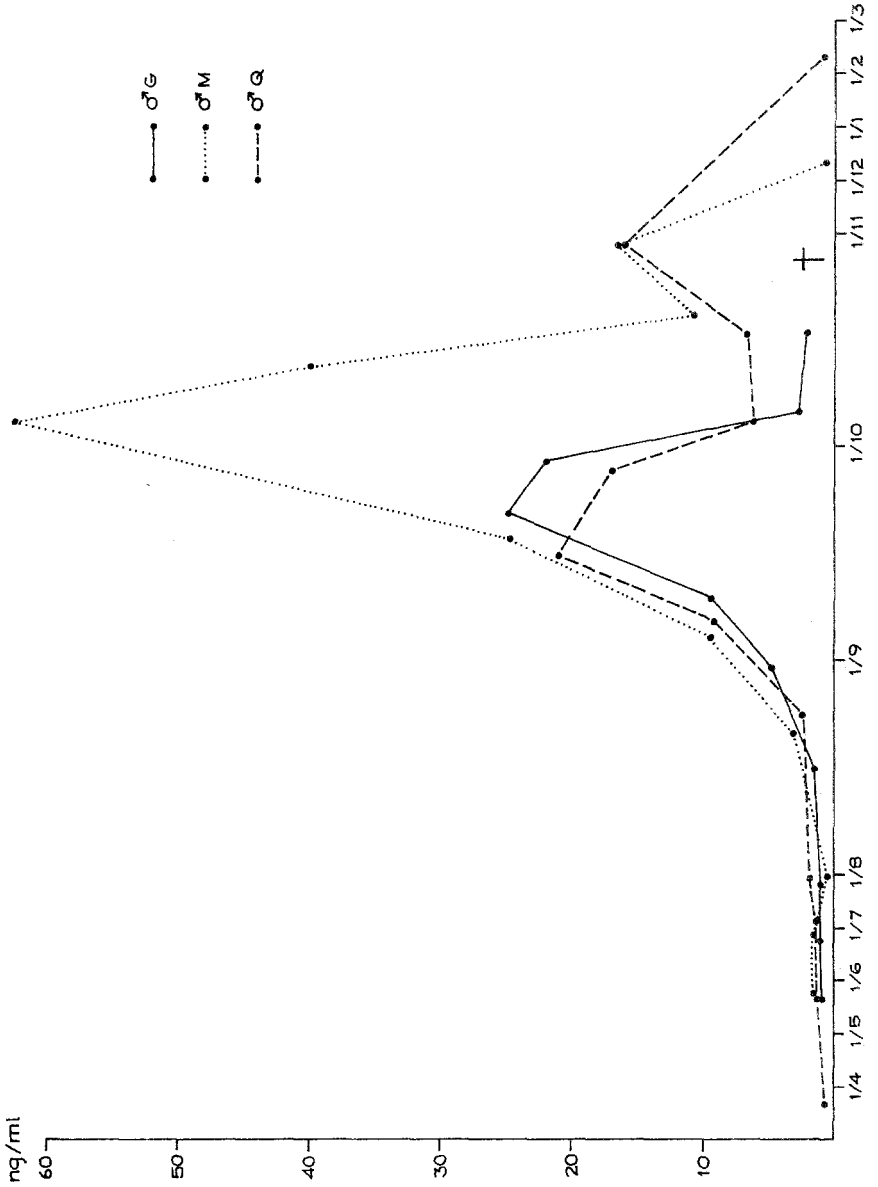

FIG. 6. Variations in amounts of testosterone in three adult reindeer bulls during the rutting period in 1978.

hindlegs (t-u). Apart from this and a high frequency of other aggressive displays, no other behavioral activity associated with rutting was observed in male Q.

DISCUSSION

The results of this study suggest that the behavior of the rutting bull is influenced by several interacting factors, although photoperiod is the most important controlling factor for total reproductive behavior. Its regulatory effect upon the gonads in Cervidae is described by Lincoln (1977), Sanford et al. (1978), and West and Nordan (1978). The sequence of events observed during consecutive rutting seasons, and the concurrent onset of androgen production in 1978, indicate strict temporal regulation of external factors. Subsequent hormonal output and behavioral development then depends on other factors. Mutual behavioral and physiological stimulation between the sexes and the supression of subordinates are probably such factors.

Androgen affects the animal in many ways, but the most striking effect is an enhanced general social aggressiveness. In the reindeer bull, the ability to perform sexual or aggressive behavior is clearly not dependent on the androgen level per se. The behavior examined was, to a varying extent, restricted to the rutting period. Low-stretch displays, and especially flehmen, persisted for long periods; in some cases, these took place after the antlers had been shed. The performance of tramping and urinating is obviously related to an elevated level of androgen and aggression, since this behavior could also be induced by the injection of large quantities of androgen.

The development of the physiological characters was confined to periods when there were relatively high levels of androgen. Widening of the preorbital pocket in agonistic situations was not restricted to the rutting period, although the secretion was only seen for a short time during rut. This secretion consists of low-volatile fats and high-volatile ketones (Andersson, 1977); the latter are also found in the urine at this time (Brundin, 1977). The secretory epithelium is most developed in intact males during rut (Mossing and Källquist, in preparation), indicating an androgen dependency. Urine and salivary odors were confined to the peak period of androgen production and during its decline. It thus appears, in the case of the various types of behavior, that androgen acts by inducing behavioral and physiological changes which can then persist more or less independently of the actual hormonal level.

Throughout the rut, variations in the frequency and intensity of the various behaviors were observed. Unlike urine and breath odors, the different behaviors slowly developed from imperfect to more perfectly performed motor patterns. The degree of motivation and the nature of the stimuli appear to determine the behavioral output. This is demonstrated by variation

in the duration of flehmen which is presumably dependent on the degree of excitement. Flehmen is common in ungulates, and its association with the vomeronasal organ is generally accepted (Estes, 1972). Although both sexes of reindeer have anatomically and histologically similar vomeronasal organs (unpublished results), flehmen is mainly restricted to mature males.

Smell is very important in the social life of reindeer (Müller-Schwarze et al., 1979), especially during the rut. Urine, in particular, is important in the social life of mammals and is frequently used in the rutting behavior of Cervidae (Grau, 1976). The strong odor of urine from a rutting bull is distributed to his hindlegs during tramping and urinating behavior. Thus, it is a self-marking display. The motor pattern itself is associated with aggression and dominance, and the odor is most likely a part of this. The tramping and urinating of the reindeer bull can be compared to the use of wallowing pits by moose and elk (deVos, 1967) and the urinating of Père David's deer (Shaller, 1978). These types of behaviors also result in self-marking with urine. The conspicuous odor of the breath in reindeer may serve a function similar to the odoriferous substance emitted via the submaxillary gland of the boar. This odor, which is of steroid nature, is panted into the face of the sow, causing her to more readily assume a proper mating stance (Signoret, 1970). Since the appearance and disappearance of odors in the breath and urine of rutting reindeer occur in a certain relation to each other, it is probable that they are caused by the same physiological process although their origins and chemical structures may differ. These odors are more or less constantly emitted from the rutting bull, which makes him an olfactory signpost. Self-marking may be necessary since reindeer do not possess territories in the strictest sense of the term but are constantly on the move. It is difficult to define the exact behavioral functions of the odors, but they may act as attractants to females and as hostile signals to other males.

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FEEDING PREFERENCES OF SPRUCE BUDWORM (*Choristoneura fumiferana* Clem.)[1] LARVAE TO SOME HOST-PLANT CHEMICALS[2]

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Abstract—Single-choice and three-choice tests were used to determine the relative importance of host-plant chemical extracts in eliciting feeding by spruce budworm larvae. Water-soluble components of the host trees are the most important and, of these, the sugar and glycoside fractions are the most stimulating. The amino acid and organic base fractions have no apparent effect. The organic acid fractions deter feeding slightly.

Key Words—*Choristoneura fumiferana*, *Lepidoptera*, *Tortricidae*, *Abies balsamea*, feeding stimulants, feeding deterrents, feeding preference, sugars, glycosides, amino acids, organic acids.

INTRODUCTION

The eastern spruce budworm, *Choristoneura fumiferana*, is a lepidopterous pest which feeds on various species of fir and spruce in North American forests (Morris, 1963). The second-instar larva, after overwintering in diapause, begins to feed on the developing buds or newly opened shoots of the host tree in early May. In eastern Canada, balsam fir (*Abies balsamea*) is the preferred species, followed by white spruce (*Picea glauca*), red spruce (*Picea rubens*), and black spruce (*Picea mariana*). This preference may in part be related to the host-tree phenology, which coincides with the emergence of the larva from its hibernaculum in early May. Balsam fir budburst occurs first, about four days prior to that of white spruce, and some thirteen days before that of red

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and black spruces. Black spruce remains relatively untouched, probably due to its late bud opening (Swaine and Craighead, 1924; Blais, 1957).

Physical stimuli such as light, temperature, and humidity are important in modifying the budworm's orientation to a suitable feeding site (Wellington, 1948, 1949, 1950). Subsequent feeding activity presumably must rely on the sensory information supplied to the larva's chemosensilla from the host-plant chemicals (Heron, 1965; Albert, 1980). The levels of various chemicals in a given tree or tree species may account for their preference to larvae. Balsam fir, for example, has higher levels of amino acids than white spruce (Kimmins, 1971). Heron (1965) showed that certain amino acids, either alone or in combination with sucrose, appeared to act as phagostimulants for spruce budworm larvae.

It is commonly known that insects are capable of perceiving a wide variety of chemical substances from plants. The evolution of this insect–host-plant interaction as it applies to feeding deterrents has been reviewed recently by Dethier (1980). Barbosa et al. (1979) studied the host-plant preferences of gypsy moth larvae, and studies of this type have provided valuable information on the behavioral and phytochemical bases of feeding preferences in leaf-feeding insects.

The purpose of the present study is to determine which host-plant chemicals act as phagostimulants to spruce budworm larvae and to test the relative acceptability of extracts from various species of evergreens.

METHODS AND MATERIALS

Experimental Animals

The animals were obtained as second-instar larvae from the Maritimes Forest Research Center, Fredericton, Canada. They were reared in the laboratory on an artificial diet (McMorran, 1965). For the single-choice tests, penultimate (fifth) instar larvae ranging in weight from 25 to 70 mg were used. They were starved for 6 hr prior to testing. For the three-choice tests, fifth-instar larvae $\leq$1 day old were starved for 24 hr before testing. It was determined from a series of two-choice tests that feeding preferences remained the same for animals starved for 3, 6, 21, and 24 hr. The longer the animals were starved, the more quickly they completed the tests.

Bioassay

Single-Choice Tests. The basic medium into which the test chemicals were incorporated was prepared from a mixture of 2% agar-cellulose in 0.08M NaCl. The NaCl was used so that the results of the present experiments could

be correlated to future electrophysiological experiments on individual taste sensilla, in which the salt would serve as the electrolyte. Test media were prepared 6 hr prior to testing.

Following mixing, 6 ml of the mixture were poured into small glass containers and allowed to cool. At the time of testing, cardboard lids were used to seal the containers which were placed in a 22° C incubator for the duration of the 48-hr test period. A single larva was placed in each test container. Tests were done in replicates of eight. Any larva which molted before conclusion of the test was discarded.

Five sugars were tested at final concentrations in the diet of 0.10 and 0.50 M. Twelve DL-amino acids were tested at 0.02 and 0.10 M. Plant extracts were tested at 20% v/v concentrations (volume extracted–volume of diet mixture). Media containing 0.08 M NaCl alone served as controls. Total frass production during the 48-hr test period was used as a measure of the amount of feeding (Ma, 1972). The frass pellets were oven-dried for 12 hr at 90° C, and then weighed.

Three-Choice Tests. This choice test was modified after Jermy et al. (1968). Animals were tested using 6.5-mm-diam feeding disks punched from Millipore HA 0.45-μm cellulose filters. Disks were placed on pins 2 mm above the floor of a styrofoam sheet and arranged to form a circle in an a b c, a b c, etc., fashion. Each circle consisted of 9 disks, three for each of three different test chemicals, and covered with a 3.5-cm-diam by 1-cm-high Petri dish. The disks were wetted with 15-μm aliquots of the chemical or extract solution to be tested. Disks treated with deionized water alone served as controls. Animals which ate $<50\%$ or $>250\%$ of the total disk area (max. 300% possible) for the preferred group of disks were discarded.

Tests averaged about 25 hr duration.

Plant Collection and Extraction

Single-Choice Tests. Foliage samples of balsam fir (*Abies balsamea* (L.) Mill.), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco), Norway spruce (*Picea abies* (L.) Karst.), and Red spruce (*Picea rubens* Sarg.) were collected in late October 1974 from the MacDonald College Arboretum, Ste-Anne-de-Bellevue, Quebec. Samples of eastern white pine (*Pinus strobus* L.) were collected at Lost River, Quebec, in early November 1974. All samples were stored at −18° C. Needles from the distal portion of the branches (current year's growth) were used for extraction. They were oven-dried, and the various water, 80% ethanol, and petroleum ether extracts were prepared as follows, using a modified version of the method described by Davis (1961).

Twenty-eight grams of dry needles were boiled for 30 min in 300 ml of dist. H_2O. The resulting solution was filtered and the filtrate concentrated by vacuum evaporation to a final volume of 100 ml. This was termed the water

extract. The plant residue from this extraction was boiled for 15 min in 200 ml of 80% ethanol, the solution was filtered, and the filtrate concentrated to a final volume of 25 ml. Petroleum ether was added to the concentrated ethanol extract in a separatory funnel, and phasing was allowed to proceed overnight. The ethanol phase was removed and brought to a final volume of 50 ml. This was termed the 80% ethanol extract. The petroleum ether phase was added to the plant residue from the 80% ethanol extraction, in addition to a further 250 ml of petroleum ether. This was allowed to stand overnight, filtered, and concentrated to a final volume of 75 ml. This was termed the petroleum ether extract. The plant residue from this extraction was discarded.

Three-Choice Tests. Foliage samples of balsam fir were collected from Harrington, Quebec, in mid-July 1978. Current and 2-year-old needles were removed from the stems and freeze-dried within 4 hr of collecting. They were stored at −18° C over Drierite and subsequently extracted as follows, using a method supplied by Dr. E.W. Underhill, NRC Prairie Regional Lab, Saskatoon, Canada.

Twenty grams freeze-dried needles were boiled 10 min in 200 ml 90% methanol. The solution was filtered, and the residue was ground in a blender with 50 ml 80% methanol, and boiled 10 min after adding a further 150 ml 80% methanol. After filtering, the needles were again boiled 10 min in a further 200 ml 80% methanol, and filtered again. All methanol extracts were combined and concentrated. The residue was discarded.

The methanol extracts were separated with chloroform into an aqueous and organic fraction. The aqueous fraction was passed through an Amberlite IR-120 (H^+) resin column, which was washed with dist. H_2O until pH 4–5. The effluent and washings were saved. The column was eluted with 1.5N NH_4OH until the pH became basic, then eluted with two more column volumes of NH_4OH. The eluate and washings were concentrated to yield the fraction containing amino acids and bases. The effluent and washings were passed through an Amberlite IR-4B (OH^-) resin column, then washed with 2–3 column volumes of dist. H_2O. The effluent and washings were combined and concentrated; this fraction contained the sugars and glycosides. The column was eluted with 1 N H_2SO_4. At pH 1, one more column volume of 1 N H_2SO_4 was added. The eluate and washings were treated with $Ba(OH)_2$ to precipitate the SO_4^{2-}. The solution was filtered and passed through a fresh IR-120 (H^+) column. The free-acid effluent and washings were combined and concentrated. This fraction contained the organic acids.

All fractions were evaporated to dryness and stored at −18° C over Drierite for later use in behavioral tests. The following amounts of material were recovered from each gram of freeze-dried needles (f.d.n.): (1) amino acids–bases, current needles, 6.87 mg; (2) amino acids–bases, 2-yr-old needles, 7.35 mg; (3) sugars–glycosides, current needles, 218.20 mg; (4) sugars–

glycosides, 2-yr-old needles, 191.05 mg; (5) organic acids, current needles, 35.37 mg; (6) organic acids, 2-yr-old needles, 19.03 mg.

A small portion (15.58 mg/g f.d.n., or 7.1%) of the material recovered in the sugar–glycoside fraction from the current year's needles was insoluble in 100% methanol. This was filtered and dissolved in deionized H_2O to be tested as a separate fraction. All extracts were dissolved in deionized H_2O for use in behavioral tests.

Statistical Analysis

Single-Choice Tests. Results of these tests were analyzed using a multiple comparisons test (Sokal and Rohlf, 1969).

Three-Choice Tests. The Scheffe test for significance between means was performed on the results of these experiments (Bruning and Kintz, 1977).

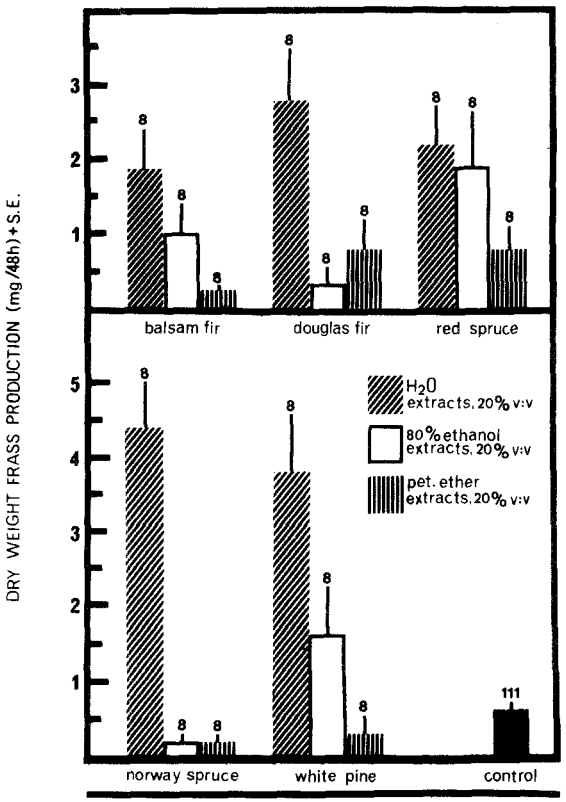

FIG. 1. Dry weight of frass (mg ± SE) produced by larvae reared on diets containing various tree extracts. Numbers above histograms = N animals. Multiple comparisons: H_2O vs. total extracts. Significant difference between Norway spruce and balsam fir. No other significant differences ($P = 0.05$).

RESULTS AND DISCUSSION

Single-Choice Tests

An initial step in this investigation was to test the relative acceptability of several evergreen tree extracts (Figure 1).

Petroleum Ether Extracts. Larvae given any of the media treated with the various petroleum ether extracts did not feed more than those on the control media. This confirms previous evidence that the lipid components of the needles do not play an important role in determining preference for host-plant species (Heron, 1965).

Ethanol Extracts. When treated as a group, the 80% ethanol extracts were more effective than the petroleum ether extracts in stimulating feeding ($P < 0.05$). The responses to these extracts, however, were lower than those for the water extracts.

Water extracts. These extracts showed a higher feeding response than the petroleum ether or the ethanol extracts ($P < 0.001$). Significant differences in feeding stimulation were found among the aqueous extracts of Norway spruce, white pine, red spruce, and Douglas fir. Balsam fir, although most preferred in nature, was not the most preferred species in these tests. This may be due to the time of year when the foliage was collected (October), when the nutrient levels may have been lower than those of other evergreen species.

Spruce budworm attack on white pine trees has been observed, but only on rare occasions (Bean and Waters, 1972). This may be the result of physical rather than chemical factors, since larvae showed some preference for the water extract of this species. Needles on this tree are very long and slender compared to fir and spruce needles, and they may not be suitable for the proper establishment of feeding sites by young larvae.

Among the numerous compounds which can be extracted from plant tissues with water are two principal groups, amino acids and sugars. These two groups of organic compounds occur in appreciable quantities in plant tissues and are known to stimulate feeding by phytophagous insects (e.g., Heron, 1965; Ma, 1972; Mitchell, 1974). The effects of some pure amino acids and sugars were thus investigated. The amino acids could be sorted into two distinct groups, one group evoking significantly greater responses than the other. The group consisting of 0.02 and 0.10 M glutamic acid, 0.10 M proline, 0.02 M citrulline, and 0.02 and 0.10 M arginine did not elicit responses significantly different from each other, but they each differed from any member of the other group (Figure 2).

Proline was one of the most stimulating amino acids. It has also been found to be important for other insects (Ma, 1972; Mitchell, 1974) as well as for the spruce budworm (Heron, 1965). The members of the higher response-evoking group are those same amino acids which occur at the highest concentration in

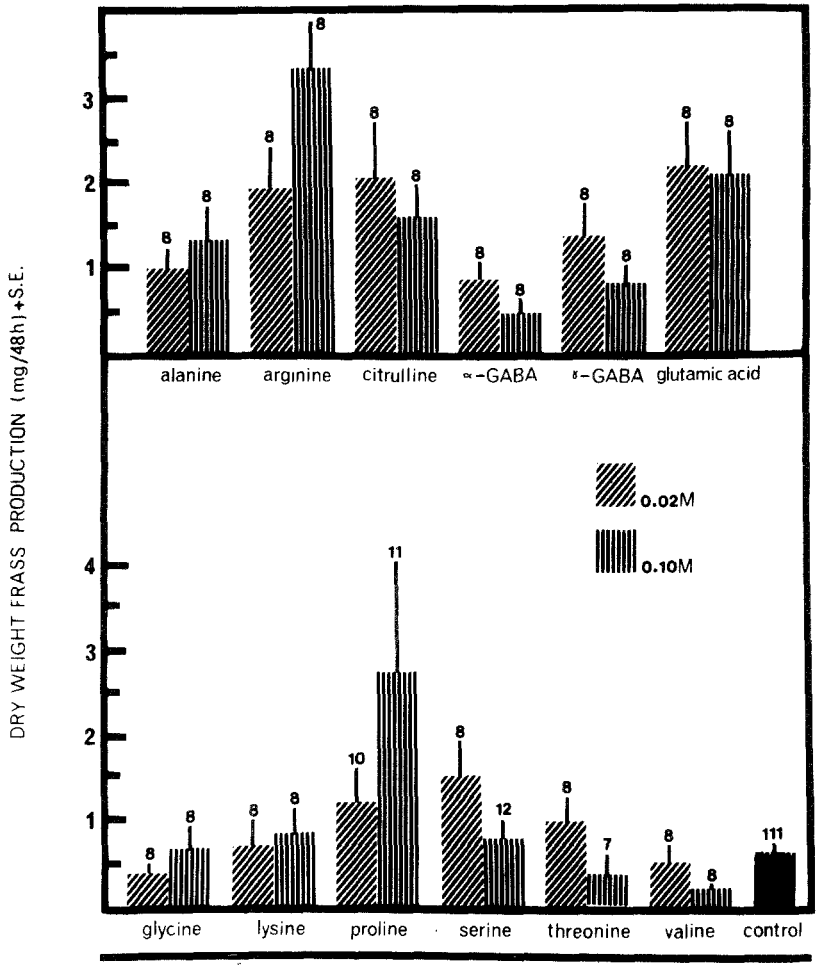

FIG. 2. Dry weight of frass (mg ± SE) produced by larvae reared on diets containing various DL-amino acids. Numbers above histograms = N animals. Multiple comparisons among six amino acids having the highest means (0.1 M arginine, 0.1 M proline, 0.02 M glutamic acid, 0.1 M glutamic acid, 0.02 M citrulline, and 0.02 M arginine). No significant differences ($P = 0.05$).

white spruce foliage at the time of budworm emergence in early May. They are also found in even higher levels in balsam fir (Kimmins, 1971).

Sugars were more stimulating than any of the extracts or the pure amino acids (Figure 3). Sucrose, glucose, and fructose were all found to be stimulatory to other insects (Ma, 1972) as well as sucrose and fructose to the spruce budworm (Heron, 1965).

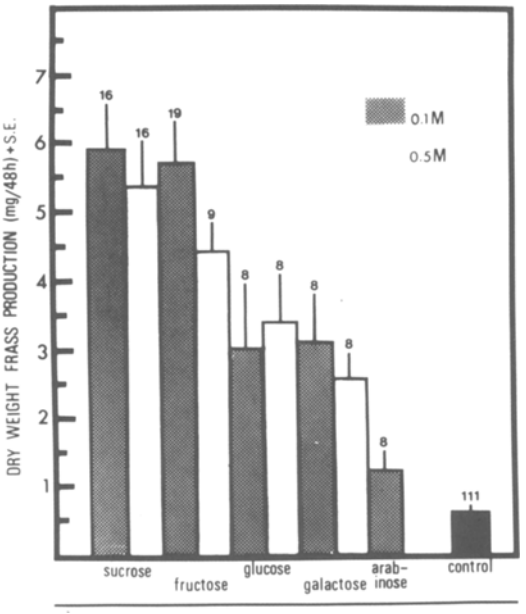

FIG. 3. Dry weight of frass (mg ± SE) produced by larvae reared on diets containing various sugars. Numbers above histograms = N animals. Multiple comparisons among all sugars. Significant differences between 0.1 M sucrose and 0.1 M arabinose; 0.1 M fructose and 0.1 M arabinose; 0.1 M sucrose and 0.5 galactose; 0.5 M sucrose and 0.1 M arabinose; 0.1 M fructose and 0.5 M galactose. No other significant differences ($P = 0.05$).

Three-Choice Tests

The water extracts of the various evergreen species were shown to contain the most stimulating chemicals in single-choice feeding tests. In order to study the role of these chemicals in more detail, samples of balsam fir, the spruce budworm's most common host plant in eastern Canada and the U.S., were extracted and purified by column chromatography for further behavioral testing. Three-choice tests were chosen for this experiment since they proved to be more sensitive than the single-choice tests for budworm larvae. In each test, deionized H_2O was used as the control so that the data from individual tests could be normalized to a common H_2O control. With the extracts prepared from current year's growth and one-year-old needles, 21 possible three-choice test combinations were performed. Results are presented in Table 1 and Figure 4.

It is apparent that the sugars–glycosides fraction is by far the most stimulating, especially from current year's needles. This confirms the results found in the single-choice tests.

TABLE 1. MEAN PERCENT CONSUMPTION ± SE OF DISKS TREATED WITH BALSAM FIR EXTRACTS IN THREE-CHOICE TESTS[a]

		Mean Consumption ± SE						
Test No.	N	A Control (deion. H_2O)	B Extract			C Extract		
1	20	34.6 ± 5.1	1AA	39.3 ± 5.2	—[b]	1OA 26.1 ± 3.3	—	O[c]
2	21	11.2 ± 3.3	1AA	7.9 ± 2.1	—	$1S_1$ 80.9 ± 4.5	*	X
3	20	24.8 ± 4.3	1AA	17.9 ± 3.0	—	$1S_2$ 57.3 ± 5.1	*	X
4	20	33.2 ± 4.6	1AA	29.0 ± 4.8	—	2AA 37.8 ± 5.2	—	O
5	23	41.7 ± 4.5	1AA	37.0 ± 4.5	—	2OA 21.3 ± 4.1	*	X
6	23	12.1 ± 2.7	1AA	10.4 ± 1.8	—	2S 77.5 ± 3.7	*	X
7	20	6.2 ± 1.9	1OA	10.0 ± 2.2	—	$1S_1$ 83.8 ± 3.2	*	X
8	23	19.3 ± 2.5	1OA	16.0 ± 3.6	—	$1S_2$ 64.6 ± 4.1	*	X
9	20	39.8 ± 4.3	1OA	29.2 ± 4.1	—	2AA 31.0 ± 3.5	—	O
10	21	43.1 ± 4.6	1OA	22.1 ± 3.2	*	2OA 34.8 ± 4.0	—	O
11	20	10.8 ± 1.8	1OA	11.6 ± 2.1	—	2S 77.6 ± 3.4	*	X
12	19	7.2 ± 2.0	$1S_1$	83.1 ± 4.3	*	$1S_2$ 9.7 ± 3.0	—	X
13	20	8.8 ± 1.9	$1S_1$	83.5 ± 2.8	*	2AA 7.7 ± 1.7	—	X
14	23	7.5 ± 2.1	$1S_1$	83.5 ± 3.9	*	2OA 9.0 ± 2.3	—	X
15	21	4.0 ± 0.9	$1S_1$	72.8 ± 3.7	*	2S 22.4 ± 3.8	*	X
16	20	13.4 ± 2.5	$1S_2$	64.7 ± 4.3	*	2AA 22.0 ± 3.3	—	X
17	20	22.9 ± 4.1	$1S_2$	60.3 ± 4.5	*	2OA 16.8 ± 2.0	—	X
18	21	12.6 ± 3.0	$1S_2$	11.2 ± 2.6	—	2S 76.2 ± 4.0	*	X
19	21	43.8 ± 6.0	2AA	28.2 ± 4.3	—	2OA 28.0 ± 4.8	—	O
20	20	11.5 ± 1.6	2AA	9.7 ± 2.4	—	2S 78.8 ± 3.1	*	X
21	20	12.2 ± 2.7	2OA	6.9 ± 1.6	—	2S 80.9 ± 3.8	*	X

[a] All tested at concentration of extract equal to amount of chemical recovered from 0.5 mg of freeze-dried needles dissolved in 15 μl of a H_2O solution. 1AA: current foliage, amino acids and bases; 1OA: current foliage, organic acids; $1S_1$: current foliage, sugars and glycosides (part 1, not methanol soluble, see text); $1S_2$: current foliage, sugars and glycosides (part 2); 2AA: 2-year-old foliage, amino acids and bases; 2OA: 2-year-old foliage, organic acids; 2S: 2-year-old foliage, sugars and glycosides.

[b] —, not significantly different from A (Scheffé test; $P = 0.05$); *, significantly different from A (Scheffé test; $P = 0.05$).

[c] O, not significantly different from B (Scheffé test; $P = 0.05$); X, significantly different from B (Scheffé test; $P = 0.05$).

The amino acids-organic bases fractions have no effect on larval feeding responses while the organic acids fractions actually deter feeding slightly, but significantly ($P = 0.05$).

From the above one concludes that spruce budworm larvae prefer the water-soluble components of balsam fir foliage and other evergreen species over the ethanol or the petroleum ether-soluble components. Sugars and/or glycosides are a highly stimulating group of chemicals for the larvae, and some

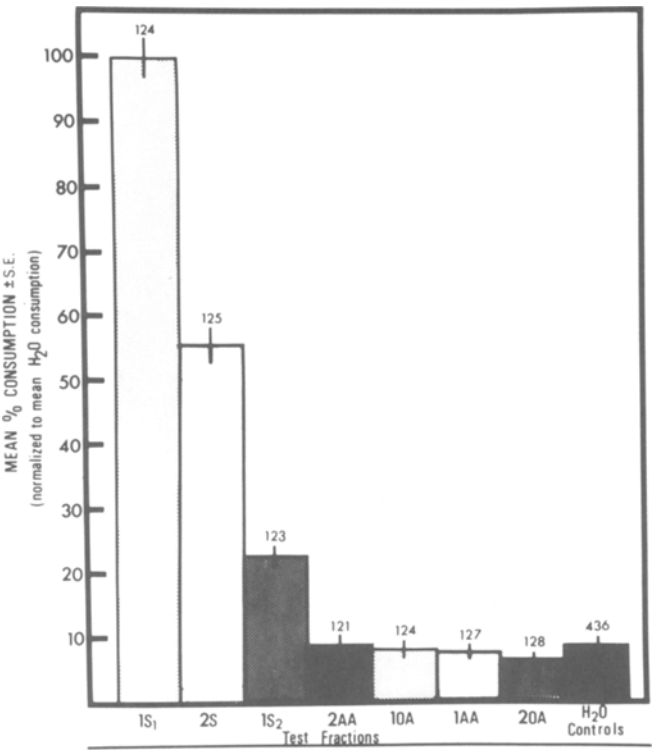

FIG. 4. Relative percent consumption ±SE of disks treated with extracts of balsam fir at concentrations of extract equal to the amount of chemical recovered from 0.5 mg of freeze-dried needles dissolved in 15 μl of solution. Data normalized from Table 1. Three-choice tests. Numbers above histograms = N animals. See Table 1 for legend to test fractions. $1S_1$, $2S_1$, and $1S_2$ were each significantly different from all others (Scheffé test, $P = 0.05$).

amino acids (e.g., arginine, glutamic acid, and proline) are also stimulatory. The organic acids fractions from balsam fir needles show some feeding deterrency.

The different feeding responses to the water extracts of the various evergreen species would suggest that it may be worthwhile to investigate them further. By collecting foliage from each type of tree at the same time of year when the larvae are actively feeding, and by quantifying and qualifying the chemical compounds shown here to influence feeding behavior, one may get a more accurate representation of the insect–host-plant interaction. It would appear to be deceptively simple that the sugars alone account for the larval preferences. The glycoside content of these extracts may be of even more importance, especially if some specific glycosides are found to occur in one

host species and not in another. A specific amino acid may act as a synergist with the sugars. It is known for example, that budworm larvae prefer a mixture of sucrose and *l*-proline than sucrose alone in single-choice tests (Heron, 1965). Other water-soluble compounds from the needles may also play an important role, and further purification of the plant extracts will be needed to determine their activity.

The physiological basis of the above behavioral responses is at present unknown. Albert (1980) described the morphology and innervation of the mouthpart sensilla of budworm larvae and suggested that the sensilla styloconica on the galea might be of importance since they contain sugar- as well as amino acid-sensitive cells. Further behavioral and electrophysiological experiments will be undertaken using the water-soluble components of the various hosts. Two- or three-choice behavioral tests will be used because of their greater sensitivity. The extent of feeding-preference induction (Jermy et al., 1968) will also be determined for diet-reared and foliage-reared animals.

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DUFOUR'S GLAND SECRETION IN THE CELL LININGS OF BEES (HYMENOPTERA: APOIDEA)[1]

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Abstract—The volatile chemicals, detectable by coupled capillary gas chromatography–mass spectrometry, in the nest cell linings of four species in the bee genera *Colletes*, *Andrena*, and *Lasioglossum* are identical with, and limited to, the volatile components present in the Dufour's gland of females, suggesting this source for some of their cell-lining constituents. The extractable material that lines the cells of *Trachusa byssina*, in contrast, is derived from *Pinus* resin, to which it can be chemically traced.

Key Words—Dufour's gland secretion, *Colletes*, *Lasioglossum*, *Andrena*, *Trachusa*, macrocyclic lactones, nest cell lining.

INTRODUCTION

Of those species of bees whose nests are subterranean, most provide membranes or waxen hydrophobic linings of variable thickness for their nest cells. Possible exceptions are species of *Dasypoda* (Melittidae) and *Perdita* (Andrenidae), whose nest cell walls appear uncoated (Michener, 1964; Rozen, 1967). Some nest cell linings are insoluble in most organic solvents (Jakobi, 1964; Batra, 1972) and resist degradation by acidic and basic hydrolyses (May, 1974; Hefetz et al., 1979).

The long-suspected origin of this lining material, in the Colletidae, Andrenidae, Halictidae, and some Anthophoridae, is the Dufour's gland of the sting apparatus. This is evidenced in these bees by correlation of glandular enlargement with nesting activity (Bordas, 1895; Semichon, 1906; Lello,

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1971a,b; May, 1974), female cell construction behaviors (Batra, 1968; Roberts, 1969; May, 1974), and the potential for ready polymerization of some Dufour's gland exudates, such as the macrocyclic lactones (Bergström, 1974). May (1974), using IR spectrometry, indicated the presence of wax esters in both the Dufour's gland secretion and nest cell lining of *Augochlora pura* (Halictidae). The nest cell linings of three North American *Colletes* species are formed from polyesters composed of ω-hydroxy acid units derived from the corresponding macrocyclic lactones in the bee's Dufour's gland secretions (Hefetz et al., 1979). The glandular origin and chemistry of secreted cell linings have only been demonstrated for these three species of *Colletes*, for *Anthophora abrupta* (Norden et al., 1980), and for *Augochlora pura pura* (Duffield et al., 1980).

Most Megachilidae nest in above-ground substrates, ranging from modified preexisting cavities, especially plant stems, to various fabricated structures of mud, leaves, plant resins, and hairs, etc. (Michener, 1974). *Trachusa* spp. (Megachilidae) seem intermediate in nesting habits, as they excavate subterranean nests but coat their cell surfaces with plant resins and leaves (Hachfeld, 1926). No megachilid bee is known to use secreted nest cell linings (Michener, 1974), based on morphological and behavioral evidence.

METHODS AND MATERIALS

Biological Materials. The bees used in this study were all obtained from the Swedish island of Öland. Dufour's glands were excised from live, chilled, recently collected bees (Bergström and Tengö, 1974), whose own nest cell linings were also to be analyzed. Bees were collected directly from their nests. Previously identified Dufour's gland components were in all cases verified (Bergström, 1974; Bergström and Tengö, 1974). Single, whole glands or their extracts were placed in a thermally programmed precolumn and the volatiles were directly analyzed with a combined capillary gas chromatograph–mass spectrometer (Bergström and Tengö, 1974).

Nest cell linings were obtained only from unprovisioned cells. Volatile lining components of cells were made available for GC-MS analyses either by precolumn thermolysis of cell lining fragments or by chemical extraction and concentration in either methylene chloride, pentane, or hexane. Additionally, for *Andrena marginata*, methylene chloride extracts were prepared both from five pollen provision balls and fifty flowers of *Campanula rotundifolia* (Campanulaceae) at which this species was observed foraging.

Instrumentation. Chemical analyses were performed with the aid of a combined GC-MS (Pye Unicam gas chromatograph, LKB-2091 mass spectrometer) modified to receive splitless precolumn injections (Bergström, 1973). An FFAP/OV-17, 25-m-long glass capillary column was selected for the GC-MS system, while an OV-101 25-m-long column in a Perkin-Elmer 900 GC with flame ionization detection provided comparative chromatograms.

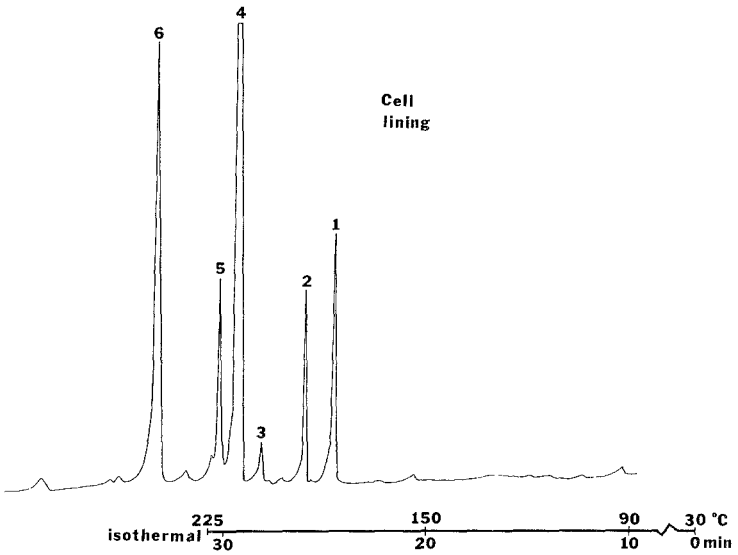

FIG. 1. Capillary gas chromatogram of 5-min precolumn thermolysis of a cell lining fragment of *Colletes cunicularius*. Precolumn 175°C. 1 = heneicosane; 2 = unknown; 3 = tricosane; 4 = 18-octadecanolide; 5 = 18-octadecenolide; 6 = 20-eicosanolide.

Chemical Identification. Identifications of the volatile components of glands, cell linings, and pollen balls were made by comparisons with reference spectra and retention times of previously published or available synthetic compounds (Bergström, 1974; Bergström and Tengö, 1974; Tengö and Bergström, 1975). Two to five glands and cell linings per species were each analyzed independently by GC-MS to determine constancy of chemical constituencies. All chromatograms and spectra are deposited with the Ecological Station at Uppsala University.

RESULTS

In each of the following species, the predominant volatile compounds isolated from the nest cell lining were identical with and limited to the chemicals of the Dufour's gland secretion of the respective bee species. Thus, macrocyclic lactones predominate in the Dufour's gland secretion (Bergström, 1974, their Figure 1) and cell linings (Figure 1) of *Colletes cunicularius* as well as *Lasioglossum albipes* (Figure 2A and B). Farnesyl derivatives characterize the *Andrena haemorrhoa* Dufour's gland secretion (Bergström and Tengö, 1974, their Figure 11) and nest cell linings (Figure 3). Three farnesene isomers are present in similar proportions for both the nest cell lining and Dufour's gland secretion (Figure 4) of *Andrena marginata.*

The predominant volatile components isolated from the pollen balls of *Andrena marginata* are identical with and limited to those chemicals present

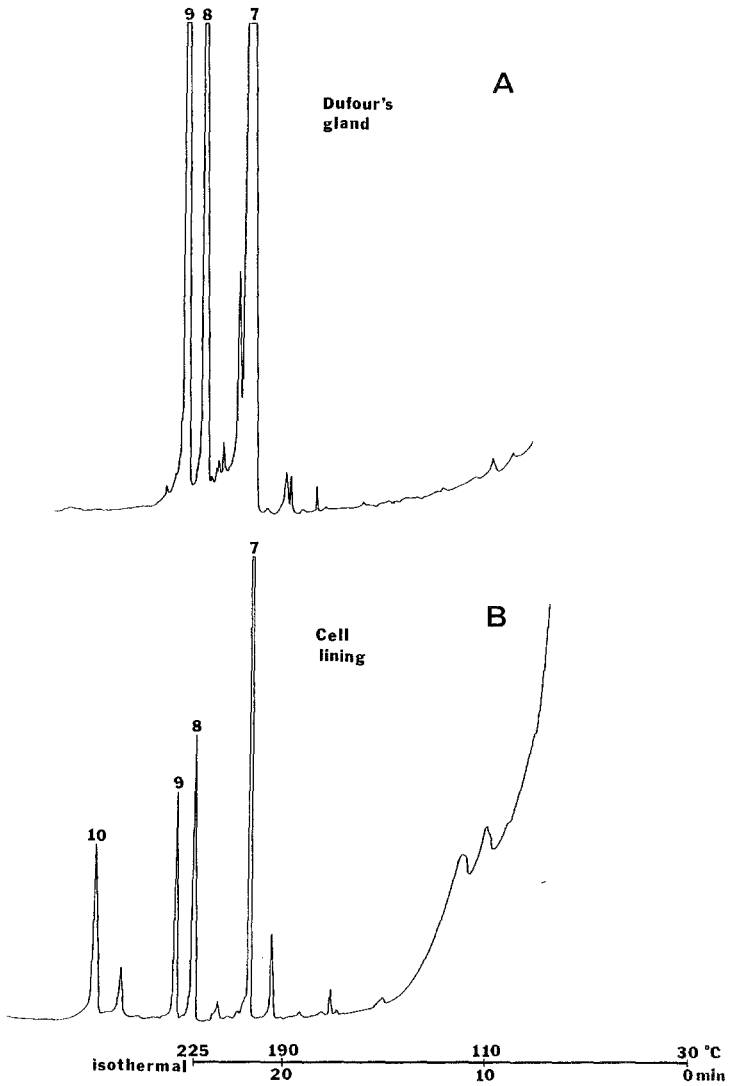

FIG. 2. Capillary gas chromatograms of 5-min precolumn thermolyses of the Dufour's gland (A) and pentane extract of the nest cell lining (B) of *Lasioglossum albipes*. Precolumn 200°C. 7 = 16-hexadecanolide; 8 = 18-octadecanolide; 9 = 18-octadecenolide; 10 = 20-eicosanolide.

in its Dufour's gland reservoir (Figures 4A and B). They are not those volatiles extracted from *Campanula rotundifolia* nectar or pollen, as judged from the pattern of GC retention times.

The origin of the volatiles extracted from the nest cell walls of *Trachusa byssina* (Megachilidae) was chemically traced both to the resin balls carried by

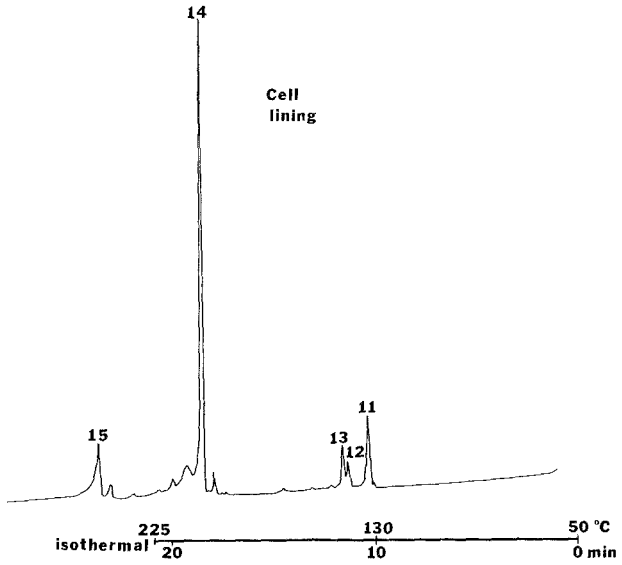

FIG. 3. Capillary gas chromatogram of 5-min precolumn injection of a cell lining fragment of *Andrena haemorrhoa*. Precolumn 200° C. 11, 12, 13 = farnesene isomers; 14 = farnesol; 15 = farnesyl hexanoate.

the females and the exuding bud resin of nearby *Pinus sylvestris* (Pinaceae). They all contain α-, β-pinenes, Δ-3-carene, and limonene in like proportions (Figure 5).

DISCUSSION

It is likely that Dufour's gland secretions are present in the nest cell linings of other burrowing bees. There is a close infrageneric chemical similarity between the nest cell linings described here and the Dufour's gland secretions previously reported for species of *Colletes* (Bergström, 1974; Hefetz et al., 1979), *Andrena* (Bergström and Tengö, 1974; Tengö and Bergström, 1978; Cane, unpublished), and *Lasioglossum* (*sensu lato*) (Bergström, 1974; Hefetz et al., 1978). Similar macrocyclic lactones are present in the Dufour's gland secretions of other Halictinae, such as *Halictus* (Bergström and Tengö, 1979), *Agapostemon*, and *Augochlorella* (Hefetz et al., 1978). Thus, the Dufour's gland secretion may be expected in their cell linings too, as Duffield et al. (1980) have shown for *Augochlora pura pura*.

Hachfeld's (1926) observation that *Trachusa byssina* females visit conifers to obtain resins for cell linings is chemically confirmed. Only Correia (1977) has previously analyzed any other resinous megachilid cell lining chromatographically, using *Heriades truncorum*. Volatile coniferous leaf oil

terpenes have been chemosystematically analyzed (Rudloff, 1975) and so can provide evidence for the cell lining origins of other megachilids.

Although the secreted cell linings studied here include chemicals derived from the Dufour's gland in *Colletes*, *Andrena*, and *Lasioglossum*, they may not be limited to this glandular source. Suggested origins have included not only the Dufour's gland (*Colletes*—Hefetz et al., 1979; *Andrena*—Lello,

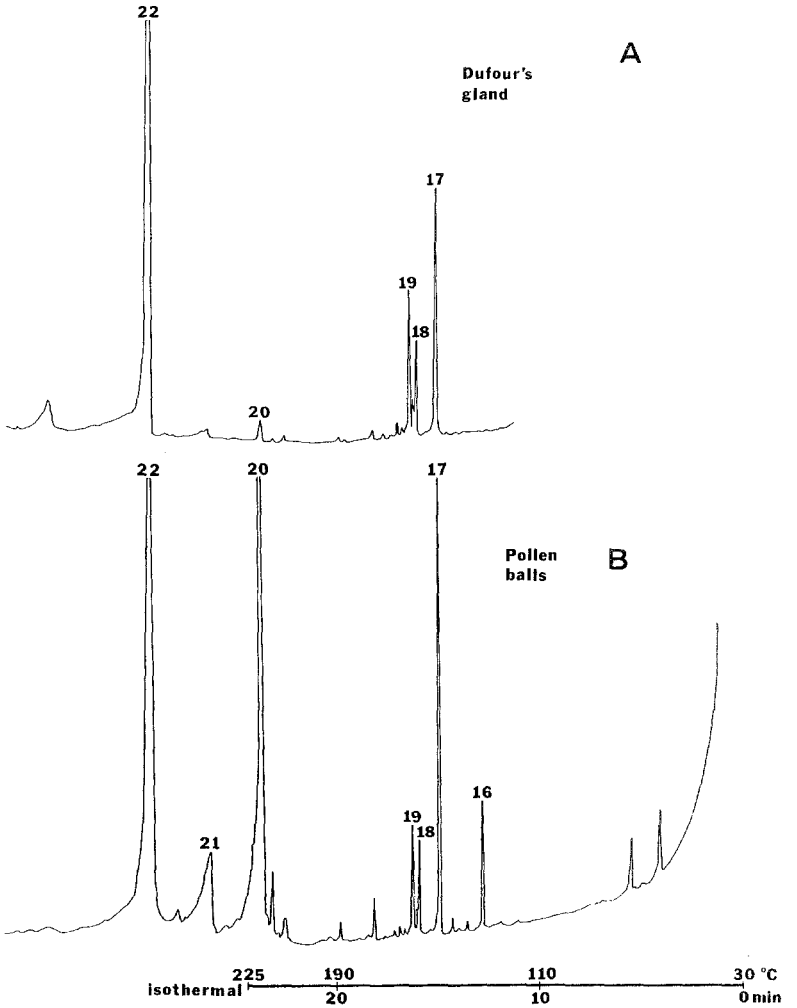

FIG. 4. Capillary gas chromatograms of 5-min programed precolumn thermolysis of the Dufour's gland (A) and splitless injection of methylene chloride extract of five pollen balls (B) of *Andrena marginata*. Precolumn 200°C. 16 = dihydrofarnesene; 17, 18, 19 = farnesene isomers; 20 = farnesol; 21 = tricosane; 22 = farnesyl hexanoate.

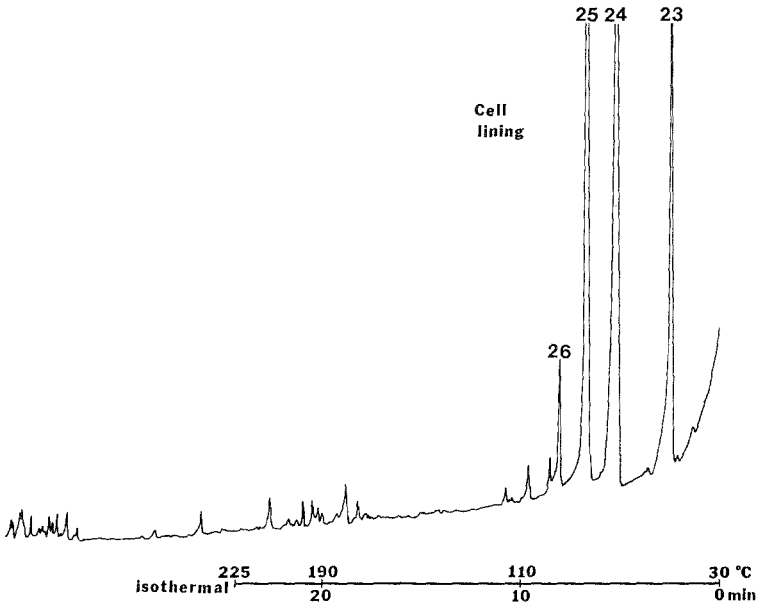

FIG. 5. Capillary gas chromatogram of a splitless injection of pentane extract of cell lining fragment of *Trachusa byssina*. 23 = α-pinene; 24 = β-pinene; 25 = Δ-3-carene; 26 = limonene.

1971a; Bergström and Tengö, 1974; *Augochlora*—May, 1974; *Anthophora*—Semichon, 1906; Malyshev, 1928), but also the salivary glands (*Colletes*—Claude-Joseph, 1926; Batra, 1972; Malyshev, 1968; *Andrena*—Batra, 1972), and intersegmental glands (*Andrena*—Altenkirch, 1962).

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ONION FLY[1] TRAP CATCH AS AFFECTED BY RELEASE RATES OF *n*-DIPROPYL DISULFIDE FROM POLYETHYLENE ENCLOSURES[2]

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Abstract—*n*-Dipropyl disulfide loadings of at least 1 μl and 10 μl in polyethylene enclosures were required to effect significant trap catches of onion fly, *Hylemya antiqua* (Meigen), females and males, respectively. Corresponding release rates for this threshold for trap catch were ca. 100 μg/hr. Catches increased with loadings up to 100 μl/capsule but then plateaued. As determined gravimetrically, the release rate from enclosures containing a reservoir of chemical remained constant under isothermal conditions but increased exponentially as a function of temperature.

Key Words—Host-plant attractants, *Hylemya antiqua*, onion flies, onion maggot, *n*-dipropyl disulfide, polyethylene enclosures, attractant release rates.

INTRODUCTION

n-Dipropyl disulfide (*n*-Pr_2S_2), a major onion volatile found in the head-space of cut onions (Carson and Wong, 1961; Boelens et al., 1971), has been identified as an oviposition stimulant and "attractant" for the onion fly, *Hylemya antiqua* (Meigen) (Matsumoto and Thorsteinson, 1968; Matsumoto, 1970). Subsequent behavioral studies (Dindonis and Miller, 1981a) have proven that onion odors are indeed attractants of *H. antiqua* since they elicit movement directed upwind and toward the source. In field tests, traps baited with relatively large amounts of *n*-Pr_2S_2 caught more flies than traps

[1]Diptera: Anthomyiidae
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baited with other onion volatiles (Matsumoto, 1970), fresh and rotting onion juice (Loosjes, 1976), or healthy and decomposing onion plants (Dindonis and Miller, 1980), suggesting that this single compound may be a useful trap bait for monitoring onion fly populations.

The efficacy of a trap bait, however, may be dependent on the release rate at which the compound is dispensed. For example, significantly more cabbage root fly, *Hylemya brassicae* (Bouché), females flew upwind when presented with an optimal (104 mg/hr) release rate of allyl isothiocyanate (Hawkes and Coaker, 1979). Similarly, numerous trapping studies employing pheromones show that optimal release rates are necessary to produce maximal trap catches (Roelofs and Cardé, 1977).

In the present experiments, we investigated the effects of five different *n*-Pr_2S_2 loadings on *H. antiqua* trap catch and established a range for maximal catch. Furthermore, release rates of *n*-Pr_2S_2 dispensed from saturated polyethylene capsules were characterized as a function of time and temperature.

METHODS AND MATERIALS

Trap Catch as Affected by Disulfide Loadings. Responses of onion flies as measured by trap catch were tested in the field to five loadings of *n*-Pr_2S_2, an onion half, and an unbaited control. *n*-Pr_2S_2 was released from size 3 BEEM™ polyethylene embedding capsules (Pelco Electron Microscopy Supplies, Ted Pella Co., Tustin, California) as follows: 1 μl, 10 μl, and 100 μl loadings were dispensed as neat *n*-Pr_2S_2 ($\leqslant$99% pure by GLC determination); a 0.1-μl loading was dispensed in hexane which was allowed to evaporate before the capsule was closed; and the largest amount dispensed (10 $\times$ 100 μl), was achieved by grouping 10 capsules each containing 100 μl of *n*-Pr_2S_2. An empty capsule was used for the control. Capsules were held upright and 2 cm above ground by perforated platforms made from paper cup bottoms. All treatments were inserted into brown paper sleeves to reduce possible degradation due to sunlight. The covered treatments were then secured by wire stakes beneath acetate cone traps (Dindonis and Miller, 1980).

The treatments, replicated five times, were set out June 5–July 10, 1979, on the border of a large commercial muck soil onion field at Stockbridge, Michigan. A linear randomized complete-block design was used with treatments spaced 6 m apart and 10 m from the onion field. During each rerandomization, all blocks were shifted 3 m in the same direction. The new trap positioning reduced the possibility of inflating a trap catch due to *n*-Pr_2S_2 persisting from a previous treatment; this problem was suspected in previous experiments using very high release rates of *n*-Pr_2S_2. The two highest loadings were renewed every 4–6 days to maintain a visible reservoir within the

capsules, whereas the three lowest loadings and the onion half were replaced every 48 hr.

Characterization of n-Pr_2S_2 *Release from Polyethylene Capsules.* *n*-Pr_2S_2 release rates from the BEEM polyethylene capsules were quantified gravimetrically over time. Four capsules filled with neat *n*-Pr_2S_2 were suspended by a wire within individual 50-ml beakers. A water bath maintained the air temperature within the beakers at 33 ± 1°C as measured by a telethermometer probe placed adjacent to the capsule of the center beaker. Capsules were weighed 3–4 times daily until no visible reservoir remained; at this point, capsules were weighed once a day until they reached their original weight.

The effect of temperature on the release rate of the disulfide was quantified similarly. Three *n*-Pr_2S_2-containing capsules were monitored gravimetrically for each temperature investigated (see Figure 3). For determinations at lower temperatures (<38° C), capsules were suspended in beakers in water baths, whereas vented GLC ovens were used for measurements at high temperatures (≥38°C). Capsules were weighed every 2–12 hr; the frequency of weighings was dependent on temperature.

RESULTS AND DISCUSSION

Traps baited with 10 μl, 100 μl, and 10 × 100 μl of *n*-Pr_2S_2 caught significant numbers of female and male onion flies; however, no significant trap catch differences were found among these three loadings (Figure 1). Females flies were also trapped in significant numbers by traps baited with 1 μl. As determined gravimetrically, the release rates from 1 μl and 10 × 100 μl of *n*-Pr_2S_2 were 60 μg/hr and 9 mg/hr, respectively, demonstrating that comparatively high release rates of this single component are required for attracting onion flies and that these flies are responsive to a wide range of *n*-Pr_2S_2 emission rates.

Although no single loading produced a distinctly optimal catch, there was a trend toward larger trap catches with increasing *n*-Pr_2S_2 loads. Increasing the levels of *n*-Pr_2S_2 emitted may extend the active space and, thus, effect responses from more flies. At the 10 × 100-μl loading, however, trap catch plateaued. The effect of extending the distance of communication via the tenfold increase of the release rate from 900 μg/hr to 9 mg/hr may have been negated by an attendant close-range repellency or arrestment of the incoming flies. For cabbage flies, upwind-oriented flight at 4–5 m from the source is mediated by allyl isothiocyanate release rates ranging from 3 to 300 mg/hr (Hawkes and Coaker, 1979). However, a repellent effect during landing and inhibition of oviposition has been reported for even lower allyl isothiocyanate concentrations (Nair and McEwen, 1976). Similarly, Matsu-

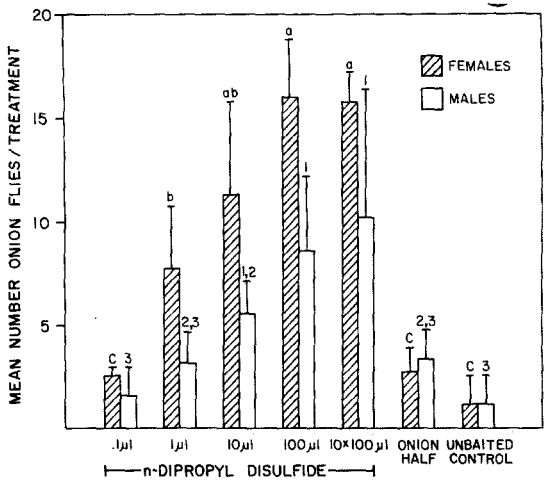

FIG. 1. Mean trap catches and 95% confidence intervals for male and female onion fly responses to different loadings of *n*-dipropyl disulfide and an onion half. Treatments represented by the same letters (females) or numbers (males) are not statistically different based on a two-way ANOVA followed by a planned F test for mean separation on data transformed to $(X + 0.5)1/2$.

moto (1970) reported that onion fly oviposition is inhibited by sand dishes treated with 10 µl of *n*-Pr_2S_2, whereas this and greater loadings produced significant trap catches in our experiment. Thus, higher release rates of a single compound may elicit long-range flight but deter landing at the source. Alternatively, high concentrations of the stimulus have been reported to induce flight arrestment in Lepidoptera before the source is reached (Farkas and Shorey, 1974).

Neither the traps baited with 0.1 µl of *n*-Pr_2S_2 nor the onion half caught more flies than the control. Apparently, the concentration of the odor stimulus from these treatments was subthreshold or effective for only a short time. In retrospect, we believe that the sliced onion treatment should have been replaced daily or even more frequently for a more valid measure of attractiveness.

Although more seed corn flies (*H. platura*) than onion flies were caught in the *n*-Pr_2S_2-baited traps, no seed corn fly catch by *n*-Pr_2S_2 was significantly greater than control. However, a significant catch was obtained in the traps baited with sliced onion; a mean of 65.2 and 38.0 seed corn flies, 2/3 of which were females, were caught in onion-baited and control traps, respectively.

Characterization of n-Pr_2S_2 *Release from Polyethylene Capsules.* Polymeric macroencapsulation is reported to be an effective dispensing system for controlled release of volatile compounds (Glass et al., 1970; Kuhr et al., 1972; Campion et al., 1978; Marks, 1976). The mechanism of transport

and release rate characteristics of polymeric delivery systems are reviewed by Lewis and Cowsar (1977), Kydonieus (1977), and Rogers (1977). Laminated polymeric vessels and some polyethylene enclosures have been characterized as having constant release rates for an extended period. Steady-state release appears to be contingent upon maintaining an internal chemical reservoir and, hence, saturated capsule walls. We felt that this type of system would effectively regulate the release of relatively low-molecular-weight plant volatiles.

Under isothermal conditions, the filled polyethylene BEEM capsules released *n*-Pr_2S_2 at a constant rate (Figure 2). Latency for the onset of steady emission was approximately 2 hr, during which time *n*-Pr_2S_2 was emitted at ca. 0.55 mg/hr. Thereafter, the rate averaged 0.9 mg/hr for about 8 days, decreasing only after no visible reservoir remained. At this point, the release kinetics became first order and within the next 30–40 hr, emission dropped below amounts detectable by weighing.

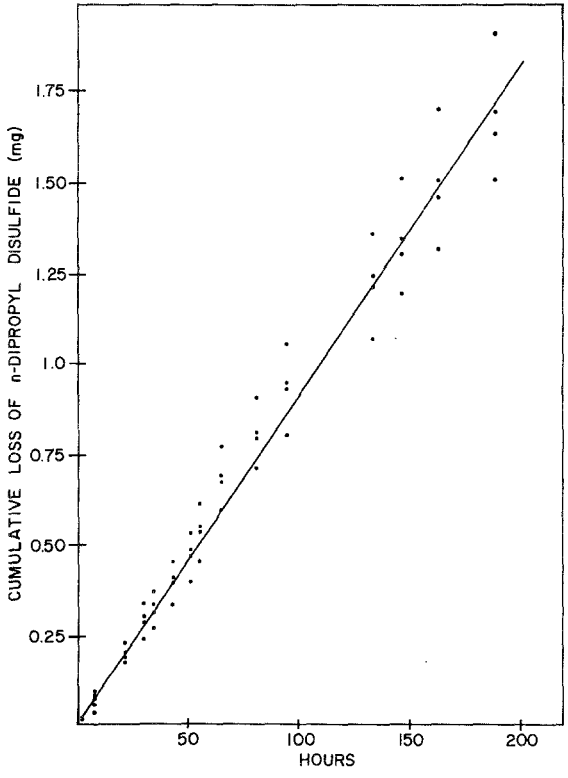

FIG. 2. Release profile of *n*-dipropyl disulfide from BEEM size 3 polyethylene capsules as a function of time at 33° C.

The release rate of *n*-Pr_2S_2 increased exponentially as a function of temperature (Figure 3). Fortunately, however, inflection was not too severe over the range of temperatures commonly encountered in the field. From 18° to 40° C, the release rates ranged from 0.29 mg/hr to 2.0 mg/hr. The emission rates at temperatures exceeding 65° C varied extremely, probably due to changes in the polymeric material; at temperatures $\geqslant$69.5° C the capsules emit by-products from a parting layer formed as a result of the casting process (T. Turnbull, Ted Pella, Inc., personal communication) and at ca. 80° C the capsule walls begin collapsing.

These results demonstrate that polyethylene macrocapsules are ideally suited for dispensing highly volatile plant attractants for insects. By appropriately adjusting the size of the reservoir and the surface area of polyethylene exposed to the atmosphere, a constant release rate can be maintained for an extended time with relatively small deviation due to natural air temperature fluctuations. Shielding such capsules from direct sunlight is advisable.

The present data and those from Dindonis and Miller (1980) suggest that size 3 BEEM polyethylene enclosures (or some equivalent) saturated with *n*-Pr_2S_2 might serve as useful baits for *H. antiqua* monitoring traps since they elicited ca. 8–11 times higher fly catch than controls. However, such high release rates of a single onion volatile do not seem to be an ideal bait. At certain times, *n*-Pr_2S_2-baited traps have not caught well when placed in fields

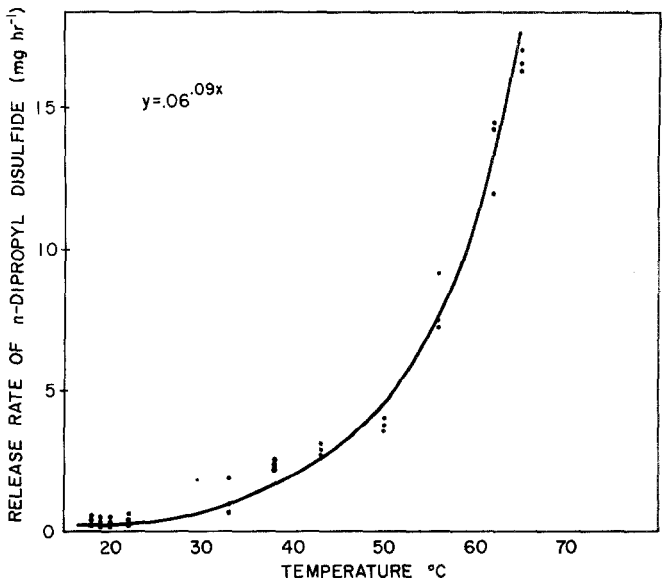

FIG. 3. Release rate profile of *n*-dipropyl disulfide from BEEM size 3 polyethylene capsules as a function of temperature.

containing high numbers of maggot-infested and decomposing onions (Dindonis and Miller, unpublished) or when compared with some rather potent food attractants (Miller and Haarer, unpublished). A bait for population monitoring should, over time, be uniformly attractive to individuals in the population; hence, measured increases and decreases in catch would reflect real increases and decreases in the population. Identifying the chemical signals emanating from those treatments which out-compete *n*-Pr_2S_2 and sliced onions should lead to improved *H. antiqua* attractants and an improved understanding of the role chemicals play in allowing this fly to locate its host plant.

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ONION FLY[1] AND LITTLE HOUSE FLY[2] HOST FINDING SELECTIVELY MEDIATED BY DECOMPOSING ONION AND MICROBIAL VOLATILES[3]

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(Received May 23, 1980; revised July 19, 1980)

Abstract—Responses of onion flies, *Hylemya antiqua* (Meigen), to various synthetic onion and microbial volatiles as well as volatiles from microbial cultures and decomposing onions were tested to characterize the most effective host-finding stimuli. Of nine onion and microbial volatiles tested individually, only the known attractant, *n*-dipropyl disulfide, caught significant numbers of flies. However, a blend of these volatiles attracted more flies than any single chemical, including *n*-dipropyl disulfide. In another experiment, agar plates inoculated with microorganisms from decomposing onions did not attract onion flies. However, cut onions inoculated with microorganisms and conditioned 4 days caught more onion flies than freshly cut onions and *n*-dipropyl disulfide. These results suggest that a blend of chemicals, rather than a single key chemical, is the more effective host-finding stimulus, and that microbial activity enhances the attractancy of a blend of onion volatiles. Large numbers of *Fannia canicularis* (L.), the little house fly, responded to the microbial cultures, demonstrating the existence of a potent attractant for this muscid.

Key Words—Onion fly, *Hylemya antiqua*, little house fly, *Fannia canicularis*, host finding, host-plant attractants, microbial attractants.

[1]Diptera: Anthomyiidae.
[2]Diptera: Muscidae.
[3]Paper No. 9470 of the Michigan State University Agricultural Experiment Station. Received for publication May 23, 1980.

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INTRODUCTION

In field tests conducted in commercial onion fields, both female and male onion flies, *Hylemya antiqua* (Meigen), were attracted to point sources of damaged onions (Dindonis and Miller, 1980, 1981a). The rotting process seemed to enhance the attractiveness of onions (Dindonis and Miller, 1980, Loosjes, 1976). Moreover, a major volatile of crushed onions, *n*-dipropyl disulfide (*n*-Pr_2S_2), has been identified as a host-finding stimulant for *H. antiqua* (Matsumoto, 1970). This chemical trapped more onion flies than potted onion plants (Dindonis and Miller, 1980) or onion juice (Loosjes, 1976); however, relatively high release rates of *n*-Pr_2S_2 (ca. 1 mg/hr) were required to achieve these catches (Dindonis and Miller, 1981b).

The available data suggest that the chemical nature of the optimal host-finding stimulus for *H. antiqua* may be: a particular onion volatile, a mixture of onion volatiles, a mixture of onion volatiles in combination with volatiles from associated microorganisms which cause onion rot, or a particular microbial volatile(s). Quantitative changes in each proposed signal could also be expected to affect host finding.

This paper reports experiments intended to help characterize the optimal host-finding stimulus for *H. antiqua*. Attraction to various combinations of microbial by-products and onion volatiles was tested to determine the relative effectiveness of volatile blends. Another experiment assessed onion fly attraction to microorganisms removed from their onion substrate, and concurrently compared the attractiveness of freshly cut and decomposing onion bulbs.

METHODS AND MATERIALS

Experiment 1: Attractancy of Blends of Onion and Microbial Volatiles. The attractancy of six metabolic by-products of soil-inhabiting microorganisms (Pelczar et al., 1972) (*n*-butanol, 2,3-butanediol, *n*-butyric acid, 85% aqueous acetyl methyl carbinol, hexanoic acid, propionic acid) and two volatiles common to onions (Whitaker, 1976) and microbes (Pelczar et al., 1972), (ethanal and isopropanol) were tested individually and collectively. In addition, the oviposition stimulant and attractant, *n*-Pr_2S_2, was dispensed alone and in combination with the chemicals. A freshly cut onion half (replaced every 3–4 days) and a control trap baited with an empty dispensing vial completed the treatments.

n-Pr_2S_2 was dispensed from BEEM™ size 3 polyethylene enclosures charged with 100 μl of neat material (purity $\geqslant$99%). Although this system released optimal rates of *n*-Pr_2S_2 (Dindonis and Miller, 1980c), these capsules were not suitable for release of some of the other compounds due to their high

volatility. The objective was to achieve a continuous release of each chemical; however, no attempt was made to maintain equal release rates. Hence, 6 ml of all other compounds were dispensed from 8-ml glass miniscintillation vials capped with white polyethylene stoppers (Brockway Glass, Chicago, Illinois). These dispensers could hold a large reservoir of chemical while the hard polyethylene prolonged release by retarding diffusion through the 1-mm-thick cap. Vials containing ethanal were embedded cap upward in the soil to reduce further the release rate and pressure of this highly volatile compound. All other vials were placed horizontally within cylindrical brown paper sleeves to reduce chemical degradation by direct sunlight. Onion halves were similarly covered to retard dehydration. During the experiment, the treatments were rerandomized three times, onion halves were replaced every 4th day, and the chemicals (always detectable by the human nose) were replenished whenever necessary to maintain a visible reservoir.

The test was conducted September 29 to October 13, 1978, in Hudsonville, Michigan. The treatments were placed beneath acetate cone traps (Dindonis and Miller, 1980) spaced 3 m apart on the border of a muck soil onion field. The four replicates were arranged in a linear randomized complete-block design.

Experiment 2: Attractancy of Microbial Cultures, Freshly Cut and Decomposing Onions. The rate of *H. antiqua* larval development on onion is accelerated by the presence of bacteria transmitted by the larvae (Friend et al., 1960, Zurlini and Robinson, 1978). Thus, we hypothesized that volatiles specific to these or other microorganisms from decomposing onions might be involved in host finding by *H. antiqua* females. In an attempt to determine the relative effects of microbial volatiles, inoculum from maggot-infested and decomposing onions was cultured on medium, and the attractancy of such plates and various onion treatments was compared.

The eight treatments were: an agar plate with and without *n*-Pr_2S_2, an agar plate inoculated with microorganisms from decomposing onions also tested with and without *n*-Pr_2S_2, *n*-Pr_2S_2 alone, a freshly cut onion half, a decomposing onion half, and an empty plate as a control.

The decomposing onion treatment was prepared by placing 15 field-collected 2nd–4th instar onion fly larvae on the cut side of an onion half. The infected onion halves were pressed into nonsterilized muck soil and watered daily. Onions conditioned for 4 days were used to inoculate the agar plates and then were placed in the field as the decomposing onion treatment.

Potato dextrose agar in 50-mm plastic Petri dishes was inoculated with decomposing onion microorganisms by pressing the cut side of the conditioned onion half into the agar medium for 3 sec. One onion half was used for every five plates. The inoculated plates were incubated at 21°C for 48 hr.

Before deploying the plates in the field, the Petri dish covers were replaced with two layers of brown paper toweling tautly secured by a tight-

fitting plastic ring. This cover allowed volatiles to emanate from the microorganisms and agar while excluding foreign microorganisms as evidenced by the absence of visible colonization of control agar plates during the experiment.

n-Pr_2S_2 was released at ca. 900 μg/hr from size 3 BEEM polyethylene embedding capsules. The capsules were suspended 1.0 cm above the agar and microbial plates by a wire attached to the plate.

Treatments were placed within brown paper sleeves and secured beneath acetate cone traps placed on the border of a heavily *H. antiqua*-infested onion field in Stockbridge, Michigan. The test ran from July 5 to 18, 1979, and consisted of four replicates deployed in a linear randomized complete-block design with a 6-m intertreatment spacing. To maintain relatively constant volatile release rates, the microbial and agar plates were replaced every 24 hr, the decomposing and cut onions every 48 hr, and *n*-Pr_2S_2 was replenished as necessary to maintain a visible reservoir within the capsules. Also, treatments were rerandomized within blocks every 4th day, at which time all blocks were moved 3 m in the same direction to guard against contamination due to odors persisting from a previous treatment.

RESULTS

Experiment 1: Attractancy of Blends of Onion and Microbial Volatiles. With the exception of *n*-Pr_2S_2, no trap baited with a single compound caught more flies than the control trap (Table 1). However, the combination of the eight chemicals (without *n*-Pr_2S_2) elicited a significantly greater female response than any of the chemicals tested individually, except *n*-Pr_2S_2. Moreover, the combination of eight volatiles plus *n*-Pr_2S_2 produced a female catch significantly greater than either the 1–8 combination or *n*-Pr_2S_2 alone. The pattern in male response was similar, although fewer significant treatment differences were evident (Table 1).

Experiment 2: Attractancy of Microbial Cultures, Freshly Cut and Decomposing Onions. The greatest trap catch of female and male onion flies occurred in traps baited with decomposing onion halves (Table 2). Fewer yet significant numbers of female flies were also caught by all traps containing *n*-Pr_2S_2. Male flies responded similarly to *n*-Pr_2S_2. However, with the addition of microbial plates (treatment 7), *n*-Pr_2S_2-baited traps caught no more males than control; furthermore, the microbial cultures alone caught significantly fewer males than the unbaited control trap. Female response to the microbial culture was indistinguishable from that to control.

In contrast to the low catch of onion flies, traps baited with the microbial plates caught large numbers of female *Fannia canicularis* (L.) (Table 2). Moreover, the catch to the microbial plates was further elevated by the

TABLE 1. ONION FLY RESPONSES TO MICROBIAL BY-PRODUCTS, ONION VOLATILES, AND THEIR COMBINATIONS

Treatments	Mean number onion flies caught per treatment[a]	
	Female	Male
1. Ethanal	13.3d	11.0c
2. *n*-Butanol	14.3d	9.8c
3. 2,3-Butanediol	9.8d	8.8c
4. *n*-Butyric acid	21.3cd	9.8c
5. Acetyl methyl carbinol	9.3d	7.5c
6. Hexanoic acid	8.0d	6.8c
7. Isopropanol	15.0d	12.3c
8. Propionic acid	8.0d	10.3c
9. Combination of 1-8	32.8bc	16.5abc
10. *n*-Dipropyl disulfide	43.3b	25.8ab
11. 9 + 10	73.5a	30.8a
12. Onion half	27.0bc	18.6abc
13. Control	16.5cd	14.3bc

[a]Means followed by same letters within columns are not statistically different as determined by 2-way ANOVA followed by a planned F test for mean separation of data transformed to $(\chi + 0.5)^{1/2}$

addition of *n*-Pr_2S_2, which by itself caught no *F. canicularis*. Virtually no females were trapped by any of the other treatments tested, and no male flies were caught in any of the traps.

DISCUSSION

H. antiqua. Although additional investigation is necessary, the present experiments have aided our attempt to characterize the chemical composition of an effective host-finding stimulus for *H. antiqua.* The hypothesis that a blend of chemicals, rather than a single key chemical, is the more effective host-finding stimulus is supported by the significantly larger catch by the combination of eight chemicals (Experiment 1) compared to any of the chemicals tested individually. Further support is provided by the even greater catch to the combination of the eight chemicals and *n*-Pr_2S_2, and the significantly greater onion fly response to the decomposing onion treatments than to optimal release rates of *n*-Pr_2S_2. Previous investigations (Dindonis and Miller, 1981b) demonstrated that onion flies were equally responsive to a wide range of *n*-Pr_2S_2 release rates, 150 μg/hr to 9 mg/hr, further suggesting that once above threshold, quantitative differences of a stimulus may be of less importance than qualitative differences.

TABLE 2. FLY RESPONSES TO MICROORGANISMS REMOVED FROM ONION SUBSTRATE, AND TO FRESHLY CUT AND DECOMPOSING ONIONS

Treatments	Mean number flies caught per treatment[a]			
	Hylemya antiqua		*Fannia canicularis*	
	Females	Males	Females	Males
1. Decomposing onion half	32.8a	25.2a	0.4c	0
2. Freshly cut onion half	13.4bc	13.4bc	0.2c	0
3. *n*-Dipropyl disulfide	18.4b	13.8b	0.0c	0
4. Agar plate	12.4bcd	8.4cd	0.2c	0
5. 3 + 4	18.0b	10.8bc	0.0c	0
6. Microorganism culture	8.4cd	4.4e	43.6b	0
7. 3 + 6	14.0bc	7.2de	72.0a	0
8. Control	7.6c	8.0d	0.0c	0

[a]Means followed by same letters within columns are not statistically different as determined by 2-way ANOVA followed by a planned F test for mean separation of data transformed to $(\chi + 0.5)^{1/2}$

An onion half, renewed every 4 days, did not catch more flies than control (Table 1), whereas an onion half replaced every 2 days did effect a significant catch (Table 2), suggesting a time-dependent decrease of onion volatiles. The decomposing onion, although cut 4 days before exposure, effected the greatest male and female catch, strongly suggesting that the microbial activity on onions produced a blend of volatiles even more stimulating than volatiles from a freshly damaged onion.

The import of microbial activity in releasing onion fly behavior has recently been demonstrated by Ellis et al. (1979), who found that reduced oviposition occurred on onion seedlings grown in a relatively sterile medium. The levels of alkyl sulfides released from onions grown in sterilized sand were undetectable (Coley-Smith and King, 1969). Furthermore, soil bacteria are capable of enzymatically cleaving onion volatile precursors which may be exuded by the roots, producing alkyl disulfides, such as n-Pr_2S_2 (King and Coley-Smith, 1969). Although damaged onion cells emit high levels of sulfur-containing compounds, the characteristic odor of a healthy onion may be due, in part, to the onion's microbiota. Microorganisms are strongly implicated in production of volatiles stimulatory to the onion fly.

The microorganisms removed from their onion substrate and allowed to flourish on agar medium did not enhance onion fly host finding. This may indicate that microbial volatiles alone are not stimulatory. However, the disparate carbon sources and the different physicochemical natures of the media may have altered the expression of certain metabolic pathways with the

result that differing by-products were produced. Furthermore, the microorganisms emitting the attractive volatiles may have been selected against by the culture medium, or they may have been outcompeted by other microorganisms. That the inoculated plates were actually releasing volatiles was substantiated not only by human perception of an odor (not unlike a rotting vegetable) but also by the *F. canicularis* trap catch.

F. canicularis. The little house fly is reported to breed in a variety of decaying animal and plant matter, including onions (Chillcott 1960). However, in the present study, practically no *F. canicularis* were caught by traps baited with the decomposing onions which were attractive to *H. antiqua.* Possibly, a more severely decomposed onion may be a suitable oviposition site. Conversely, traps baited with the microbial plates caught high numbers of *F. canicularis*, but not onion flies. Although of decomposing onion origin, the microbial plates must have produced a volatile profile different from that of decomposing onions and one quite specific for *F. canicularis.*

The behavioral response of onion flies to microbial volatiles appears to be limited to onion–microorganism associations, whereas the muscid fly may respond to microbial volatiles produced from nononion substrates. However, the response of *F. canicularis* was significantly greater to the microbial plate and *n*-Pr_2S_2 combination than to the microbial plates alone, indicating that *F. canicularis*, like the onion fly, may perceive and respond preferentially to a stimulus consisting of a blend of chemicals. In contrast, the attraction of *F. canicularis* to fermented sucrose solutions was reportedly due solely to one volatile constituent, ethanol (Hwang et al., 1978). In finding and selecting sites for oviposition, insects capable of integrating the information contained in multicomponent signals, such as those emanating from a decomposing onion, should realize an adaptive advantage if they can select the host or host conditions which will be nutritionally and ecologically optimal for their offspring.

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PHEROMONAL CUES DIRECT MATE-SEEKING BEHAVIOR OF MALE *Colletes cunicularius* (HYMENOPTERA: COLLETIDAE)

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Abstract—Males of the vernal ground nesting bee, *Colletes cunicularius*, actively search their nesting aggregations for emerging virgin females. Males often detect and unearth preemergent virgin males and females which have not yet dug up to the soil surface. Field experiments indicate that the odors from one unmated individual are sufficient to both excite males and direct their excavation of the virgin bee. A volatile component of this species' mandibular gland secretion, linalool, increases flight activity and directs local search behavior in aerially patrolling males. Linalool is detectable by coupled gas chromatography–mass spectrometry in association with the digging activities of either sex.

Key Words—Sex pheromone, mandibular gland, linalool, *Colletes*, mate attraction.

INTRODUCTION

Among the solitary ground-nesting wasps and bees occur numerous taxa whose nests are commonly aggregated (Evans and Eberhard, 1970; Michener, 1974). Males may localize their search for mates over such aggregations (references in Alcock et al., 1978). The resulting increases in competition between males have been implicated in explaining the associated "enhanced search mobility" noted in the monandrous species (Parker, 1978). Males of some species of *Andrena* (Malyshev, 1926; Vleugel, 1947), *Centris* (Alcock, Jones, and Buchmann, 1976), *Colletes* (Bergström and Tengö, 1978), and *Bembix* (Tsuneki, 1956; Schöne and Tengö, 1980), have been observed to

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cluster and dig excitedly downward at points within their aggregations where virgin females are soon to emerge. In such cases, olfactory cues seem to function as both releasing stimuli of the males' overall excitation (Tengö, 1979a) and orientation stimuli directing male search behavior for emerging conspecifics (Alcock et al., 1976). The identity and origin of these suspected olfactory chemical signal(s) were unknown until now.

METHODS AND MATERIALS

Study Site. Two nesting aggregations, both with more than 2000 nests of *Colletes cunicularius*, were studied during April 1979. One aggregation was used for experiments, while the other was reserved for undisturbed observations. Both were located on the Baltic island of Öland near the southeastern Swedish coast (56°43′ N × 1°33′ E). Nests were concentrated along sandy wheel tracks paralleling the southern edge of *Pinus sylvestris* stands.

Behavioral Analyses. Direct observations of male mate-seeking ethology were supplemented with cinematography and emergence screens (Linsley et al., 1952). Screens were placed over ground where males had aggregated and dug to collect the emerging bees.

All behavioral experiments consisted of controlled, repeated, two-choice tests performed at the aggregation on different days. The responses of patrolling males within a 10-cm radius of an odor source were recorded using Kullenberg's (1973) behavioral classifications for quick inspection (type 1), local search by hovering inspection (type 2), and approach (type 4). The last implies actual alighting or visitation (Figure 1). The total number of male passes within the 10-cm radius was taken as a measure of flight activity, suggesting the excitory capacity or general attractiveness of the substance (Σ

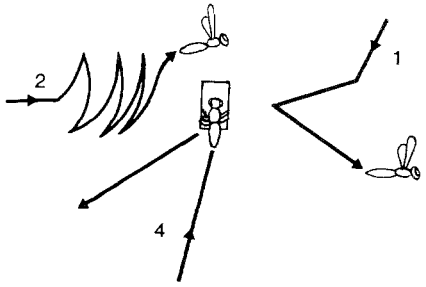

FIG. 1. Classification of the behaviors exhibited by patrolling *Colletes cunicularius* males in response to test stimuli: type 1 = quick inspection; type 2 = local search; type 4 = copulatory pounce; Σ types 1, 2, and 4 = flight activity within the 10-cm radius around the stimulus. Type 3 behaviors (Kullenberg, 1973) were grouped with type 2.

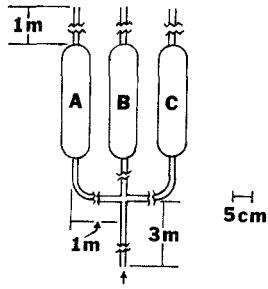

FIG. 2. Apparatus for presenting odors of active female bees to patrolling males. An airstream originating from a common source (arrow) passes through three glass cylinders in parallel containing live virgin female bees (A), postmating female bees (B), or no bees as a control (C). These airstreams subsequently emanate from the ends of 1-m-long tubes opening at ground level. The cylinders (with bees) and tubing are interchanged between tests to control for any differences in flow rates or outlet positions.

types 1, 2, and 4). Individual bees for use as odor sources were classified by sex and as either freshly emerged, in copula, immediately postmating, or male-rejecting (for females). Meteorological conditions and overall male activity were monitored during experimentation, allowing us to select similar test periods on different days.

Live individuals were presented to patrolling males in screen-capped glass tubes either at the surface or slightly buried (1–3 cm). The numbers of approaching males were recorded. The volatile odors from active bees in glass cylinders were presented independently to patrolling males (Figure 2) by passing an airstream over these enclosed bees. This airstream emanated from a tubing outlet 1 m away. Controls consisted of identical tubes and airstreams, but without bees.

Extracts of body parts of conspecifics or known synthetic chemicals (99% purity) were diluted in spectrometric-grade hexane. Responses of searching males were tested using uniform black velvet rectangles (6 × 10 mm) for evaporative release (Tengö, 1979b). Additionally, hexane extracts of *Ophrys* '*arachnitiformis-sphecodes*' flower labella were tested, as they are known to be pollinated by male *Colletes* during their "pseudocopulatory" movements on the flower labellum (B. Kullenberg in Tengö, 1979b). Each velvet received the equivalent of one half of an extracted labellum. A saturated piece of velvet initially contains approximately 100 μl of extracted or synthetic solution. Control dummies with only solvent on velvets were used simultaneously in all tests. Each test lasted 5 min.

Differences in male flight activities at control dummies (Σ types 1, 2, and 4) between test periods of different days were compared with a Kruskal-Wallis test. We consider this as a comparative measure of undisturbed male

patrolling activity. The same test was also used to detect differences in male flight activity associated with various chemical stimuli. The relative effectiveness of these stimuli for increasing flight activity, local search, and/or approach behaviors was then tested by Wilcoxan rank sums. All tests are one-tailed, as we only considered increased frequencies of behaviors towards test substances. Low numbers of test replicates (n) were sufficient to demonstrate statistical significances for our experiments, indicative of the keen olfactory discrimination elicited from these patrolling male bees.

Chemical Analyses. To obtain the predominant odors present during male or female digging activity or copulation at the aggregation, bees were enclosed in glass tubes loosely packed at both ends with glass down into which they would dig (Figure 3).

Volatile odors were collected from an airstream that was sucked through such tubes and over a packed Tenax® polymer adsorbant (Lundgren et al., 1978; Cane and Jonsson, 1980). Less volatile chemicals remaining on the glass surfaces were extracted with pentane and concentrated by microdistillation. The digging behaviors of the bees (i.e., leg and mandible movements, backing, thoracic vibration) under these conditions in the field were indistinguishable from observed natural digging behaviors and were unlike defensive behaviors.

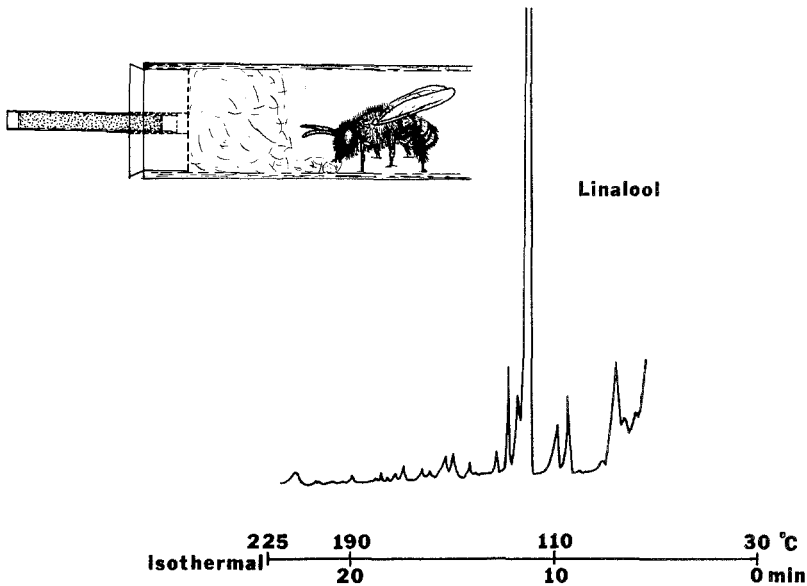

FIG. 3. Gas capillary chromatogram of digging odors of three sequentially sampled virgin female *Colletes cunicularius* bees. Odors are collected while bee digs into a glass down plug within a glass cylinder, as illustrated. Total sample time is 85 min. Desorbed from Tenax polymer (see Methods and Materials).

However, there was a tendency for the glass down to cling to their hind legs. Control samples were obtained by simultaneous airflow samples through identical tubes without bees.

Chemical identification of the adsorbed volatiles was by means of a precolumn thermal degassing directly coupled to a combined GC-MS instrument with an FFAP/OV-17 capillary column and LKB 2091 spectrometer (Bergström, 1973; Groth et al., 1977). Chemical identifications were established by comparison with retention times and spectra of synthetic reference compounds and previously identified glandular chemical components (Bergstrom, 1974; Bergström and Tengö, 1978). Chromatograms and spectra are deposited with the Ecological Station of Uppsala University on Öland.

RESULTS

Phenology of Patrolling Male C. cunicularius. Male bees began emerging at the ground surface a week before the females at both aggregations, confirming Malyshev's (1927) observations. By the first day of observed female emergences, large numbers of males were already patrolling over the aggregation, responding to both our tests and one another. Male bees would daily commence flying one or more hours before virgin females began emerging, and would continue to fly after emergences had ceased each day. Their flight activity is strongly associated with both surface temperature and solar insolation (Tengö, unpublished). It seemed that males primarily searched those areas of the aggregation where emergences had already occurred. Areas with the first male and female emergences were associated with both the earliest male searching and also the first female nest starts, suggesting the overall qualities of those areas. We did not observe males patrolling outside the limits of the aggregations.

Observational and Experimental Evidence that Olfactory Cues Direct Male Mate-Seeking Behaviors. Patrolling males of *Colletes cunicularius* aggregate on the surface above preemergent virgin males and females. Evidence includes (1) our repeated emergence cage captures, (2) the ability of males to locate and unearth buried screen-capped tubes with virgin female bees, and (3) our own success in digging up the emerging male and female bees at points where males were observed to be clustered and excavating. Emerging bees could thus be found at a depth of 1–3 cm.

Odors extracted from the bees are sufficient to release and direct local search and approach behaviors in aerially patrolling males ($P \leqslant 0.05$, $N = 6$) (Figure 4). In the presence of such odors, supplied either naturally or experimentally, the frequency of male homosexual copulation attempts and pouncing on inanimate objects often increases, suggesting associated aphro-

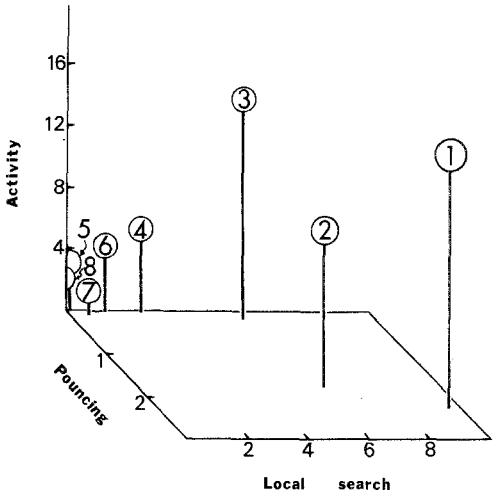

FIG. 4. Mean frequencies of mate-searching behaviors of patrolling *Colletes cunicularius* males within a 10-cm radius of odor source. Test period is 5 min, using velvet rectangles for evaporative release. All frequency values calculated by subtracting the frequencies of males responding to simultaneously presented controls using only solvent. Behavior types as in Figure 1. 1 = bee body extracts; 2 = *Ophrys "arachnitiformis-sphecodes"* extracts (1/2 labellum equivalent); 3 = linalool (10 μl); 4 = linalool (0.1 μl); 5 = linalool (0.001 μl); 6 = docosane (10 μl); 7 = 16-hexadecanolide (= dihydroambrettolide) (10 μl); 8 = 1-octanol (10 μl). Axis scales are number of males/5 min, as calculated above.

disiac qualities. Patrolling males were more attracted (type 2) to the airstream blowing over three virgin females ($\bar{X} = 10$ males, $N = 3$) than to a simultaneous nearby airstream blowing over three male-rejecting females ($\bar{X} = 0$ males, $N = 3$) (Figure 2). Males were not attracted to either the transparent cylinders containing the bees nor to a control airstream. Male bees were also attracted to emergence sites and dug there even after the female had departed *in copula*. Soil from the entrance of a fresh emergence tunnel remained similarly attractive when removed elsewhere within the aggregation (17 males digging), while displaced neighboring soil remained unattractive (0 males digging).

Sampling and Identification of Odors Present during Digging Behavior. Odor samples collected from digging males, digging virgin females, digging male-rejecting females, and copulating pairs were all dominated by linalool in the adsorbed volatile fraction (Figure 3) and heneicosane and tricosane in the pentane extracts of the glass down. We also identified nonanal and decanal in trace amounts (less than 3% area of linalool peak) in the volatile samples from the virgin and male-rejecting females. An opaque abdominal exudate released

by immediately postmating females and collected on filter paper contained nonadecane, heneicosane, heneicosene, and tricosane by GC-MS analysis. In the field, we could smell linalool-like odors around mating pairs and especially above the clusters of males gathered around virgin females. Indeed, at peak female emergence time, a faint linalool-like odor was present in the airspace over the entire aggregation. Linalool is the dominant volatile component of the mandibular gland secretion in this species (Bergstrom and Tengö, 1978).

Although our older GC-MS instrumentation precludes peak area integration, some estimate of linalool concentration for our samples can be made. The maximum secretory capacity of the bees is limited by their glandular volumes, which Bergström and Tengö (1978) calculated to be 10 μl of linalool for each female's mandibular glands. We also obtained GC peaks equivalent in size to the linalool peaks of single female *Colletes* samples while using our adsorption system to sample over 0.4 μl of undecane contained in an aliphatic reference blend. These figures serve to estimate the range over which the female bees secrete linalool during digging behavior.

Role of Linalool in Directing Male Mate-Seeking Behaviors. Male activity at controls did not vary significantly between test periods of different days ($P > 0.05$), allowing us to pool test results from these days. Pentane extracts of the body parts (head, thorax, and abdomen) evaporated from velvets attracted more patrolling males, caused more local searching, and elicited more copulatory pouncing (all $P \leqslant 0.05$, $N = 6$) when compared with controls. Linalool, in amounts of as low as 0.1 μl/velvet, was sufficient to enhance attraction ($P \leqslant 0.05$, $N = 6$) and elicit more local search behavior ($P \leqslant 0.05$, $N = 6$) when presented to patrolling males (Figure 4). Still more convincing results were obtained when quantities of 10 μl of linalool were tested. Both general activity ($P \leqslant 0.01$, $N = 7$) and local search ($P \leqslant 0.005$, $N = 8$) were significantly more frequent when compared with controls. Equal concentrations of 1-octanol, docosane, or 16-hexadecanolide, the last a component of the female's Dufour's gland secretion (Bergström, 1974) failed to release significant responses ($P > 0.05$). Linalool rarely elicited male excavating and pouncing behavior when presented in this manner ($P > 0.2$, $N = 23$), even when combined 1:1 with dihydroambrettolide. *Ophrys* "*arachnitiformis-sphecodes*" labellum extracts elicited increased rates of both local search ($P \leqslant 0.005$, $N = 18$) and copulatory pouncing ($P \leqslant 0.005$, $N = 18$) as well as enhancing attraction ($P \leqslant 0.001$, $N = 18$) (Figure 4).

DISCUSSION

Patrolling male *Colletes cunicularius* are able to accurately locate preemergent virgin male and female conspecifics. Olfactory cues, as extracts

of the adult bees, are alone sufficient to elicit these local search and approach components in patrolling males.

We conclude that the dominant mandibular gland volatile, linalool, is present during a bee's digging activity and is a pheromone enhancing attraction and eliciting and directing local search behaviors of patrolling males for emerging virgin females (Figure 4). Linalool is present in the digging odors of both sexes. Predictably, then, emerging males as well as females are commonly unearthed. Such digging up of virgin males has no apparent adaptive value, since the male has already completed 95% of its trip to the surface, and the subsequent homosexual mauling by patrolling males delays its flight and may even cause injury. However, the release of linalool during mandible use may have potential benefits for both sexes during defensive behaviors. Such reasoning may suggest the evolutionary origin of such a sex pheromone.

The general presence of linalool around the aggregation during emergence periods may stimulate the heightened excitation (or lowered behavioral thresholds) of patrolling males for precopulatory behaviors. It may also limit patrolling to the aggregation itself. Butler (1965) noted similar phenomena for *Andrena flavipes* that he attributed to undetermined aggregation odors.

Our experiments indicate that male pouncing behavior and the ability of patrolling males to distinguish virgin from postcopulatory females may also involve some olfactory cues originating from the female bees. Aphrodisiac pheromones have been demonstrated experimentally for *Lasioglossum zephyrum* (Barrows, 1975). Long-chain hydrocarbons, such as those detected in the digging odors or postcopulatory secretions of female *Colletes cunicularius*, could serve in this role as they do for stable flies (Sonnet et al., 1979). Kullenberg and Bergström (1976) propose a similar dual-volatility model for male attraction to *Ophrys* orchid odors.

Female *Colletes* of other species are attracted to disturbed, netted conspecific females at their foraging sites (Linsley and Zavortinck, 1977; Rajotte, 1979; Hefetz et al., 1979), suggesting an aggregation pheromone. Such female interactions were not observed nor induced by the experiments at our aggregations. During nest defense, when the mandibular secretion is released (Cane and Jonsson, 1980), only patrolling males responded and approached a female's burrow entrance. These were subsequently repulsed by the nesting female.

The generality of these results awaits more detailed mating observations and controlled field experimentation with other species. Kullenberg (1973) provides evidence that the cephalic extracts of certain female bees (*Eucera*, *Bombus*) and wasps (*Campsoscolia*, *Gorytes*) attract and sexually excite patrolling conspecific males, as do extracts of specific *Ophrys* orchid labella. Female and male mandibular gland secretions or their synthetic mimics are

particularly attractive to patrolling conspecific *Andrena* males (Tengö, 1979b). Linalool, sometimes in combination with citral, has been shown present in the mandibular gland secretions of a number of European and American *Colletes* species (Bergström and Tengö, 1978; Hefetz et al., 1979), many of which are characterized by male patrolling at the nest aggregation site (Friese, 1912; Jones, 1930; Stephen, 1954; Hurd and Powell, 1958; Guichard, 1974).

Particularly important will be both the documentation of the presence of the suspected pheromone and testing with comparable quantities in the known context of release (Butler, 1970), as even single-chemical secretions can be multifunctional. Furthermore, male mate-seeking activities may also include density-dependent alternative behaviors, where males could utilize the visual presence of other digging males as well as pheromonal cues for locating likely female emergence sites.

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A MULTICOMPONENT FEMALE SEX PHEROMONE OF *Dacus oleae* Gmelin:[1,2]

Isolation and Bioassay

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Abstract—The sex attractant pheromone produced by female *Dacus oleae* Gmelin is a mixture of four compounds, two of which are found in the rectal gland and the other two elsewhere in the insect body. The ratio of these compounds in the pheromone blend was measured. Biological activity of all four compounds and their combinations was studied in lab and field cage tests. The most abundant compound in the mixture (55.7%) shows the highest biological activity. Recombination of all compounds significantly increases activity of the main compound.

Key Words—Pheromones, sex attractants, *Dacus oleae*, Diptera, Tephritidae, olive fruit fly, sex pheromones.

INTRODUCTION

Sexual attraction in the olive fruit fly, *Dacus oleae* Gmelin, has been observed in laboratory and field tests (Haniotakis, 1974, 1977). This attraction has been associated with the release of an airborne female sex pheromone which functions as a male attractant (Haniotakis et al., 1977). Preliminary experiments indicated that this pheromone is a blend of more than one substance. Although *Dacus oleae* is the only known species of tephritid fruit flies that utilizes a female sex attractant for sex communication, multicomponent male-produced sex pheromones have been found in other species. Fletcher (1969) presents evidence that the pheromone of *Dacus tryoni*

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(Froggatt) is multicomponent. Jacobson et al. (1973) isolated from air condensates and identified two compounds from *Ceratitis capitata* (Wiedemann) males that were attractive to females in laboratory tests but not in the field. Field-cage attraction was achieved only when the inactive acidic fraction of the condensate was added to the two compounds. Nation (1975) has characterized four components of the *Anastrepha suspensa* (Loew) male sex pheromone blend.

The purpose of this work was to isolate the various components of the olive fly sex pheromone blend, to bioassay them individually and in combination, and to establish the combination and ratio of components for maximum male attraction.

METHODS AND MATERIALS

Insects Used. All insects used in this study were obtained from a colony originating from Tatoi Attikis, Greece, and maintained at "Demokritos" N.R.C., Athens, Greece, on artificial diets for about 15 generations (Tsitsipis, 1977). Flies 1–2 days old were immobilized by chilling at 2–4° C for 1–2 hr and separated according to sex. They were maintained in screen cages 270 cm^3 in artificial light (3000 lux intensity) and 12:12 hr light–dark regime. Temperature was 25 ± 2° C and relative humidity 65 ± 5%.

Pheromone Collection. Pheromones were obtained by a "total condensation cold trap" (Haniotakis et al., 1977). An active extract was also obtained by extracting excised rectal glands (Vita et al., 1979) from 5- to 8-day-old female flies. Rectal glands are easily removed by applying pressure to the abdomen and pulling the ovipositor with fine forceps. Excised glands were extracted with diethyl ether for 24 hr or more. Both "cold-trap condensate" or rectal gland extracts were filtered through Whatman No. 1 PS filter paper and concentrated under atmospheric pressure through a 20-cm Vigreux column. Material collected by the first method from 1000 females or by the second method from 500 females was concentrated to a final volume of about 600 female equivalents/ml.

Fractionation. Concentrated pheromone crude material was clean enough to be injected into a GLC without further purification. A Varian GLC model 1400 with flame ionization detector was used. A 95:5 splitter and a temperature gradient glass capillary collector, kindly built by J.H. Tumlinson, was installed into the chromatograph. The collector was cooled with liquid nitrogen. The following stainless-steel columns and conditions were routinely used: (1) 5% OV-101 2 m × 1.8 mm (ID) at 80° C for 10 min, and then programmed to 240° C at 6° C/min; and (2) 5% Carbowax 20M 2 m × 1.8 mm (ID) at 90° C and 140° C. Occasionally columns of 5% DEGS, 2 m × 1.8 mm (ID), and 5% OV-101, 5 m × 1.8 mm (ID), were also used. N_2 was used as

carrier with a flow of 20 cc/min. The GLC was coupled with an integrator (Hewlett Packard model 3370B) for quantitative measurements.

Bioassays. Bioassays were conducted in a 95 × 70 × 40-cm screen cage in the laboratory and in a 4 × 4 × 2.5-m field cage.

Usually 100 5- to 8-day-old male flies were introduced into the laboratory cage at least 2 hr prior to the test. The cage was placed in front of an operating hood and, by means of a small fan, a weak airstream was directed through the cage along its long axis. In tests where biological activity of individual components was compared, concentrations of 1 and 10 female equivalents were used, respectively, in lab and field cage tests, corresponding to minimum concentrations for maximum response. In laboratory tests where activity of combinations of the various components was compared, the concentration for minimum positive response was used to avoid saturation effects. This corresponds to 0.1 female equivalents (about 30% of maximum response). The ratio of the components used in these tests was approximately that occurring in cold-trap condensates.

The appropriate amount of each sample dissolved in 300 μl of solvent was poured on 7.5 cm^2 of Whatman No. 1 filter paper which was then hung by a 3-cm wire from the top of the cage near the side towards the fan. The number of males visiting the paper was recorded for 10 min. During each testing period, samples were tested individually with 5-min intervals between tests. The number of males responding to each sample was expressed as percent of the total number of males responding to all samples. The experiment was repeated five times on five different days, with different insect batches. Samples were tested in random sequence. The same amount of solvent alone on similar paper was used as controls. Bioassays were conducted one at a time during the last 2 hr of the photophase, the period with the highest mating ratio (Zervas, 1980).

For field cage tests, 300–400 males were released inside the cage several hours before the test. The appropriate amount of test material was dissolved in 1 ml solvent in a 10-ml glass vial which was hung inside a cylindrical sticky trap (Haniotakis et al., 1977). The traps were suspended from the ceiling of the cage for 2 hr. One test was conducted per day at dusk. More than one compound was tested at the same time.

RESULTS AND DISCUSSION

When cold-trap condensates were injected in an OV-101 2-m GC column, four peaks were found to have various degrees of male attraction. The numbers 2, 4, 6, and 8 were assigned to these peaks, indicating their sequence in the chromatogram. Reinjection of each of these four peaks in a Carbowax 20 M column produced one peak (peaks 6 and 8) or three peaks (peaks 2 and

4). In the latter case there were one major and two minor peaks, but activity was associated with the major peak. Reinjection of each of the four active peaks from Carbowax 20 M in DEGS and 5-m OV-101 columns produced a single peak. Routine collection, therefore, of active compounds for biological tests was made from the 2-m OV-101 column for peaks 6 and 8 by injection of air condensate or gland extracts and for peaks 2 and 4 from Carbowax 20 M by reinjection of peaks 2 and 4 from the former column. Thus any mention of these peak numbers refers to the substances obtained as above.

Following the same procedures, from rectal gland extracts only two active peaks were obtained, which coincided in all columns with peaks 6 and 8 of the cold-trap extracts. It is possible that substances in peaks 2 and 4 are produced elsewhere in the insect body. Nation (1974) suggests that a multicomponent male sex pheromone of *Anastrepha suspensa* may be produced in two different sites, i.e., the pleural gland located at the 3, 4, and 5 abdominal segments and the portion of salivary glands which corresponds to insect abdomen.

Table 1 gives Kovats' indices (Kovats, 1961) for all four active peaks in columns OV-101 2 m and Carbowax 20 M.

When cold-trap condensates were injected in an OV-101 2-m column, the four active peaks collected and reinjected in a Carbowax 20 M column, and the active peaks measured with an integrator, the following ratio was found: peak 2 = 20.6% ± 2.5; 4 = 4.8% ± 1.5; 6 = 55.7% ± 1.4; 8 = 18.9% ± 0.5. Numbers are means of three replicates ± SD. Quantitative measurements of active peaks gave highly variable results. Rectal gland extracts, for example, in one series of measurements of peak 6, which we have found to be the major pheromone component, gave an average of 64.4 ng/female fly, and in another series of measurements, 127.8 ng/fly. There were individual measurements, however, which showed concentrations of up to 300 ng/fly. Baker et al. (1980) estimated that the amount found in the rectal gland of what they refer to as the major pheromone component was at the level of 300 ng/fly. They identified

TABLE 1. KOVATS' INDICES FOR ACTIVE PEAKS OF OLIVE FRUIT FLY SEX PHEROMONE ISOLATED FROM COLD TRAP (CT) AND RECTAL GLAND EXTRACTS (GE).

	Columns			
	5% OV-101 2 m × 1.8 mm (ID)		5% Carbowax 20 M 2 m × 1.8 mm (ID)	
Compounds	CT	GE	CT	GE
2	956		1065	
4	1063		1380	
6	1139	1145	1380	1380
8	1527	1534	1864	1858

TABLE 2. MALE OLIVE FLY RESPONSE TO COLD TRAP/(CT) AND RECTAL GLAND CRUDE EXTRACTS/(GE) AND ACTIVE PEAKS[a]

Treatment	Males responding per treatment as % of total number responding in all treatments		
	CT Lab Cage Tests	CT Field Cage Tests	GE Lab Cage Tests
Crude	26.9 ± 0.4a	30.9 ± 2.9a	24.6 ± 0.5a
Peak 2	12.7 ± 1.7b	12.3 ± 1.2b	6.0 ± 0.5b[b]
Peak 4	9.8 ± 1.4c	12.1 ± 1.2b	4.7 ± 0.5b[b]
Peak 6	30.5 ± 0.9d	25.1 ± 2.3c	40.1 ± 0.6c
Peak 8	18.7 ± 0.8e	15.9 ± 2.1b	18.7 ± 0.7d
Control	1.0 ± 0.8f	3.7 ± 2.3d	5.6 ± 0.7b

[a] 10-min observations. Means of 5 replicates ± SD. Concentrations equivalent to 1 female for lab cage tests and 10 females for field cage tests were used. Means in each column followed by the same letter are not significantly different, Duncan's multiple range test, $P = 5\%$.
[b] To test the possibility of presence of peaks 2 and 4 in gland extracts in nondetectable concentrations, collections and bioassays were made at the appropriate sites of chromatograms.

this component as 1,7-dioxaspiro(5.5)undecane. It is not known whether these differences are due to actual differences in the gland contents of different insect batches or to poor standardization of the techniques used.

Table 2 shows the biological activity of all four active fractions and crude extracts collected by air condensation and rectal gland extraction in laboratory and field cage tests. It should be pointed out that the biological activity of the same concentration of peaks collected from an OV-101 column was the same as that of the same peaks reinjected in Carbowax columns. Peak 6 is the most abundant and most active of all. The increased attractiveness of peak 6 in relation to crude extract in lab cage tests is probably due to the different evaporation rates of active substance from the two samples. This becomes evident from the observation that maximum attractiveness of peak 6 in the concentration tested occurs during the first 2–3 min, disappearing almost completely after 10 min, while the same concentrations of crude material do not show any attraction during the first 2–3 min, and maximum attractiveness is extended to over 10 min. This prolonged action may also be the explanation of the higher attraction of crude extract which was observed in the field cage where the duration of the test was 2 hr.

Table 3 shows male response to various combinations of all four active peaks. Attractiveness of peak 6 is increased in combination with peaks 2, 2 + 4, 2 + 4 + 8, but not 4 alone. Most attractive is the combination of all four peaks. Any combinations in the absence of peak 6 are significantly less attractive than peak 6 alone.

Table 4 shows data from additional tests of various combinations of active peaks, designed to verify existing indications that the substance in peak

TABLE 3. MALE OLIVE FRUIT FLY RESPONSE TO VARIOUS COMBINATIONS OF FOUR ACTIVE PEAKS OF COLD-TRAP EXTRACTS[a]

	Combination							
	6	6 + 4	6 + 2	6 + 2 + 4	6 + 2 + 4 + 8	2 + 4 + 8	2 + 4	Control
Male Response[b]	12.5 ± 0.6a	12.0 ± 1.2a	14.1 ± 1.0b	16.7 ± 1.5c	23.4 ± 0.8d	9.9 ± 0.6e	9.6 ± 1.3e	1.8 ± 0.7f

[a]Laboratory cage tests. Concentrations corresponding to 0.1 female equivalents. 10-min observations. Means of four replicates ±SD.
[b]Male response per treatment as percent of total number of males responding in each test. Means followed by same letter are not significantly different, Duncan's Multiple Range Test, $P = 5\%$.

TABLE 4. MALE OLIVE FRUIT FLY RESPONSE TO VARIOUS COMBINATIONS OF ACTIVE PEAKS OF COLD-TRAP EXTRACT[a]

Treatments	Males responding/treatment as % of total males responding in all treatments (period of time from start)		
	0–5 min	5–10 min	0–10 min
6	22.6 ± 6.6a	11.1 ± 3.4a	18.4 ± 3.6ab
8	9.4 ± 2.0b	15.5 ± 4.8a	11.8 ± 3.5ad
6 + 8	24.8 ± 7.2a	25.6 ± 4.2b	24.4 ± 3.2bc
2 + 4 + 6 + 8	32.6 ± 3.7a	33.6 ± 6.9b	33.3 ± 4.3c
Control	10.6 ± 3.5b	13.7 ± 2.3a	12.1 ± 2.0ad

[a] Laboratory cage tests. Concentrations 2, 0.6, 6, 2 ng/test, corresponding to 0.1 female equivalent for peaks 2, 4, 6, and 8, respectively. Means of 5 replications ± SD. Means in each column followed by the same letter are not significantly different, Duncan's multiple range test, $P = 5\%$.

8 affects the evaporation rate of the substance in peak 6, thus prolonging its effectiveness. Attractiveness of peak 6 actually was prolonged by the addition of peak 8. Again the combination of all four peaks gave the highest attraction. Peak 6 was less attractive than combination of all four active components of the mixture (Tables 3 and 4) while crude extract, which also contains all these compounds, was less attractive than peak 6 in lab cage tests (Table 2). This may be due to impurities contained in crude extract.

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ALKYLPHENOLS IN THE DEFENSIVE SECRETION OF THE NEARCTIC OPILIONID, *Stygnomma spinifera* (ARACHNIDA: OPILIONES)

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Abstract—The opilionid, *Stygnomma spinifera*, secretes from a pair of exocrine glands on the cephalothorax between the first and second pair of legs a major volatile component, 2-methyl-5-ethylphenol, and two minor components, 2,3-dimethylphenol and 2,3-dimethyl-5-ethylphenol. These alkylphenols are constituents of a defensive secretion and repel ants, a natural predator of opilionids. The probable significance of this secretion in the biology of *S. spinifera* and in the defensive strategies of opilionids in general is discussed.

Key Words—*Stygnomma spinifera*, Stygnommatidae, daddy longlegs, defensive secretion, phenols, defensive strategy, 2-methyl-5-ethylphenol, 2,3-dimethylphenol, 2,3-dimethyl-5-ethylphenol.

INTRODUCTION

Opilionids (Arachnida: Opiliones), commonly called "daddy longlegs," possess a pair of well-defined exocrine glands opening on the cephalothorax between the first and second pair of walking legs (Juberthie, 1961a,b). The secretions of these glands have been the subject of recent biological and chemical investigations which report the identification of methylated quinones (Eisner et al., 1971, 1977, Estable et al., 1955; Fieser and Ardne, 1956),

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phenols (Jones et al., 1977), and a variety of short-chained acyclic ketones (Blum and Edgar, 1971; Jones et al., 1976, 1977; Meinwald et al., 1971).

Opilionids are frequently encountered in meadows and woods throughout the summer and fall. We have observed that a number of species are also active during the winter (October–March) in southern Florida. One species, *Stygnomma spinifera*, when disturbed emits a strong cresol-like odor similar to that emitted by the millipede *Abacion magnum* (Eisner et al., 1963). This study reports that the exocrine secretion of *S. spinifera* is dominated by one compound, 2-methyl-5-ethylphenol. It also discusses the different defensive mechanisms of opilionids.

METHODS AND MATERIALS

The opilionids were collected in Everglades National Park, Florida, in November 1977 and March 1978, from solitary retreats on the undersides of coral rocks. They were easily spotted because their light orange color contrasted with the coral background.

Defensive exudates of 16 specimens were absorbed on filter paper squares and extracted several times with methylene chloride. The volatiles were analyzed on a Finnigan 3200 computerized gas chromatograph–mass spectrometer (GC-MS), utilizing a 1.8-m 10% SP-1000 column on 60/80 Supelcoport, temperature programed from 60 to 200° C at 10° / min. Quantification of the major compound was based on two samples of six and ten specimens.

Behavioral interactions of *S. spinifera* were observed in the field near colonies of two local ant species, *Camponotus floridanus* and *Solenopsis invicta*, or in laboratory cages with anoles, *Anolis sagrei*. The chemical basis for these behaviors was determined by exposing the ants to 8-mm filter paper disks impregnated with 1 mg of either 2-methyl-5-ethylphenol or 2,3-dimethylphenol.

We are depositing a series of *S. spinifera* under our label (R.M.D.) and accession number (R.M.D. # 1–78) in the collections of the National Museum of Natural History, Smithsonian Institution, Washington, D.C.

RESULTS

Chemical Analysis of Defensive Secretion. Gas–liquid chromatographic (GLC) analysis of the extract from 16 specimens showed one major component which had a molecular weight of 136. This volatile had a mass spectrum similar to that reported for 2-methyl-5-ethylphenol [(1) in Structure I] (Jones et al., 1977). Samples of this phenol, synthesized from p-methyl-

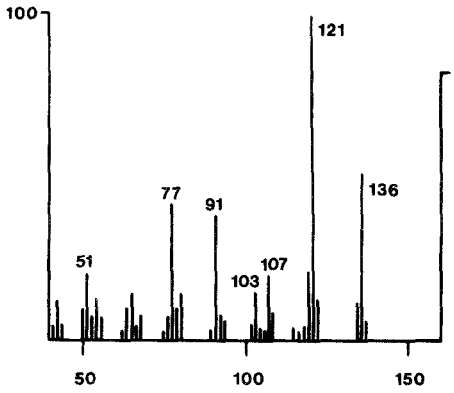


FIG. 1. Mass spectrum of 2-methyl-5-ethylphenol.

acetophenone by the method of Morgan and Pettet (1934), showed mass spectra (Figure 1), infrared spectra, and retention times identical to those of the natural product. A sample of the isomeric 2-ethyl-5-methylphenol prepared from 3-hydroxy-4-methylbenzoic acid exhibited a significantly different mass spectrum with a loss of ethyl rather than methyl as the first significant loss. Thus, even though the infrared spectra of the isomeric phenols have been used to differentiate them (Shrewsbury, 1960) their mass spectra are quite different and may also be used.

Two samples, one from six and the other from ten specimens, were

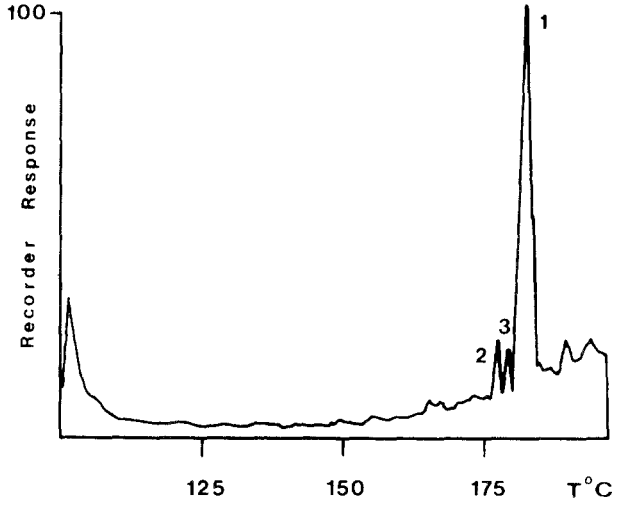


FIG. 2. Gas chromatogram of *S. spinifera* extract (1.8 m 10% SP-1000 column; 60–200°C at 10°/min). (1), (2), and (3) correspond to components illustrated in Structure I.

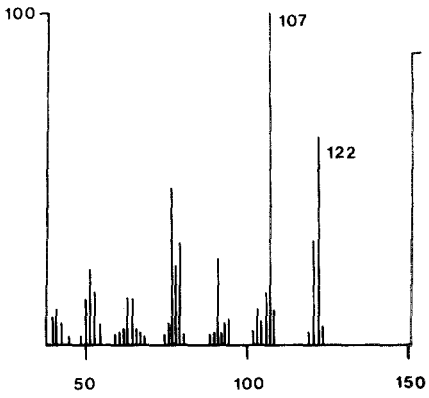


FIG. 3. Mass spectrum of 2,3-dimethylphenol.

compared chromatographically with 2-methyl-5-ethylphenol standards. The average amount was determined to be 250 μg per individual by peak area integration.

Two minor components (each 1%) were also observed in these secretions (Figure 2). Comparison with authentic samples of dimethylphenols demonstrated that the component eluting first (molecular weight 122) was 2,3-dimethylphenol [(2) in Structure I] (Figure 3). The second component, eluting prior to the major compound had a molecular weight of 150 (Figure 4). A sample of 2,3-dimethyl-5-ethylphenol [(3) in Structure I] synthesized by a method analogous to that of Morgan and Pettet (1934) from *o*-xylene had a mass spectrum and retention time identical to those of the third component. The intermediates of this synthesis and the 2-ethyl-5-methylphenol cited above had infrared, nuclear magnetic resonance, and mass spectra consistent with the assigned structures.

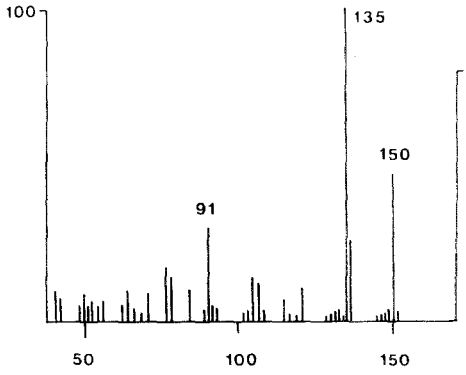
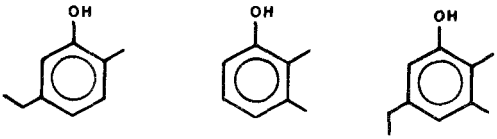


FIG. 4. Mass spectrum of 2,3-dimethyl-5-ethylphenol.



STRUCTURE I.

Field Studies of Defensive Behavior. The defensive capabilities of *S. spinifera* were tested against two ant species, *Camponotus floridanus* and *Solenopsis invicta*, both found nesting at the collection site and both well known for predatory behavior. In the field, single specimens of *S. spinifera* were placed in exposed ant colonies. When the specimen was placed in the *C. floridanus* colony, the first ant to encounter it immediately began to bite it and spray it with formic acid. Other conspecifics soon converged at the disturbance and joined the attack. At this point, the opilionid released some of its chemical arsenal, as demonstrated by the appearance of a strong phenolic odor, and the ants immediately retreated. After that, the ants ignored the opilionid. The same sequence of events was observed each of the ten times a specimen of *S. spinifera* was placed in the ant colony.

Opilionid specimens were attacked in the *S. invicta* colonies as quickly as in the *C. floridanus* colonies. As soon as the opilionid released its defensive secretion, the ants ceased attacking and the opilionid walked slowly away. If the escaped specimen was gently placed on a piece of paper, a strong phenolic odor could be detected. Consistent with these behaviors, the ants also ignored filter paper disks treated with the standard compounds.

Specimens of *S. spinifera* were placed in cages with anoles (*Anolis sagrei*) also caught at the collection site. The anoles did not eat the opilionids even though mealworms and flies placed in their cages were quickly consumed.

S. spinifera employs a novel method for the dispersal of its defensive exudates. When a specimen is held by a leg with forceps, a clear droplet appears near each ozopore (A, Figure 5). These droplets may be of enteric origin, and dissection of preserved specimens suggests that they may travel from the mouth area to the ozopores by means of well-defined channels between the first and second leg coxae. The exocrine secretion is released into these droplets, which, under high magnification, appear to be formed of two colorless, immiscible fluids. Seconds after the initial release, a second droplet appears at each posterior lateral corner of the scutum. The anterior droplets diminish in size as the posterior droplets are formed. It is presumed that the secretion flows, by capillary action, along a groove (B, Figure 5) which can be seen just above the margin of the scute extending from the region of the ozopore to the posterior lateral corner. The groove is limited ventrally by the rebordered margin of the scute itself and dorsally by a row of confluent

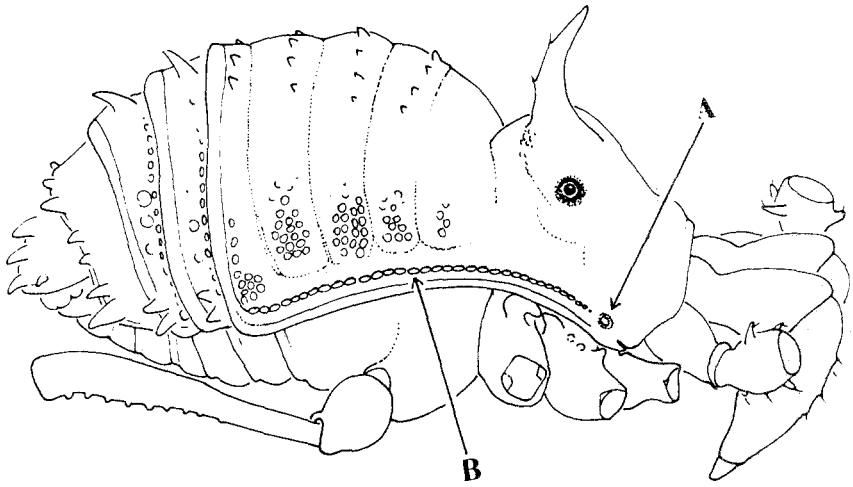


FIG. 5. *Stygnomma spinifera* (male), view slightly dorsal of lateral. A, ozopore, B, lateral marginal groove of scutum.

tubercles representing an exaggeration of the ordinary cuticular sculpture. The posterior droplets become smaller as the secretion spreads out over the free abdominal tergites, aided in part by the movements of the femora and tibiae of the fourth pair of legs.

DISCUSSION

Previous investigations have demonstrated that phenols are widespread constituents of arthropod defensive secretions (Altman and Dittmer, 1973). The phenol 2-methyl-5-ethylphenol, however, appears to be restricted to opilionids and principally to the families in the suborder Laniatores. The work of Eisner et al. (1977) has demonstrated its presence along with 2,3-dimethylphenol in a single tropical species of Cosmetidae and tentatively in a species in the Gonyleptidae where the secretion is fortified with 2,3-dimethyl-1,4-benzoquinone. The present work is the first report of 2,3-dimethyl-5-ethylphenol in arthropods.

Taxonomically, the majority of American laniatorids are now included in the family Phalangodidae even though some species probably belong to other families. The center of the distribution and the evolution of the laniatorids is in the tropics, but the taxonomic affinities of these opilionids in the Neotropical realm are unknown. Our studies (W.A.S.) suggest that the Stygnommatidae, formerly regarded as a subfamily of Phalangodidae, may be the sister group of the Gonyleptidae. Thus it is not surprising to find

2-methyl-5-ethylphenol in the defensive exudates of Neotropical representatives of both the Stygnommatidae and Gonyleptidae. Chemical data on the laniatorids may help establish clear relationships among their taxa.

Previous observations on the laniatorid genera *Cynorta*, *Paecilaemella*, and *Vonones* (Eisner, 1971; Eisner et al., 1977; Jones et al., 1977) have shown that specimens administer their defensive exocrine products by "leg dabbing." When disturbed they simultaneously regurgitate an enteric fluid from the mouth and discharge a defensive secretion from the two exocrine glands into this fluid. The legs are dipped into the fluid and the secretion is administered with deliberate strokes onto the foreign objects causing the disturbance.

There are mechanisms other than "leg dabbing" by which opilionids deliver their defensive secretions. Some laniatorids release a fine jet of defensive fluid 2 cm or more above the carapace (Lawrence, 1938). In *Leiobunum* species, a fine spray is emitted from ozopores uniformly wetting the dorsal surface of the prosoma and opisthosoma and increasing the evaporative surface (Blum and Edgar, 1971).

The delivery system of *S. spinifera* represents yet another mechanism for disseminating the glandular secretions of opilionids. Exudates mixed with enteric fluids flow posteriorly along lateral grooves in the carapace, collecting at the posterior end of the abdomen and eventually spreading over the rugose areas circumscribing the abdomen. Lawrence (1938) has made similar observations on South African laniatorid species.

While "leg dabbing" behavioral repertoires emphasize the irritant function of some defensive secretions, the delivery system of *S. spinifera* emphasizes a repellent or masking function. If the secretion of *S. spinifera* were primarily a topical irritant, it would be surprising to find species of laniatorids in which it is spread over the body. This diffuse dissemination of the secretion dramatically increases the evaporative surface area and surrounds the organism with a chemical shield.

The behaviors of phalangids both in the field and in the laboratory show that the release of the defensive exudates is a survival strategy of the last resort to evade predators. Different species often show slightly different defensive strategies. However, a number of generalizations can be made. When first disturbed, agile runners move quickly to retreat from danger. There are some species which pull in their legs, instead of running, and lie motionless. Other species, if disturbed above the ground, will drop to the ground and feign death. These behaviors are particularly effective since many species are cryptically colored and blend into the ground cover. Other opilionids, due to their very long legs, carry their bodies well above the ground with their vital organs out of reach of small predators such as ants. Eisner et al. (1971) have pointed out that many opilionids frequently regurgitate when disturbed. Thus, opilionids exhibit a number of evasive behavior patterns when faced with danger. If these evasive measures are not successful, only then will the

opilionid release some of their exocrine products, either from one ozopore or from both synchronously. Lawrence (1938) has observed that some specimens can release up to 5–6 emissions before their chemical arsenals have been depleted. Defensive secretions may be used conservatively so that adequate levels of defensive exudate are maintained for future encounters.

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CHEMISTRY OF THE DUFOUR'S GLAND SECRETIONS OF NORTH AMERICAN ANDRENID BEES (HYMENOPTERA: ANDRENIDAE)

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Abstract—The volatile components of the Dufour's gland secretions were examined in 22 Nearctic species of andrenid bees representing 10 subgenera. Farnesyl hexanoate was the dominant component in the secretion of 17 species. In others, various terpenoid esters such as geranyl octanoate, farnesyl octanoate and geranylgeranyl octanoate were the major components. Approximately 30 compounds have been identified in the secretions; many are newly identified in andrenid bees. Three different groups of secretions are discerned with "exceptions." A discussion of the possible function of the Dufour's gland secretion in the biology of, and its use in, the systematics of these bees is presented.

Key Words—Bees, exocrine product, mass spectrometry, terpenoid esters, phylogeny, nesting biology, Dufour's gland, *Andrena*, Hymenoptera, Andrenidae.

INTRODUCTION

Solitary bees belonging to the genus *Andrena* (Andreninae: Andrenidae) are among the most common spring bees in northern temperate areas. They are important for the pollination of many species of plants. The flight periods for some species are very short and can be correlated with the blooming periods of limited groups of plants. Andrenids nest in the soil, sometimes in populous aggregations (Michener, 1974). Nests consist of a main vertical shaft with short lateral tunnels leading to the brood cells. These cells are lined with a

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TABLE 1. COMPOUNDS IDENTIFIED IN DUFOUR'S SECRETIONS OF NORTH AMERICAN ANDRENID BEES[a]

Subgenus	Geranyl hexanoate	Geranyl octanoate	Geranyl decanoate	Geranyl dodecanoate	*trans*-β-farnesene	*trans,trans*-α-farnesene	Farnesyl acetate	Farnesyl butanoate	Farnesyl hexanoate	Farnesyl octanoate	Farnesyl decanoate	Farnesyl dodecanoate	Geranylgeranyl octanoate	Octyl hexanoate	Decyl hexanoate
Unassigned															
A. flexa	X	tr			tr		X	tr	M	X	X	X			
Andrena															
A. tridens	X	M	X	X					tr	tr	tr				
A. mandibularis		M								X					
Callandrena															
A. gardineri						tr	tr	tr	M	X	tr	tr			
Larandrena															
A. miserabilis		X								M					
Leucandrena															
A. erythronii									M	X					
A. placida										tr			M		
Melandrena															
A. carlini					tr	tr	tr	tr	M	tr	tr			tr	tr
A. confederata	X	X	X				tr	tr	M	X	X				
A. nivalis		tr			tr	tr		tr	M	X	tr				
A. pruni						tr	tr	tr	M	tr	tr				
A. vicina					tr	tr	tr	tr	M						X
Opandrena															
A. cressonii							tr		M	tr	tr	tr			
A. cressoni									M						
Parandrena															
A. andrenoides									M						
Scrapteropsis															
A. fenningeri							tr	tr	M		tr				
A. ilicis									M	tr					X
A. imitatrix							tr	tr	M		tr				
A. imitatrix									M	X					
Simandrena															
A. nasonii							tr	tr	M						
Thysandrena															
A. bisalicis		X			tr	tr	tr	tr	M	tr					
Trachandrena															
A. forbesii									M						
A. hippotes								tr	M						
A. hippotes									M						
A. mariae									M						
A. nuda					tr	tr	tr	tr	M						
A. nuda									M						
A. rugosa					tr	tr	tr	tr	M						
A. spiraena	X	X					tr	tr	M	tr					
Tylandrena															
A. perplexa														tr	X

[a]M = major compound, X = present, tr = trace. Reference: (1) this study; (2) Bergström and Tengö, 1978.

Octyl octanoate	Dodecyl hexanoate	Decyl octanoate	Tetradecyl hexanoate	Dodecyl octanoate	Hexadecyl hexanoate	Tetradecyl octanoate	Octadecyl hexanoate	Hexadecyl octanoate	Eicosyl hexanoate	Octadecyl octanoate	Dodecyl tetradecanoate	Docosyl hexanoate	Eicosyl octanoate	Tetradecyl tetradecanoate	Reference
															1
															1
															2
															1
															1
															2
															1
tr	tr	tr	tr	tr	tr	tr		tr							1
															1
															1
															1
tr	X	tr	X	tr											1
															1
															2
															2
															1
tr	X	tr	X	tr	tr	tr	tr	tr	tr	tr	tr				1
															1
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															1
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tr	M	X	X	X	X	X	tr	tr	tr	tr	tr	tr	tr	tr	1

coating probably secreted from the Dufour's gland of the female (Tengò and Bergström, 1977).

The Dufour's gland or basic gland is associated with the sting apparatus of females and occupies approximately half of the abdominal cavity. The freshly excised gland is opaque, has irregular transverse wrinkles, and is positioned in a U shape (Lello, 1971). In some of the large *Melandrena* species, this exocrine gland may be 3–4 cm in length and contain 1–2 μl of fluid. If the gland is ruptured under water during dissection, a clear immiscible fluid is released which floats on the surface of the water.

Previous investigations of the chemical composition of the Dufour's gland in 19 Palearctic (Bergstrom and Tengò, 1974; Tengò and Bergstrom, 1975) and 9 Nearctic (Tengö and Bergstrom, 1978) species of *Andrena* have revealed that all-*trans*-farnesyl hexanoate and geranyl octanoate are major components. Traces of other esters also appear.

We have expanded the catalog of Dufour's chemistry by analyzing the secretions of 22 species of *Andrena* representing 10 subgenera. Four of these are the same species as those analyzed by Tengö and Bergstrom (1978); however, we report here the presence of previously unidentified minor components.

Another interesting question concerns the species specificity of andrenid Dufour's chemistry. We discuss our findings and correlate them with those of Tengö and Bergstrom in an effort to answer this question. We also discuss possible roles of these components in the biology of these bees.

METHODS AND MATERIALS

The species listed in Table 1 were collected at flowers or at nesting sites in College Park, Maryland, and at the Marine Training Base, Quantico Virginia, from early April through July during 1978 and 1979. Individual specimens were placed in clean glass shell vials and stored in an ice chest. In the laboratory they were maintained at 4° C until dissection, usually one or two days later. The Dufour's glands were excised under water and extracted individually in 1-dram vials with 0.1 ml methylene chloride. Each bee was pinned and given an accession number which also identified the Dufour's extract. After the specimens were identified (W.E.L.), extracts from the same species were combined for chemical analysis.

Analysis of extracts were conducted on a computerized Finnigan 3200 gas chromatograph–mass spectrometer (GC-MS) equipped with a 1-m × 1-mm (ID) glass column utilizing 3% OV-17 or 10% SP-1000 as a stationary phase. The column was programed at 10° C/min from 60° to 200° C (or 300° for OV-17). Individual compounds were identified by comparison of their mass spectra and retention times to those of known standards.

The farnesyl and geranyl esters and their alkyl counterparts were synthesized from the corresponding alcohols and fatty acids using the *N,N'*-carbonyl diimidazole (CDI) method of Staab (1962) or a mixture of sodium acetate and the corresponding acid anhydride. The latter method gave better than 90% of the corresponding esters with a minimum of side products. Purification was achieved by column chromatography over neutral alumina using mixtures of pentane and methylene chloride as solvents.

RESULTS

The compounds identified from the 22 species of andrenid bees analyzed are listed in Table 1. As is apparent, three major types of secretions are involved. The first is the predominant type in which the secretions have only terpenoid esters based on the alcohols farnesol and geraniol. Figure 1 shows a typical gas chromatogram of methylene chloride extracts from *A. flexa*. The major component is farnesyl hexanoate, easily recognized by the base peak at m/z 69, appreciable ions at 203 and 204, the molecular ion at 320, and the C_6 acylium ion (RCO^+) at m/z 99. The other terpenoid esters show similar fragmentation ions with appreciable acylium ions, base peaks of m/z 69, and weak molecular ions. Two types of bis-homolog esters are apparent: Farnesyl esters of even-carbon-numbered fatty acids from C_2 to C_{12} in addition to geranyl esters of even-carbon-numbered fatty acids from C_6 to C_{12}. There is

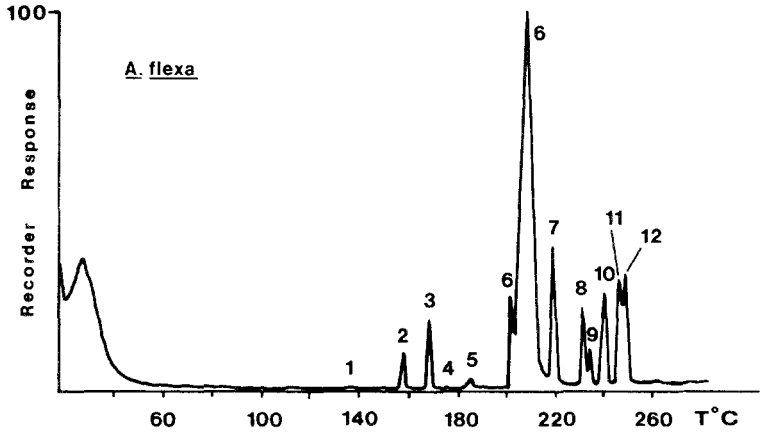

FIG. 1. Gas chromatogram of *Andrena flexa* Dufour's secretion. Peaks correspond to (1) *trans*-β-farnesene, (2) geranyl hexanoate, (3) farnesyl acetate, (4) geranyl octanoate, (5) farnesyl butanoate, (6) farnesyl hexanoate, (7) farnesyl octanoate, (8) unknown ester, (9) farnesyl decanoate, (10) unknown ester, (11) unknown ester, (12) farnesyl dodecanoate. (Drawing from originals.)

no indication of isomers other than the all-*trans* farnesol and geraniol in any of these esters.

The second type is exemplified by the extract of *A. perplexa* which shows no trace of these terpenoid esters (Table 1). It releases a complex mixture of straight-chain esters which are mostly hexanoates and octanoates, ranging from C_8 to the C_{20} alcohol. Although the gas chromatogram from *A. perplexa* (Figure 2) has only six peaks, each of these peaks is in reality a mixture of isomeric esters. The peaks eluting earlier are simpler mixtures than the later peaks. These isomeric mixtures were demonstrated by mixing authentic samples of the esters and analyzing for two different peaks in each ester. The first of these is the acylium ion (RCO^+) as in the terpenoid esters, and the second is the $RCO_2H_2^+$ ion formed by the alkyl ester cleavage and transfer of two hydrogens. Each of these peaks is prominant for the alkyl esters and can be used to quantify the amount of that ester present in the mixture. Although other acids are used to prepare these esters, the bees depend upon the C_6 and C_8 acids predominantly.

The third type of andrenid species has both the terpenoid esters and the straight-chain esters in its Dufour's secretion. *A. vicina* (Figure 3) is a typical example.

All of the andrenid species analyzed fall into the three categories listed above, with three exceptions. These are: *A. mandibularis* whose major component is the monoterpenoid ester geranyl octanoate; *A. miserabilis* whose major component is farnesyl octanoate; and *A. placida*, the only

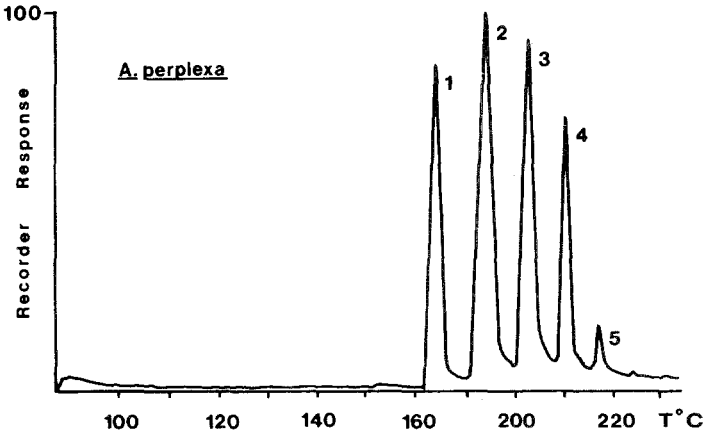

FIG. 2. Gas chromatogram of *Andrena* (*Tylandrena*) *perplexa* Dufour's secretion. Peaks correspond to (1) decyl hexanoate and octyl octanoate, (2) dodecyl hexanoate and decyl octanoate, (3) tetradecyl hexanoate and dodecyl octanoate, (4) hexadecyl hexanoate and tetradecyl octanoate, (5) octadecyl hexanoate and hexadecyl octanoate. (Drawing from originals.)

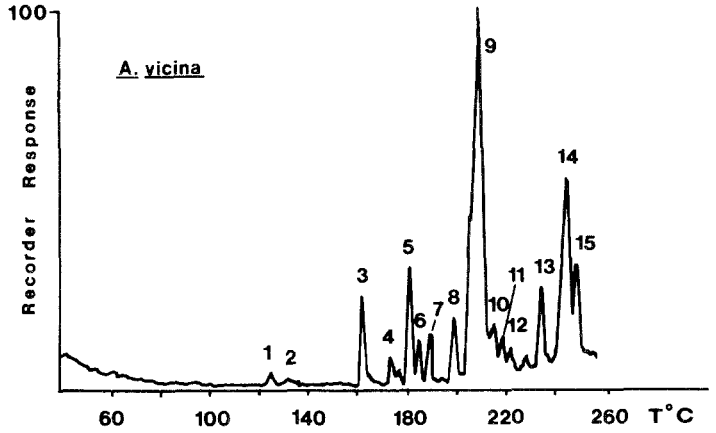

FIG. 3. Gas chromatogram of *Andrena* (*Melandrena*) *vicina* Dufour's secretion. Peaks correspond to (1) *trans*-β-farnesene, (2) *trans,trans*-α-farnesene, (3) decyl hexanoate and octyl octanoate, (4) farnesyl acetate, (5) dodecyl hexanoate and decyl octanoate, (6) C_{21} alkane, (7) farnesyl butanoate, (8) tetradecyl hexanoate and dodecyl octanoate, (9) farnesyl hexanoate, (10) hexadecyl hexanoate and tetradecyl octanoate, (11) C_{25} alkane, (12) farnesyl octanoate, (13) farnesyl decanoate, (14) farnesyl dodecanoate, (15) unknown. (Drawing from original.)

species with a terpene larger than farnesol. Its major component is geranylgeranyl octanoate, and the only other terpene detected is farnesyl octanoate.

DISCUSSION

Chemistry of Dufour's Gland Secretion. The chemistry of the Dufour's gland secretion has been studied in taxa representing five families of solitary and social bees. Dufour's extract of *Melissodes desponsa* (Anthophoridae) contains *n*-tetradecyl acetate, dihydrofarnesyl acetate, and farnesyl acetate (Batra and Hefetz, 1979); however, its function is unknown. In contrast, *Xylocopa virginica* (Anthophoridae) has Dufour's exudates dominated by long-chained hydrocarbons (Vinson et al., 1978). Species in the Halictidae produce homologous series of saturated and unsaturated macrocyclic lactones ranging from C_{16} to C_{26} (Andersson et al., 1966; Bergström, 1974; Bergström and Tengö, 1979; Duffield et al., 1981; Hefetz et al., 1978). Similarly, species in the Colletidae have Dufour's gland secretions dominated by macrocyclic lactones (Andersson et al., 1966; Bergström, 1974; Duffield et al., 1980; Hefetz et al., 1979). Octadecyl butanoate as well as other esters are major components in the Dufour's gland exudates of *Melitta* (Melittidae) (Tengö and Bergstrom, 1976). Species in the Andrenidae produce exudates

dominated by farnesyl hexanoate, geranyl octanoate, or a variety of other esters (Bergstrom and Tengö, 1974; Tengö and Bergström, 1975, 1978).

As indicated in Table 1, by far the most prevalent compound identified in the Dufour's glands of Nearctic bees is farnesyl hexanoate, identified as the major compound in 17 species as well as being present in one additional species. This substantiates the findings of Bergström and Tengö on European andrenids.

This study has gone beyond previous work to suggest three different classifications of secretions in andrenid bees. Some species produce terpenoid esters. These may be either farnesyl hexanoate or geranyl octanoate, as the major constituents of the Dufour's secretions (Table 1), or two other terpenes. Thus farnesyl octanoate dominates the Dufour's secretions of *A. miserabilis* (*Larandrena*) and geranylgeranyl octanoate those of *A. placida* (*Larandrena*). Other esters are also present in minor amounts in each species (Table 1).

The second type is exemplified by the Dufour's gland secretions of *A. perplexa* (*Tylandrena*) which contain no terpenoid esters at all. Instead, this species uses octanoic and hexanoic acids as bases, but aliphatic alcohols rather than geraniol or farnesol. Mixtures of isomers predominate (e.g., *n*-octyl octanoate and *n*-decyl hexanoate both with molecular weights of 256). Although it is not possible to separate these isomeric esters on the gas chromatography columns used, the composite mass spectra indicate distinctly what the components are from the RCO^+ and $RCO_2H_2^+$. Some of these chromatographic peaks have been found to contain as many as six isomeric esters. Synthetic mixtures have been used to verify these isomeric mixtures.

The third variation is typified in the species *A. vicina*, *A. ilicis*, and *A. nasonii*, which secrete mixtures of two types of components: farnesyl esters and straight-chain hexanoates and octanoates.

Methodology. Four species listed in Table 1 were investigated both in this study and by Tengö and Bergström (1978). Traces of additional compounds not reported by Tengö and Bergström were detected in all four species. We believe this is the result of differences in technique. While the Swedish group employed extracts of 1–2 glands and analyzed them employing a capillary column, we analyzed extracts containing as many as 20–30 glands and used a shorter length but larger diameter column.

Although it appears that the major component is the only compound detected in some cases because of concentration and sample size, analysis of a 10-fold concentrated sample of 34 Dufour's glands of *A. nasonii* showed only traces of two terpene esters in addition to the major component. It thus appears that the complexity of some andrenid Dufour's secretions and the lack of complexity of others is real.

Function of Dufour's Gland Secretion. Several workers have maintained that one function of the Dufour's gland secretions in andrenid bees is to line

the brood cells. For example Tengö (1979) states that the secretion is utilized in the lining of the nest cells; however, no comparative chemical data on the cell linings and Dufour's secretions is presented. The hypothesis is very feasible since both *Colletes* (Colletidae) (Hefetz et al., 1979) and *Augochlora* (Halictidae) (Duffield et al., 1981) line the brood cells with secretions from the Dufour's gland.

The Dufour's secretions possibly play a role in nest marking. These odors may be either species specific or specific to each individual as indicated in halictid bees by the studies of Barrows (1975; Barrows et al., 1975). Experiments suggest male *Andrena flavipes* recognize the nest site by its characteristic odor as well as by visual cues when they search for females (Butler, 1965). Females apparently remain within the nest sites where they are reared; some of these nest sites persist for years (Butler, 1965). These hypotheses are consistent with our data which show such a wide variation in Dufour's chemistry. The similarities in some species may or may not be biologically important since these may be geographically or temporally separated in their nesting behavior.

Taxonomy. Classification schemes have traditionally been based on comparisons of morphology, life history, and zoogeographical data. Here we have used the additional data from the Dufour's gland chemistry to determine whether the chemical information is consistent with previously established schemes. It must be noted that these analyses are preliminary. More species and more complete analyses of each species are required before any firm conclusions can be made.

Scapteropsis is a relatively primitive subgenus which does not occur in the Old World (LaBerge, 1971). In the three species analyzed, all secretions are dominated by farnesyl hexanoate. The chemistry of *A. ilicis*, however, appears to be quite different from *A. fenningeri* and *A. imitatrix*. *Trachandrena* is a relatively specialized subgenus most closely related and probably derived from *Scapteropsis* (LaBerge, 1973). It is thought to have arisen in the Nearctic region and later spread to the Palearctic region. The chemistry of the six species analyzed appears to be quite consistent.

Both *Melandrena* (Bouseman and LaBerge, 1979) and *Thysandrena* (LaBerge, 1977) are thought to have arisen from *Euandrena*. The Dufour's gland secretions of the five species of *Melandrena* and the one species of *Thysandrena* analyzed all are dominated by farnesyl hexanoate. In addition, some species in each subgenus also exhibit geranyl esters. *A. flexa*, which was originally placed in *Gymnandrena* [a synonym of *Melandrena* by Mitchell (1960)], differs from these species by having a series of nonterpenoid esters. This is interesting since Bouseman and LaBerge (1979) do not include *A. flexa* in *Melandrena*. One of us (W.E.L.) proposes to include it in a new subgenus with *A. macra*.

Species in the subgenus *Andrena* represent a unified group. The Dufour's secretions are dominated by geranyl octanoate; thus the chemistry supports their systematic position which is based on morphology.

The nearest relative of *Parandrena* appears to be the more primitive subgenus, *Larandrena*, which may have migrated to North America twice. The only North American representative of *Larandrena* known today is *A. miserabilis*, which is probably not derived from the same ancestor as our *Parandrena*. The chemistry of the Dufour's secretion of *A. miserabilis* is unlike any other andrenid analyzed. The only representative of *Parandrena* analyzed is *A. andrenoides*; its chemistry is very typical.

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SEXUALLY DIMORPHIC SETIFEROUS SEX PATCH IN THE MALE RED FLOUR BEETLE, *Tribolium castaneum* (HERBST) (COLEOPTERA: TENEBRIONIDAE): Site of Aggregation Pheromone Production[1,2]

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Abstract—Evidence for the existence of a male-produced aggregation pheromone secreted from the prothoracic femoral setiferous sex patch of *Tribolium castaneum* is reported. Both sexes were attracted to *ca.* 60 ng of crude secretion. Males and females perceive the pheromone on the day of emergence while perception differs between the sexes: male response reaches a maximum on day 1 posteclosion, when tested at <1, 1, and 30 days; females show a maximum response at 30 days posteclosion. Behavioral responses to pheromone odors and a complex chromatographic profile are reported.

Key Words—Sexual dimorphism, *Tribolium castaneum* (Herbst), Coleoptera, Tenebrionidae, aggregation pheromone, behavior, bioassay, red flour beetle.

INTRODUCTION

The red flour beetle, *Tribolium castaneum* (Herbst), is a well-known cereal and stored-product pest of cosmopolitan distribution (Sokoloff, 1974). When

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[2]Dedicated to Dr. Alexander Sokoloff for his monumental contributions to the studies on *Tribolium* and upon the occasion of his 60th birthday.
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this insect is disturbed, glands present on its thorax and abdomen secrete benzoquinones as a defense mechanism (Roth, 1943; Alexander and Barton, 1943; Loconti and Roth, 1953; Happ, 1968; Markarian et al., 1978). Keville and Kannowski (1975) first reported the presence of male- and female-secreted pheromones, thought to comprise 1-pentadecene, *n*-hexadecane, 1-heptadecene, and heptadecadiene, which are attractive only to male *T. confusum* Jacquelin duVal. Ryan and O'Ceallachain (1976, 1977) later discovered a male-secreted aggregation pheromone which is attractive to both sexes and a female-secreted sex pheromone which is attractive to male *T. confusum*. Suzuki et al. (1975), while working with *T. confusum* and with *T. castaneum*, identified seven unsaturated hydrocarbons (1-tetradecene, 1-pentadecene, 1-hexadecene, 1-heptadecene, 1,6-pentadecadiene, 1,8-heptadecadiene, and heptadecatriene) which, in admixture, have repellent qualities and which therefore act as alarm pheromones. Suzuki and Sugawara (1979) also reported the isolation of an aggregation pheromone, of unknown structure, from mixed species of both sexes.

In previous packed-column gas-chromatographic (GC) studies of volatile extracts from *T. castaneum* and *T. confusum*, relatively simple compositional profiles were obtained (Keville and Kannowski, 1975; Suzuki et al., 1975; Baker et al., 1978; Markarian et al., 1978); many of the major-component peaks were thought to be hydrocarbons (Keville and Kannowski, 1975; Suzuki et al., 1975; Baker et al., 1978; Markarian et al., 1978), quinones (Markarian et al., 1978), and unidentified biosolvents (Markarian et al., 1978).

Hinton (1942) differentiated the sexes of the confused flour beetle *T. confusum* and red flour beetle *T. castaneum*, by the presence of a subbasal setiferous sex patch on the ventral side of the male femur, a structure which is absent in the female. Sokoloff (1972) observed the femoral sex patch in males is encrusted with flour in old beetles and suggested that this accretion could result from a beetle secretion. Faustini (1981) confirmed the presence of a lipid secretion produced at the setiferous sex patch on the male prothoracic femur in *T. castaneum*. Since the function of this character has been overlooked by systematists, its role has been neglected with regard to the behavior of the insect. It appears in fact to be important from the standpoint of population regulation via an apparently sophisticated chemical communication system. Because of the importance of this insect with regard to cereals, we report here further studies of the biological activity of this secretion and its complex gas-chromatographic profile.

METHODS AND MATERIALS

Insects. The beetles were from the Vera Cruz wild-type strain of *Tribolium castaneum* and were obtained from Sokoloff (California State

College, San Bernadino, California) in 1977. They were maintained in 4-oz culture jars containing 20 g of medium, which consisted of whole-wheat flour and 5% brewer's yeast (19:1). The beetles were sexed as pupae by examination of genital lobes (Ho, 1969) and isolated in separate culture containers, with medium, to develop into adults. Insects were maintained on a 16:8 light–dark photoperiod with lights on at 6:00 AM EDT.

Scanning Electron Microscopy. Male beetles were mounted on Cambridge stubs using a fast-drying silver colloidal paint. In order to view the setiferous sex patch, it was necessary to place the entire insect into a hexane bath for ca. 30 min to dissolve the setiferous secretion. Preparations were coated with ca. 200 Å of a 60:40 gold–palladium mixture, using a Hummer I sputter coater, and viewed with a Hitachi S-500 SEM with an accelerating voltage of 20 kV.

Collection of Male Setiferous Secretion. Under light ether anesthesia, experimental males (100 days old) with large globular secretions on the prothoracic femur were held in place with jewelers' forceps while the secretions were removed with an insect minuten pin mounted on a wooden applicator stick. The globules (ca. 1.2 ng/globule) were then placed on the bottom of an empty glass vessel.

Bioassay. The fan-shaped multiple-choice olfactometer bioassay chamber has been previously described in detail (Burkholder, 1970). Briefly, it consists of an attractant choice available to the test insects at one or more of five alternatives equidistant from the point of release. Bioassays were performed in a room which was controlled at 27 ± 2°C and 55 ± 8% relative humidity, illumination being provided by cool white fluorescent lighting. Air flow rate for each choice was controlled at 0.25 liters/min using Gelman flow meters. Twenty-five test beetles were released into the chamber floor and their distribution in the olfactometer was recorded every minute for 16 min. The total number of beetles within each air plume was counted at each observation. Four replicates were counted for each test.

Controls consisted of five vessels containing whole-wheat flour. Treatments consisted of a central vessel of the olfactometer (vessel 3) containing either 50 globules that had been removed from males or 50 globules still remaining on the male prothoracic femora. The remaining four vessels contained whole-wheat flour. Both male and female responses were tested against these treatments. Males or females <1, 1, or 30 days old comprised the experimental groups used to monitor the activity of the male globular secretion or the secretion still intact on the prothoracic leg in 150-day males. Response for each sex to 90-day-old males was also examined for activity without the presence of a prothoracic femoral globule.

After each experiment, the total number of beetles responding to each choice was calculated and expressed as a mean value for each choice. Data were subjected to analysis by the rank sum nonparametric statistical test of

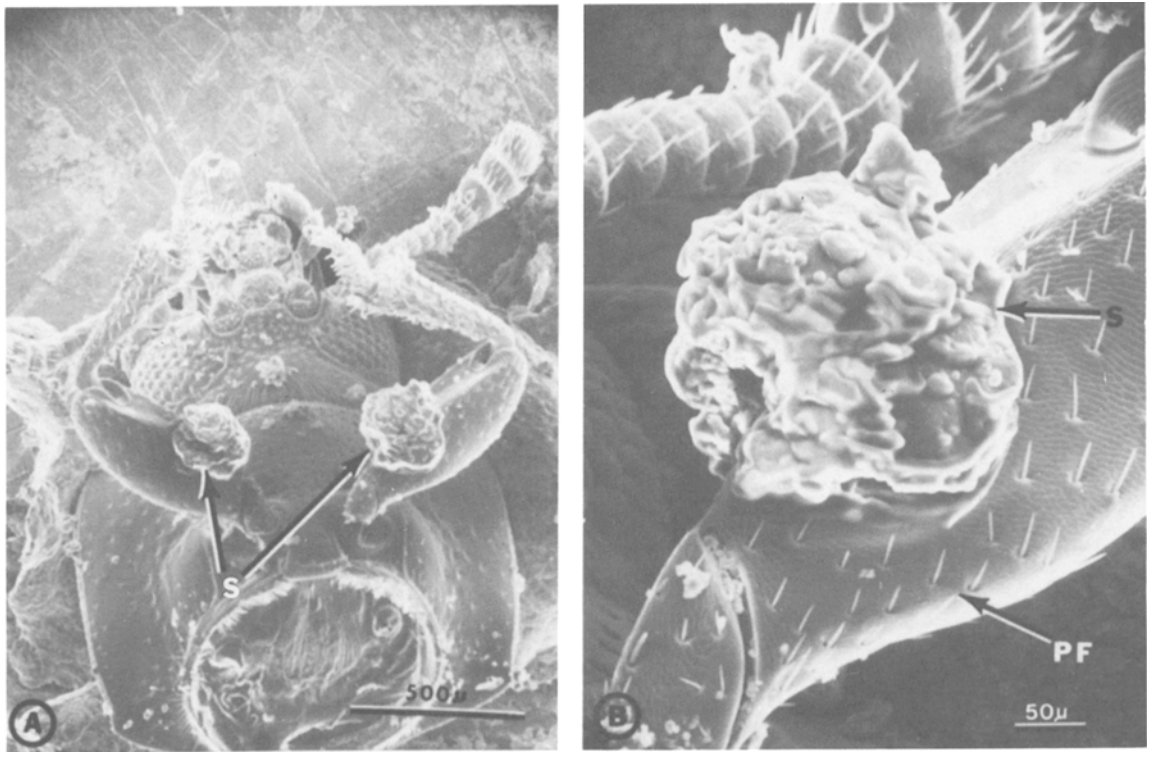

FIG. 1. (A) Ventral view of the male prothoracic femoral sex patch covered by a secretion (S). (B) Enlargement of prothoracic femur (PF) and globular secretion (S). (C) Ventrolateral view of sex patch (SP) in newly emerged male showing setae extending above cuticle. (D) Ventral view of sex patch with fluted setae (FS) and secondary reservoirs (SR) interdispersed between the setae. Area marked with circle is shown in Figure 2.A.

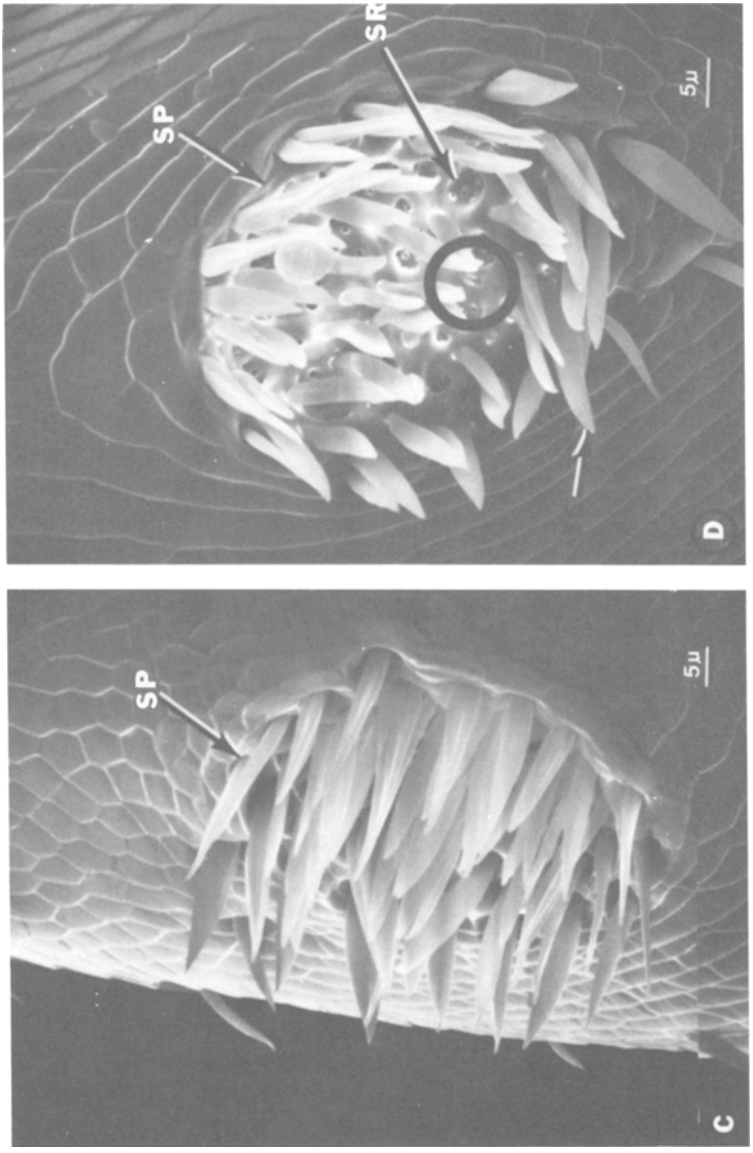

FIG. 1. (cont.)

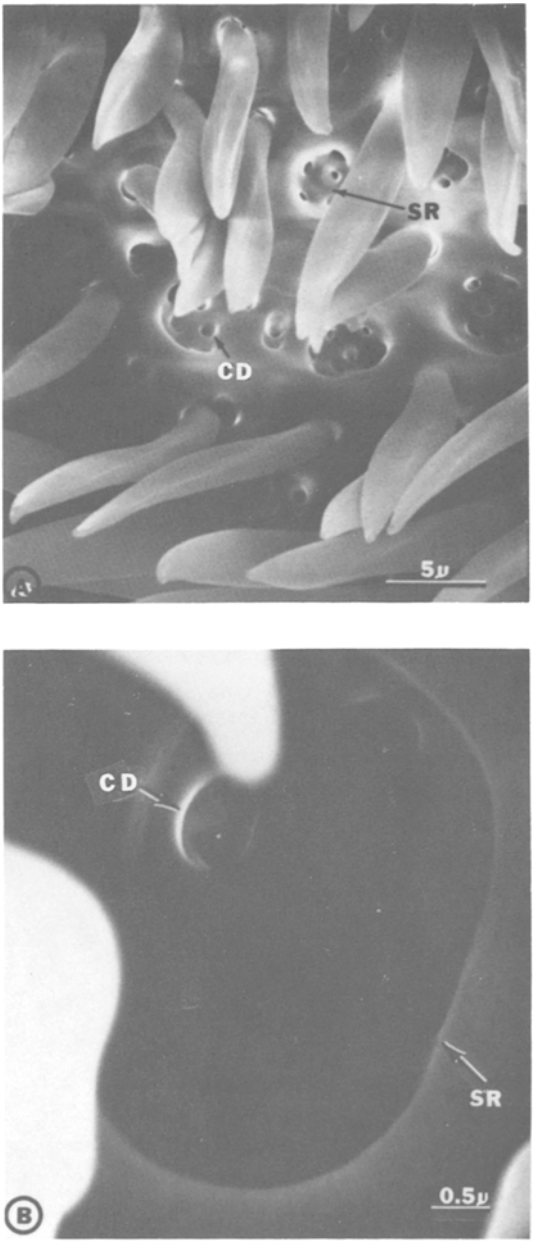

FIG. 2. (A) Enlargement of the secondary reservoirs (SR) within the sex patch. Note the cuticular ducts (CD) within each secondary reservoir. (B) Secondary reservoir containing ca. 5–7 cuticular ducts.

Wilcoxon (Hollander and Wolfe, 1973), and differences only at the 1% level or lower were accepted as statistically significant.

Gas Chromatography. The gas chromatograph was a Varian model 3700 with a laboratory-constructed capillary inlet splitting system. The open-tubular glass-capillary column, also fabricated in the laboratory contained SE-52 stationary phase and was 20 m in length by 0.25 mm (ID) Pyrex. Hydrogen was employed as the carrier gas (10 psi inlet; 25:1 split ratio). Injections of 1.5 μl were employed, whereupon the column temperature, momentarily held at 60°, was taken rapidly to 180°, then at 3°/min to 320°C.

RESULTS

Scanning Electron Microscopy. The setiferous sex patch is completely covered by a subcuticular produced secretion in a 150-day-old male beetle (Figure 1A). The irregular texture of the globule suggests that small flour particles are trapped within the secretion (Figure 1B).

To dissolve the waxy secretion for morphological studies, the entire insect was bathed in hexane for about 35 min. Removal of the secretion subsequently revealed that the sex patch contained 51 $\pm$ 4.6 (n = 50) fluted seta (Figure 1C and D), which appear smooth, short, and ovate. These structures, clearly arise from within the sex patch, which serves as a primary reservoir, and continue above the plane of the cuticle. The setae are particularly sparse within the center of the setiferous sex patch but appear dense in arrangement at its periphery. The outer margin, however, where the femoral cuticular surface forms a sculpture-like texture, is devoid of setae.

Numerous pores (secondary reservoirs) were interspersed among the fluted setae along the cuticular basement of the setiferous sex patch (Figures 1D and 2A). These secondary reservoirs measured 4.3 $\pm$ 0.17 μm (N = 25). Upon close examination, minute cuticular ducts with a diameter of 1.3 $\pm$ 0.25 μm were observed within these structures (Figure 2A and B). Each secondary reservoir contained 5.2 $\pm$ 2.1 cuticular ducts (N = 25).

The secretion accumulates as the beetle becomes older (Figure 3). The secretion migrates out of the setiferous sex patch and onto the outlying cuticle (10 days old), accumulates further in the central region of the patch (30 days old), covers all of the setae within the patch (60 days old), and extends beyond the margin of the patch and continues over the plane of the femoral cuticle (150 days old).

Female and Male Behavioral Response. Characteristic responses by either sex to the source of the male globules and males with large globules on the prothoracic leg included: (1) extension of the prothoracic legs, often

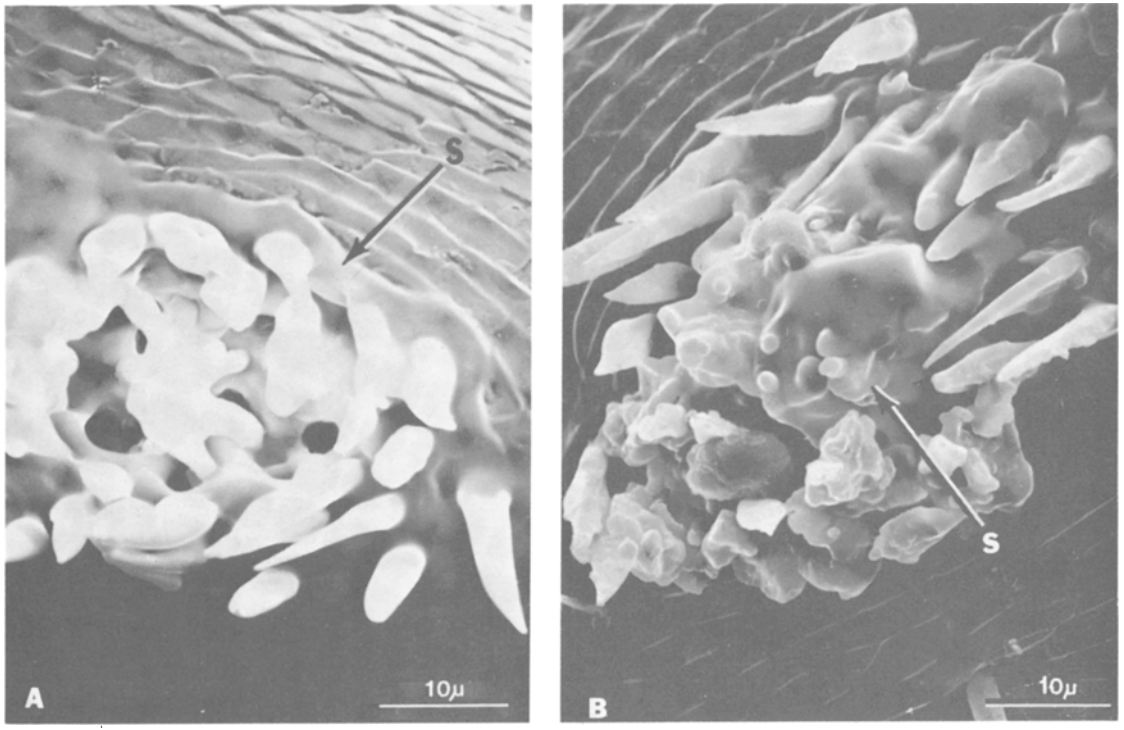

FIG. 3. (A) Ventral view of the sex patch on a 10-day-old male. Secretion (S) is deposited on the margin of the sex patch. (B) Ventral view of the sex patch on a 30-day-old male. Secretion (S) is observed increasing in mass in the central area of the patch. (C) Ventral view of the sex patch in a 60-day-old male. Secretion (S) has completely coated the setiferous structure. (D) Ventrolateral view of male prothoracic femora. The secretion has extended beyond the margin of the patch and continues over the cuticle.

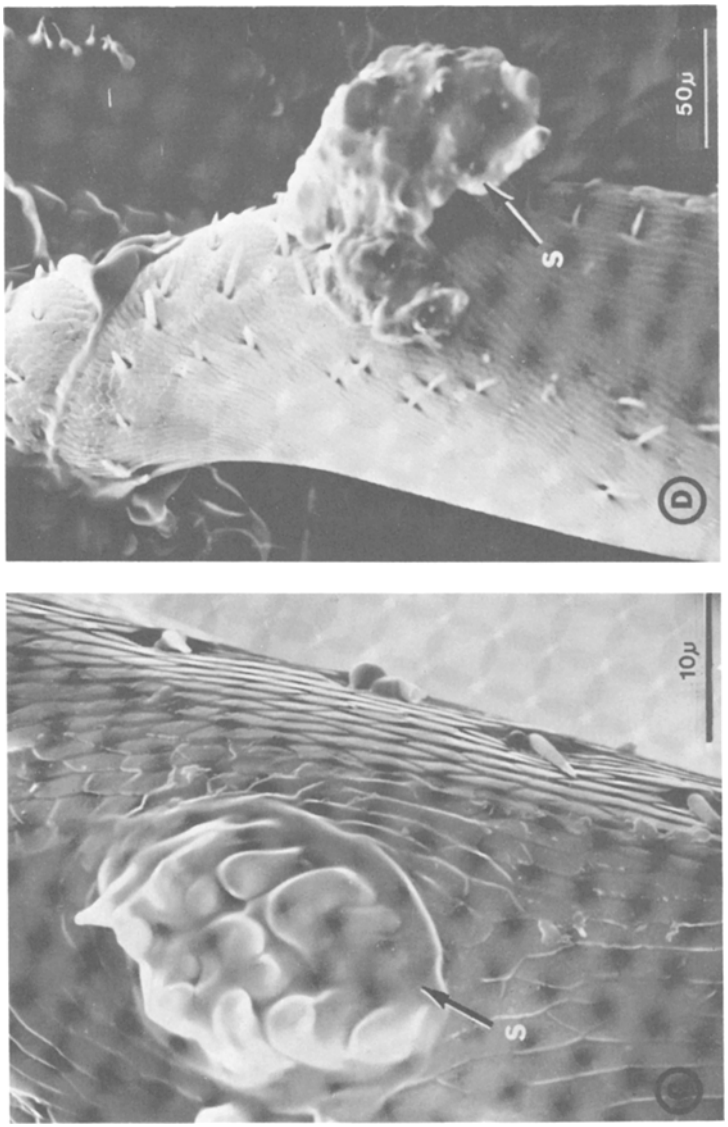

FIG. 3. (cont.)

accompanied with bobbing up and down movements; (2) antennal protraction; and (3) orthokinetic movements in a zigzag manner directly beneath the air plumes of the odorant source. In each of the bioassays, the beetles favored the walls of the olfactometer, maintaining a high degree of wall-surface contact. Thus, after 16 min, the vast majority of the insects were found to reside in slots 1 and 5. Some variation occurred between replicate runs for beetles counted within each slot, which was attributed to conflicts between the time of day during which the assays were conducted and peak biological rhythms. Thigmotactic behavior was reduced in both sexes in conditions where the male secretion was tested.

Multiple-Choice Olfactometer Bioassay: Female Response. Figure 4 summarizes the results of studies of female response to vessels containing whole-wheat flour (controls); only random movement (in several directions) was observed. When a source of 50 male globules (25 male equivalents) was provided in the central vessel, 12-hr posteclosion females responded significantly to the source of odor. Thirty-day posteclosion virgin females showed a slightly higher response than that observed by 12-hr posteclosion females (Figure 4).

Twelve-hour posteclosion female response to odors from 150-day-old males with large prothoracic femoral globules was significant. However, it was slightly lower than that obtained with 50 crude globules. One-day- and 30-day-old posteclosion female responses were greater than 12-hr posteclosion females and flour controls. Male odors, in addition, clearly excited older females (Figure 4).

When 30-day-old females were provided with 90-day-old males without prothoracic femoral globules, a nonsignificant response was found. Furthermore, where two sources of male odors were provided, females showed a stronger response to 150-day-old males with prothoracic globules than to 90-day-old males without globules.

Multiple-Choice Olfactometer Bioassay: Male Response. Results obtained for males responding to whole-wheat flour in the olfactometer were similar to those found for females (Figure 4). When 12-hr posteclosion males were exposed to odors from 50 globules, the response was significant; 30-day-old males showed a slightly higher response. The response for the latter was still significant when compared to the whole-wheat flour control.

Twelve-hour posteclosion males that were provided with an odor source from 150-day-old males with large globules on the prothoracic femora showed a strong attraction when compared to the control (Figure 4). Response increased dramatically from 1-day-old males to the older males; however, a decline in response was recorded for 30-day-old males, although it was still significant.

Similar to the behavior of females, when 30-day-old males were provided with 90-day-old males without globules, a nonsignificant response was

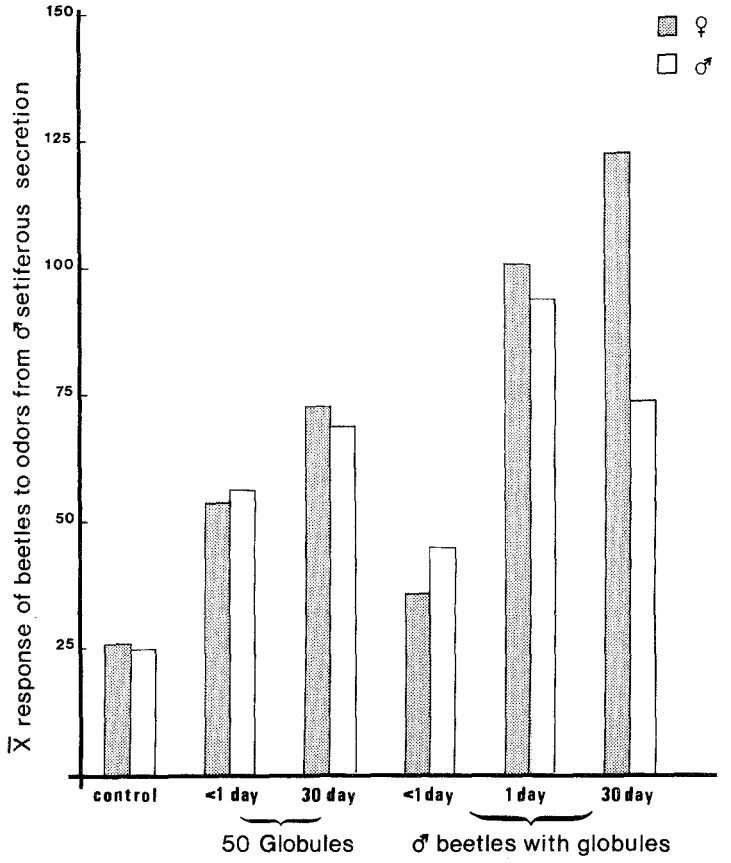

FIG. 4. The mean response to odors from the male secretion. Odors consist of two groups: (1) 50 globules (25 male equivalents); or (2) 25 males with large globules intact on the male prothoracic femora. The treatments consisted of females and males of various age groups, while controls were odors from whole-wheat flour.

obtained. Furthermore, when given a dual choice of male odors, 30-day-old males selected those emanating from 150-day-old males with large globules rather than those from 90-day-old males without globules.

As shown in Figure 4, beetles of both sexes are clearly excited by the odors emanating from the male secretion. It appears, furthermore, that 30-day-old female individuals comprise the most responsive chronological age to the aggregation pheromone. In contrast, of the male groups examined, 1-day-old males show the maximum response.

As mentioned previously, globules of 150-day-old males weighed approximately 1.2 ng/globule (2.4 ng/beetle). Choice tests were based upon

50 crude globules (ca. 60 ng). The activity observed, therefore, was elicited from nanogram levels of secreted material.

Analysis of the Secretion. The gas chromatogram of a hexane solution of combined globule material, shown in Figure 5, clearly illustrates the complex nature of the material: well over 100 peaks are visible with at least 15 major components comprising the bulk of the sample. Little, however, can be said at this time with regard to the chemical makeup of the profile, except to note that an appreciable amount of sample elutes at elevated temperatures, thus implying (and in contrast to the studies cited above) volatility considerably less than that of C_{10} to C_{20} alkanes and olefins.

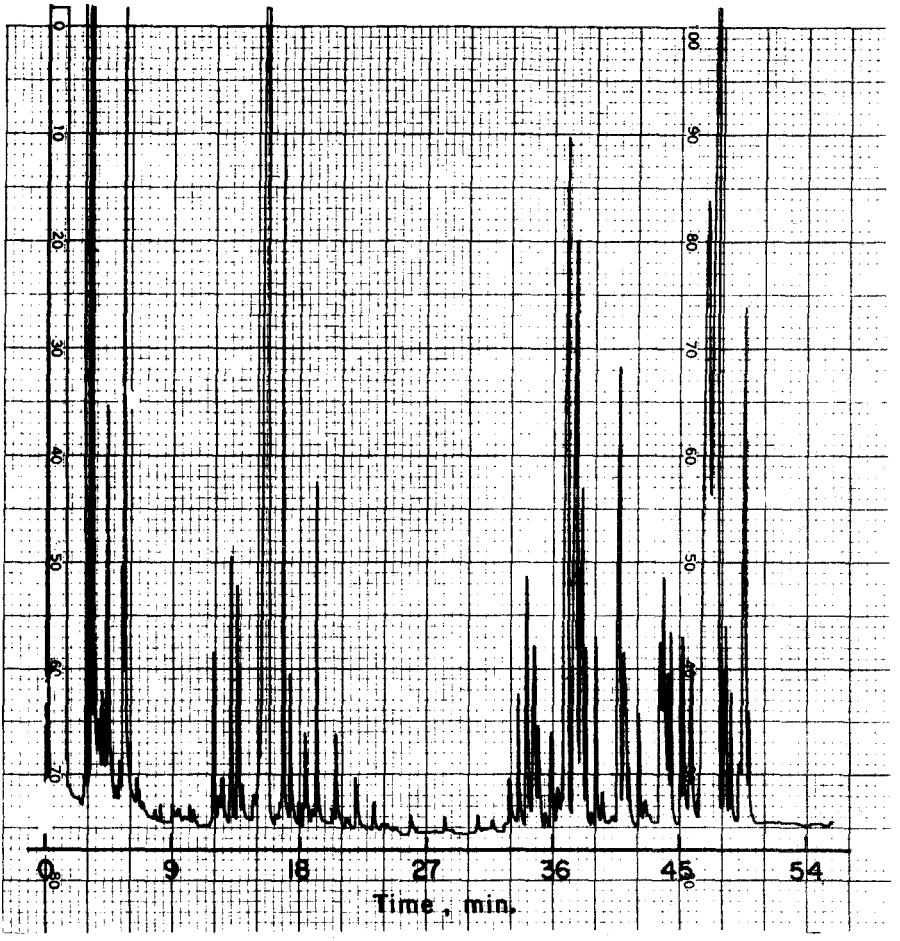

FIG. 5. Gas chromatogram of combined globule material in hexane; see Methods and Materials for column and conditions.

DISCUSSION

Evidence for the existence of a male-produced aggregation pheromone attractive to both sexes has been confirmed clearly by the responses exhibited within the multiple-choice olfactometer. This system has also demonstrated response to male odors over a distance. Both sexes responded significantly to male globules and 150-day-old males with large prothoracic femoral globules, as compared to whole-wheat flour controls. Furthermore, from the external morphology of the femoral sex patch, it appears that the secretion is produced by subcuticular secretory cells, where it is transported into the secondary reservoirs by cuticular ducts. Thus, while Ryan and O'Ceallachain (1976 1977) were the first to report the presence of a male-produced aggregation pheromone attractive to both sexes of *T. confusum*, and Suzuki and Sugawara (1979) reported that male beetles of *T. castaneum* produced an aggregation pheromone, the site of pheromone production (not previously determined) has here been clarified.

This is also the first report of female and male behavioral responses in *T. castaneum* to male-produced pheromone odors, which include leg extension, antennal protraction, and zig-zag locomotion, which are comparable in behavioral response to those of other Coleoptera (Burkholder and Dicke, 1966; Kuwahara et al., 1978; Barak and Burkholder, 1977; Abdel-Kader and Barak, 1979).

Males and females, furthermore, perceive the male-produced pheromone on the day they emerge. The sexes differ, however, in that male perception reaches a maximum between 1 and 30 days after eclosion, while female response continues to increase until 30 days posteclosion. Thus, while male pheromone secretion is apparently a continuous process within the exocrine gland, in point of fact perception of the odors differs between sexes.

Observations of sexually active beetles indicate that there is a correlation between the amount of pheromone released from the exocrine gland and the onset of sexual maturity, i.e., each species produces and perceives pheromones at characteristic ages that are related to sexual maturity (Shorey, 1974). Similar findings have also been noted in other studies. Barratt (1974) found pheromone production in *Stegobium paniceum* (L.) at 3–4 days to be related to female egg production, and O'Ceallachain and Ryan (1977) recorded female pheromone production and male responsiveness to be correlated with mating behavior in *T. confusum*. Thus, the ability of *T. castaneum* males to produce pheromone which attracts 0- to 12-hr-old females is consistent with their ability to mate 0 to 3 hr after eclosion (Dawson, 1964). Further, Good (1936) recorded *T. castaneum* males to be fertile for over 3 years, which is consistent with the present finding that females demonstrate a strong attractiveness to odors from 150-day-old males at 30 days posteclosion.

Hydrocarbon bioassay studies of *T. confusum* by Keville and Kannowski

(1975) seem to indicate that 1-pentadecene, *n*-hexadecane, and 1-heptadecene act as sex stimulants in amounts of 2–2.5 mg of each. Suzuki et al. (1975) found strong repellency in *T. confusum* using the same compounds in lower amounts (1–10 μg). These levels of biological activity appear to us to be high, based upon the amount of compound used in the assay. Thus, Suzuki and Sugawara (1979) found attractancy to the male-produced aggregation pheromone in *T. castaneum* with as little as 3.2–32 ng of isolated pure pheromonal material. In the present study, both male and female attractancy was obtained with about 60 ng of crude male aggregation pheromone. Since most pheromones are produced by the insect in nanogram and picogram amounts (Tumlinson and Heath, 1976), we regard the model used here for biologically active pheromone perception as realistic. We recognize, however, that further purification of the crude globule is likely to yield odor perception at picogram levels, especially in view of the number and concentration levels of compounds extant in the mixture (cf. Figure 5).

The fluted setae associated with the male femoral setiferous sex patch of *T. castaneum* appear to be analogous in morphology to that of the termite. For example, the trail-marking pheromone gland of the termite *Zootermopsis spp.*, localized in the region of the fourth abdominal sternite, is equipped with a distinct pattern of campaniform sensillae which function as proprioceptors (Stuart, 1964; Stuart and Satire, 1968). It has also been postulated (Stuart, 1964) that these sense organs function as part of a feedback control system of substrate stimuli which determines the quantity of trail pheromone deposited. The setae found in the femoral sex patch of *T. castaneum* hence might serve in "trail marking" since *Tribolium* routinely tunnel in farinaceous materials, thereby increasing the probability of locating an individual of the same species, although these structures probably act, in addition, as sites for increased pheromone evaporation. The fluted setae may be sensillae in *T. castaneum*, however, until TEM studies are completed the term seta appears more appropriate than sensilla.

By way of summary of the present study, the male-produced prothoracic leg secretion is perceived by olfactory receptors in both sexes. The synthesis of the male aggregation pheromone in *T. castaneum* could therefore lead to a useful tool for monitoring and controlling populations in warehouses and stored grain. Since both sexes are attracted from a distance, the pheromone could prove to be of practical value in pest management programs.

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Journal of Chemical Ecology, Vol. 7, No. 2, 1981

Letter to the Editor

THE FUNCTION OF PHEROMONES

It has recently been suggested that students of chemical communication abandon the term pheromone because of difficulty in applying the traditional criteria for defining pheromones to mammalian chemical communication systems (Beauchamp et al., 1976). Katz and Shorey (1979) have rebutted this suggestion and proposed the adoption of a broad definition of pheromone. Beauchamp et al. (1979) have responded by rightfully pointing out that Katz and Shorey's definition is too broad to be useful. Martin (1980) has offered still another critique of the initial suggestion of Beauchamp et al.; moreover, he defines pheromones as a special class of compounds that convey information between conspecifics, namely, those which are "relatively species specific, which elicit a clear and obvious behavioral or endocrinological function, and which produce effects involving a large degree of genetic programing and influenced little by experience." In this letter I hope to show that all these suggestions have missed a key point about pheromones that may permit us to define them in a useful way and retain the term as part of our working vocabulary.

All students of chemical communication can agree that pheromones are chemical signals used in communicatory interactions between animals. In fact, this was the major component of the initial definition proposed by Karlson and Luscher (1959). It follows then that pheromones should not be defined as chemical compounds with some arbitrary set of physical or biological attributes (one or a few compounds, species specific, elicit genetically programed response, etc.) but instead as compounds that function as signals in a unique set of organismic interactions, namely, those involving communication. The problem then becomes one of defining animal communication. I suggest that we adhere to the selectionist definition, outlined by Otte (1974) and widely accepted by evolutionary biologists, which defines communicatory interactions as those in which the behavior or signal of one animal (the sender) alters the behavior or physiology of another animal (the receiver) in a way that promotes the fitness or reproductive success of both

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animals. Any compound or blend of compounds that functions as a signal in such an interaction may be called a pheromone.

Again, this definition is not new but its benefits need to be reemphasized. (1) It places no physical constraints on the nature or number of the compounds that may constitute a pheromone. The complexity of a chemical substance need not play a role in our assessment of whether or not it is an olfactory signal. (2) This definition, unlike that of Karlson and Luscher (1959), does not require that pheromones be species specific in structure or effect. One can imagine two species of moths that are geographically isolated and whose females have identical and cross-reactive sex attractants. To require that pheromones be species specific in any way would erroneously exclude these signals from the definition. However, it has generally been regarded as useful to restrict the term "pheromone" to signals used in intraspecific interactions of mutual benefit. "Synomone" (Nordlund and Lewis, 1976) and "allomone" (Brown et al., 1970; Otte, 1974) have been suggested to describe chemicals mediating interspecific interactions of mutual benefit. (3) The selectionist definition can encompass chemically mediated interactions of mutual benefit in which learning and experience play a large role. As long as a chemical compound functions as a signal in a communicatory interaction, it is of little consequence whether the compound or the response to it is acquired by experience or learning. All pheromones and responses to them are affected by experience to some extent, and so attempts to define some arbitrary level of experience that will exclude a chemical from consideration as a pheromone will only lead to a great deal of fruitless debate. (4) The focus of this definition is on the behavioral effects of pheromones and not on information transfer which is difficult to define. In the final analysis we assess whether or not information transfer has occurred by the response of the receiver and so this should be at the core of any definition of pheromone. This is not to imply that we cannot document the kinds of information that are being transferred but only to emphasize that any demonstration of a pheromone is ultimately dependent upon a behavioral bioassay. (5) The stipulation of mutual benefit separates compounds that have evolved for communicatory function from those with incidental effects and those that have evolved for different purposes under different selection pressures. As pointed out by Beauchamp et al. (1979), the modified definition of Katz and Shorey fails to make this distinction. Martin's definition has the same problem. In particular, pheromones should be clearly distinguished from chemical compounds that mediate interactions in which only the receiver or only the sender benefits. For example, the marine snail, *Fasciolaria tulipa*, tracks down and eats conspecifics. Attackers locate target animals using their odor. On the other hand, the target animals may effectively respond to the odor of a pursuer by performing escape manuevers (Snyder and Snyder, 1971). In these contexts, these odors clearly are not pheromones. It must be

recognized, however, that compounds that act as pheromones may have other functions and/or effects as well. For example, the chemical produced by certain bolas spiders mimics a moth sex attractant (Eberhard, 1977). Clearly, this secretion acts as a pheromone when employed intraspecifically by the moth but not when employed by the spider. The emphasis, again, is on determining whether or not a potential pheromone elicits a behavioral or endocrinological response that is mutually beneficial to both sender and receiver.

Admittedly, it is not always easy to show that a chemical substance released by one animal alters the behavior or physiology of another in a way that is mutually beneficial; however, this is a practical and not a conceptual problem. Three of the six reported mammalian pheromones analyzed by Beauchamp et al. (1976) have clearly documented behavioral functions that qualify them as pheromones by the definition proposed here. The other three do not and so should not be elevated to the rank of pheromone without further and more detailed behavioral work as to their function. This is not to belittle the problems of developing clear-cut behavioral assays in vertebrates but to point out that if a secretion cannot be shown to have a communicatory function, it simply means it is not a pheromone, not that we should drop the term pheromone from our vocabulary.

In summary, I have here reiterated a definition of the term pheromone that is based exclusively on the criterion of communicatory function. All other criteria that have been proposed have little or no bearing on documenting the role of a chemical or mixture of chemicals in communication, and therefore tend to be arbitrary, a condition that has generated unnecessary argument. A strictly functional definition will perhaps clarify our thoughts about pheromones and permit us to continue using the term in a rigorous and fruitful fashion.

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Announcement

AMERICAN CHEMICAL SOCIETY

A symposium entitled "Chemistry and Applications of Insect Pheromone Technology" will be held at the national meeting of the American Chemical Society (Pesticides Division) in New York City, August 23–28, 1981. The subjects include: isolation and identification, synthesis, analysis, monitoring of pheromone vapors, and applications including formulations.

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PARTIAL MOLTING SYNCHRONY IN THE GIANT MALAYSIAN PRAWN, *Macrobrachium rosenbergii*: A Chemical Communication Hypothesis

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Abstract—Groups of 50 juvenile specimens of *M. rosenbergii* were observed daily for the occurrence of molting for periods up to 50 days. Each animal in a group was physically isolated from the others in one of 50 chambers assembled in a flat chamber array that was immersed in a recirculated bath of fresh water (28° C). While an average of 6.4% of the animals molted each day in each of three separate trials, molting occurred in significantly nonrandom peaks and valleys. There was no evidence that peaks in molting frequency occurred at regular intervals, nor were animals of the same size molting synchronously, although weight and molting frequency were significantly correlated. Based on intervals between molts predicted from animal weights, animals molting during a peak molted sooner than animals molting with few others. Molting frequency in a group of animals tended to rise until water was changed, at which time molting frequency dropped significantly. Within each array of animal chambers, animals molted in significant spatial aggregations that coincided with the measured pattern of water flow among chambers in the array. Those spatial aggregations were most pronounced during peaks in molting frequency. These results suggest that some waterborne stimulus, perhaps released by molting animals, is responsible for partial molting synchrony in groups of *M. rosenbergii*. The potential advantage of molting synchrony is discussed in connection with the behavioral ecology of *M. rosenbergii*.

Key Words—Molting, *Macrobrachium rosenbergii*, chemical communication, crustacean.

INTRODUCTION

The act of molting (ecdysis) and the softness of newly formed cuticle expose crustaceans to an increased risk of mortality, a vulnerability that has been

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shown to be reduced in some species by molt-associated behavioral adaptations (Passano, 1960). Part of the risk of molting for aggressive gregarious species is the danger of being killed or displaced by conspecifics. Reaka (1976) has described a novel and complex risk-reduction strategy for several species of mantis shrimps that are normally vulnerable to attack by conspecifics at ecdysis. That strategy includes not only reclusive behavior in molting animals but also control of the timing of ecdysis. In common with other aggressive crustaceans, the durations of molt-cycle stages in mantis shrimps are skewed to minimize the vulnerable (soft) period and to allow a relatively protracted premolt period, during which animals may molt in response to an appropriate stimulus (Reaka, 1975). Reaka's (1976) studies show that molting in mantis shrimp populations is significantly synchronous, with rhythmic molting episodes that are correlated with lunar or tidal cycles. Her results support the hypothesis that synchronous molting provides safety in numbers for molting animals.

A comparable risk-reduction strategy would be adaptive for species with similar life histories; for example, the Malaysian prawn *Macrobrachium rosenbergii*, a large, aggressive palaemonid native to the Indo-Pacific region. Adults of *M. rosenbergii* live in bodies of freshwater as far as 200 km from the ocean (Ling, 1969). Mated females migrate into brackish estuaries to release larvae, and the planktonic larvae develop into benthic juveniles that migrate upstream into adult habitats (Raman, 1964). The behavior of *M. rosenbergii* in its natural habitat is largely unstudied. Because *Macrobrachium* has been successfully cultured for more than a decade (Ling, 1969), however, there is a growing literature on the behavioral ecology of captive animals raised at high density in large ponds.

Aggressive behavior is conspicuous in captive *M. rosenbergii* and has been implicated as contributing to high mortality in some ponds (Forster and Beard, 1974; Peebles, 1977, 1978) and to markedly nonuniform growth rates in animals of the same age (Fujimura and Okamota, 1970; Malecha, 1977). Animals are subject to lethal attacks from conspecifics primarily at ecdysis (Peebles, 1977). Adult males are larger and have proportionally larger chelae than females (Ling, 1969). Animals forage at dawn and dusk and at other times defend home ranges that are smaller and more permanent for males than for females (Peebles, 1979). Animals that are soon to molt or that have recently molted tend to occupy less-preferred substrates, where a choice of substrate types is available.

In the course of an unrelated experiment, some degree of molting synchrony seemed to occur among juvenile *M. rosenbergii* that were maintained in the same water supply. Because the social behavior of *M. rosenbergii*, at least in captivity, suggested that molting at the same time as potential conspecific competitors could be adaptive, the possibility of molting synchrony was examined in greater detail.

METHODS AND MATERIALS

The results of three experiments are reported. Since the design of the first differed from that of the latter two in several respects, they are described separately. All were performed in the laboratories of the National Marine Fisheries Service and the University of Houston in Galveston, Texas. The water used in all animal tanks was obtained from the Galveston Municipal Water Supply (mean hardness = 84 mg/liter) and was aerated before use to remove chlorine.

Experiment 1. M. rosenbergii postlarvae from a commercial source were held for three months in a 40,000-liter tank of heated fresh water under diffuse natural illumination. One hundred animals were then transferred to a 200-liter tank in the laboratory where they were maintained for 100 days at 28° C (±0.5° C) with a light regime of 12 hr dim (500 lx) fluorescent light and 12 hr darkness. Fifty randomly selected animals were then blotted briefly, weighed (range: 0.17–2.84 g), and transferred to individual chambers, 6 cm on each side, arranged in a flat, 5 × 10-chamber array. Chambers in the array had rigid, opaque, white styrene walls, plastic screen bottoms and a removable plastic screen cover. The array was fitted over a shallow sand and gravel filter bed inside a fiberglass tank and covered with 40 liters of water. Six airlift pumps circulated water in through the tops of the chambers and out through the bottom of the filter bed at approximately 1 liter/min. Light and temperature regimes were not altered. Distilled water was added daily to replace evaporative loss, but water was not exchanged except as noted in Results. Twice daily, 2 hr before the end of the dark period and 5 hr before the end of the light period, the tray was removed from the water, the positions of molted animals were recorded, exuviae (cast exoskeletons) and dead animals were removed, and food (Tetramin flake to maintain an excess) was provided to each animal. This observation period lasted 15 min, during which room lights were on. Experiment 1 was terminated after the 50th day of observations, when 40 animals survived.

Experiments 2 and 3. For each of these later experiments, two independent 50-chamber arrays were tested concurrently for shorter periods of time. These chambers were constructed of 3.8-mm-thick grey polyvinyl-chloride sheets and were smaller than in experiment 1: 4 × 10 × 4 cm high. Each array was immersed in a shallow tray containing 16 liters of aged, aerated municipal water drawn from a common tank. Water drawn from 24 evenly spaced drains in the bottom of each tray was pumped at approximately 10 liter/min through a filter cannister containing a glass-wool element. Filtered water returned to the chamber tray through a diffuser (Figure 1). At three-day intervals glass wool filter elements were replaced. At the same time 800 cm^3 of granular activated carbon was temporarily placed in the filter cannisters and removed after 1 hr. Regular charcoal filtration

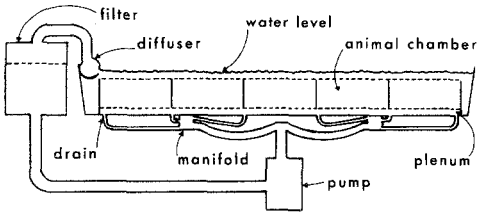

FIG. 1. Schematic diagram of water recirculating system for animal chamber arrays used in experiments 2 and 3.

substantially reduced any progressive deterioration in water quality and its attendant effects on mortality; no animal died in either experiment. The two trays were placed side by side under identical dim fluorescent illumination (14L : 10D). Water temperature in the two trays ranged from 26° C to 27° C in experiment 2 and from 26.5° C to 27.5° C in experiment 3. The two trays never differed in temperature by more than 0.2° C. During the experiments, 1 ml of water was exchanged between trays each day to minimize potential qualitative differences in tank microflora or fauna.

In the course of experiment 1, 96% of 146 observed molts occurred during the dark period. Therefore, animals were observed only once daily in experiments 2 and 3, 1 hr after the end of the dark period. In these later experiments, chamber arrays were only partly emersed during observations to reduce trauma to the experimental animals. Exuviae were removed and food added as before, except that the diet used was an experimental pelletized ration developed for penaeid shrimp by the University of Arizona. Experiments 2 and 3 were terminated after 27 and 15 days, respectively. Mean molting frequencies for groups of animals fed either diet did not differ from each other or from published molting frequencies, suggesting that the experimental diets were adequate to promote normal molting.

RESULTS

Molting Peaks. The number of animals molting each day in each chamber array, expressed as a percentage of the number of animals surviving on that day, is shown for all experiments in Figure 2. In experiment 1 (upper trace) an average of 6.3% of the animals molted each day, but molting activity appeared to occur in peaks rather than continuously. That hypothesis was confirmed by fitting a Poisson distribution to grouped (8 classes) daily molt percentages and testing goodness of fit (for H_o = no difference: $\chi^2 = 39.4$, $P < 0.005$). As Figure 2 suggests, the frequency of days when no animal molted or when more than 14% molted significantly exceeded expectations. The results of experiments 2 and 3 are similar. Mean daily molting

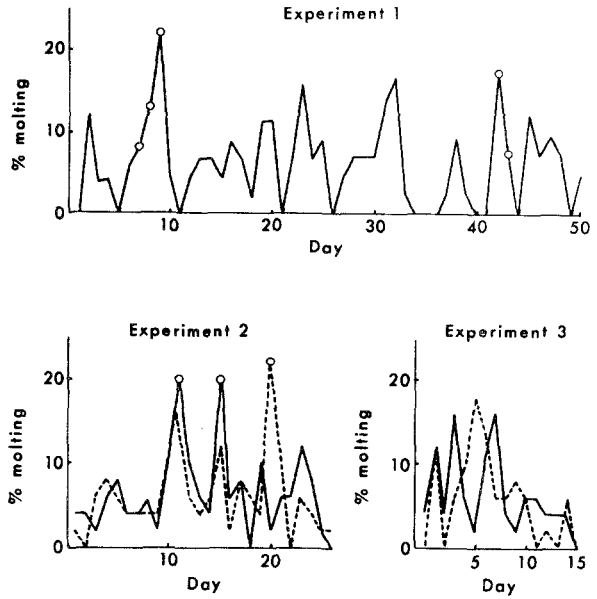

FIG. 2. Number of animals (as % of survivors) molting each day in experiments 1 (upper trace), 2 (lower left), and 3 (lower right). Dashed and solid lines in the graphs for experiments 2 and 3 indicate the response of two independent groups of animals. See text for the significance of values marked by open circles.

percentages for the two chamber arrays in experiment 2 were 6.5% and 6.2% and in experiment 3, 6.8% and 6.3%. None of these latter means differed significantly from another or from the mean in experiment 1. Experiments 2 and 3 were too short in duration (27 and 15 days, respectively) for the distributions of daily molt percentages in either experiment to differ significantly from a Poisson distribution. When all daily observations in the latter experiments were pooled, however, the distribution of pooled observations differed significantly ($\chi^2 = 36.7$, $P < 0.005$, 8 df) from Poisson expectations, again with both high and low values more frequent than expected.

Periodogram Analysis. To detect potential periodicities in the data from experiment 1, daily molt percentages were used to construct a periodogram (Enright, 1965) according to the modification suggested by Sokolove and Bushell (1978). The latter authors showed that the statistic, Qp, a measure of the degree to which hypothetical periods explain variation in a time series, has a distribution that closely approximates χ^2 with degrees of freedom equal to one less than each integral period. Qp is plotted in Figure 3 together with the appropriate $\chi^2_{0.005}$ values for periods between 2 and 16 days. The significance level of χ^2 was chosen to produce an acceptable probability of a type I error.

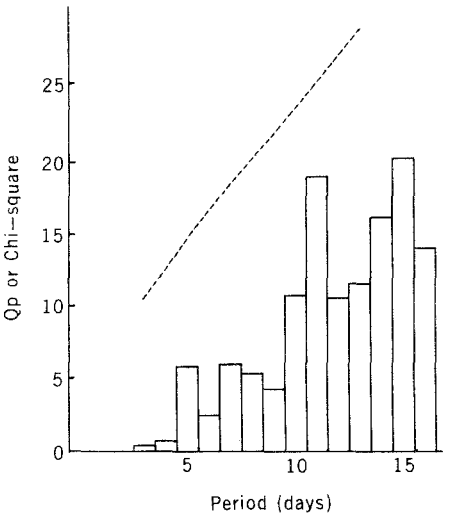

FIG. 3. Periodogram (Sokolove and Bushell, 1978) for daily molting percentages in experiment 1. The dashed line indicates $\chi^2_{0.005}$, a significance criterion.

For critical χ^2 values at the 0.005 level and 14 test periods, the probability of one or more type I errors, i.e., false peaks for which *Qp* exceeds χ^2 when the data are actually random, is approximately 0.07 (Sokolove and Bushell, 1978). Since no *Qp* value exceeds criterion, the data from experiment 1 do not support the conclusion that the peaks in daily molting frequency are periodic. Experiment 1 was too brief to permit testing for possible periods longer than 16 days, and the later experiments were too brief for any periodicity analyses.

Effect of Animal Weight on Molt-Cycle Duration. In experiments 1 and 2, time between successive molts for a given animal (intermolt duration) was significantly correlated with its initial weight ($r = 0.33$, 71 *df*, and $r = 0.54$, 64 *df*, respectively; $P < 0.01$). In experiment 1 the intermolt duration for the hypothetical animal of mean weight (0.65 g) was 14.6 days and each additional gram of weight lengthened intermolt duration by approximately 3 days (least-squares regression). For experiment 2, comparable values were 0.50 g (mean weight), 11.1 days, (mean intermolt) and 6 days (slope). No obvious explanation accounts for the difference in regression slopes between the two experiments. Experiment 3 was too brief for analysis.

Although animals of the same weight tend to molt with equal frequency, molting peaks did not appear to reflect simultaneous molting in groups of like-size animals. The weights of animals that molted in five large, well-defined peaks in experiments 1 and 2 (open circles, Figure 2) were compared by χ^2 goodness of fit to the weight distributions of all animals used for those

experiments. The probabilities that animal weights in the sample peaks could have been random samples of the overall weight distributions ranged from 0.3 to 0.9 (mean $P > 0.5$).

Effect of Peaks on Intermolt Duration. The fact that intermolt duration and weight were strongly correlated in both experiments provided a method for predicting intermolt duration: for each animal that molted at least twice, an expected intermolt duration was generated from the appropriate regression equation. Actual intermolt durations for those animals were then compared to the predicted values, and animals were scored as "late" or "early" molters, accordingly. For each day on which second (or third) molts occurred, the total number of molting animals was counted. For experiment 1 the mean for that count was 4.5 animals; for experiment 2, 4.9 animals. Days on which four or fewer animals molted were therefore scored as "few"; those on which five or more animals molted were termed "many." Contingency tables (Table 1) were then constructed for each experiment based on those scored attributes. For both experiments the two attributes were significantly nonindependent ($\chi^2_c = 4.1$ and 5.2, respectively; $P < 0.05$), and the deviations from random expectations were in the predicted direction. In general, animals molting with few others molted later than predicted on the basis of weight, and animals molting with many others (that is, in peaks) molted sooner than expected.

Effect of Water Exchange on Molting Frequency. To examine the possibility that a molt-accelerating factor accumulated in experimental water supplies, the water recirculated in experiment 1 was replaced at day 33. Potential long-term effects of that manipulation were detected by computing an 11-day moving average for daily molt percentage. The smoothed data are shown in Figure 4. In that figure open circles represent 11-day means that include only days before the water change, half-filled circles indicate means that include progressively more postchange days, and closed circles indicate means of solely postchange days. Smoothed molt frequency appears to rise slightly (although not significantly) until the water change. At day 29, as the

TABLE 1. NUMBER OF SECOND AND THIRD MOLTS IN EXPERIMENTS 1 AND 2, CLASSIFIED ACCORDING TO DURATION OF PRECEDING INTERMOLT PERIODS AND NUMBER OF ANIMALS MOLTING ON THOSE DAYS.

Number of animals molting	Molt timing			
	Experiment 1		Experiment 2	
	Late	Early	Late	Early
Few	21	19	21	14
Many	8	23	9	22

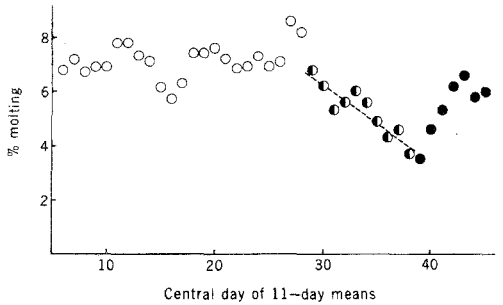

FIG. 4. Eleven-day moving averages of molting percentages in experiment 1, before (open circles), during (half-filled circles), and after (closed circles) a water change. The dashed line represents the least-squares fit to the half-filled circles.

moving average begins to include postchange days, molt frequency drops markedly. A line fitted by least squares to the half-filled circles has a significantly negative slope (Student's t test, two-tailed, $P < 0.01$). By the seventh day after the water change, smoothed molt frequency begins to rise.

Spatial Aggregation in Molting Peaks. The results of experiments 2 and 3 were examined for possible spatial aggregation in the locations of molting animals. The flat arrays of chambers used in those experiments rested on a shallow plenum (Figure 1) from which water was removed at a maximum measured rate of 10 liters/min. That rate of pumping resulted in a net flow of water downward through the chambers at a calculated velocity of from 5 cm/min when filters were clean to 3 cm/min just before the filter elements were replaced. Despite many regularly spaced drains from the plenum, that slow flow of water was not laminar, that is, water flowed laterally between chambers as well as vertically through each chamber.

The magnitude of that effect was checked in a series of dye experiments. With the recirculating pump operating at 10 liters/min, a small volume of dye solution was released into a selected chamber. Water samples (0.5 ml) were then removed from that cell and from neighboring cells at 30-sec intervals, and dye concentrations were measured photometrically. Dye concentrations were highest in nearby cells at the first sampling time (+30 sec), but even 1 min after dye release, concentrations in certain nearby cells remained well above those in more distant cells. Figure 5a shows dye concentrations recorded 60 sec after release as a percentage of the initial concentration in the central cell for two typical experiments. Two results are worthy of mention. First, dye released into corner cells dissipated more slowly than from more central cells. Second, as the geometry of the chamber array suggests, water flow between cells in the same column (centers 4 cm apart) is much greater than between cells in the same row (10 cm apart). By 3 min after dye release in most experiments, the dye was uniformly distributed in all cells in the array.

Based on the results of the dye experiments, an algorithm was developed to test the results of experiments 2 and 3 for spatial aggregation (autocorrelation) of molting within columns of chambers. That algorithm was modeled after the approach of Sokal and Oden (1978) and had the following properties:

1. In order to accumulate sufficient molts for each autocorrelation analysis, three successive days of molting records were combined into a single chamber matrix.

2. In the experiments analyzed, an intermolt period shorter than 7 days occurred only once. Accordingly, each calculation excluded animals that had molted six or fewer days before the end of the 3-day period.

3. Consistent with the dye experiments, an instance of molting in two adjoining column cells was assigned a high relative weight (1 join), and molting in two cells in the same column that were separated by a single cell was assigned a lower weight (1/2 join). No other spatial relationship between cells in which molting occurred was given weight. The number of joins expected with this weighting rule, assuming random locations for molts, was computed by the method of Sokal and Oden (1978).

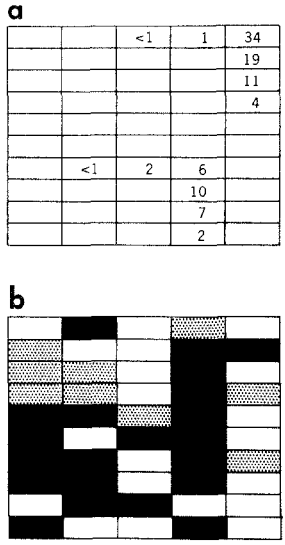

FIG. 5. (a) Dye concentrations (% of initial concentration) recorded 60 sec after dye release in two experiments. The grid is a scale representation (top view) of the chamber arrays used for experiments 2 and 3. Dye was released into the upper right corner cell and later into the cell that is 2nd from right and 4th from bottom. (b) Molting pattern for days 5, 6, and 7 in one of the trays used for experiment 3. Blackened cells indicate the locations of molting animals, and stippled cells indicate recently molted animals that were excluded from a spatial autocorrelation analysis (see text).

The operation of these rules is illustrated in Figure 5b which reproduces the molting pattern for one of the trays of experiment 3 for the 3-day period centered on day 6. During that period, 21 animals (darkened cells) molted and 9 animals (shaded cells) that had molted on days 2, 3, or 4 were excluded. A total of 16.5 joins connect the molting cells, compared with a predicted value of 11.1 joins. For the 73 three-day periods in both experiments, actual joins exceeded expected joins by an average 0.53 joins for each period. Significance tests for this excess were complicated by the fact that successive 3-day values included 2 days of molt data in common and were, therefore, nonindependent. To avoid this nonindependence, the difference between actual and expected joins for every third 3-day period, beginning with the first, was selected from each tray in both experiments, yielding a sample of 25 independent values. The mean of this reduced sample was, coincidentally, 0.53 molts, and was significantly greater than 0 (two-tailed t test, $P < 0.05$). Joins in excess of predicted values for 3-day periods were plotted as a function of the number of animals molting during the central day of each 3-day period. The two variables are positively correlated (Kendall's τ, $P < 0.05$). That correspondence is demonstrated graphically for experiment 2, tray 1, in Figure 6. Spatial autocorrelation analyses show that animals molting within 2 days of each other form significant spatial aggregations within chamber arrays and that these spatial aggregations are most pronounced during peaks in molting frequency.

Peak Timing in Parallel Experiments. The results of experiments 2 and 3 were examined for coincidence of molting peaks between duplicate animal arrays. The results as shown in Figure 2 are equivocal. In experiment 2 molting frequencies in trays 1 and 2 are significantly correlated (Kendall's τ, $P < 0.05$). The largest peak in either tray (day 20, tray 2), however, occurs on a day when molting frequency was well below average in the other tray. On the

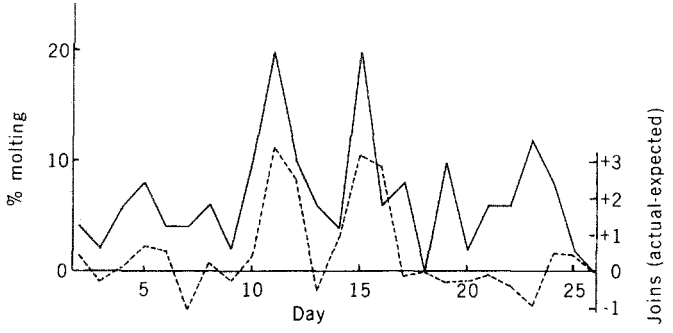

FIG. 6. Molting record (solid line, left ordinate) and number of cell joins in excess of predictions (dashed line, right ordinate) for one of the trays in experiment 2.

other hand, peaks after the first in the two trays of experiment 3 are out of phase, and daily molting percentages are not significantly correlated (Kendall's τ, $P > 0.1$).

DISCUSSION

Individually caged juvenile *M. rosenbergii*, held 50 at a time in a small volume of recirculated water were observed daily for molting. Slightly more than 6% of each group molted each day, yielding an estimate of approximately 15.8 days for the length of molt cycle for experiments 1 and 2. Those two experiments were of sufficient duration to provide instances of multiple molts by the same animal. Estimates of molt-cycle duration based on the mean interval between successive molts were 14.6 and 11.1 days for experiments 1 and 2, respectively. The difference between these estimates of molt-cycle duration has been discussed by previous investigators (Reaka, 1979) and is primarily due to the fact that for experiments that are short relative to the duration of the molt cycle, animals that molt frequently are more likely to molt more than once. No directly comparable data on molt-cycle duration for *M. rosenbergii* have been published, although Segal and Roe (1975) comment that slightly larger animals (2–5 g) held at higher temperatures (30° C) molted about every 9 days with considerable interanimal variation. Growth rates in the experimental apparatus used for experiments 2 and 3 were found in a preliminary 25-day test to be 2.01 ± 0.72% (SD) per day, a value that is comparable to some previous reports for laboratory studies of juvenile *Macrobrachium* (Segal and Roe, 1975; Minamizawa and Morizane, 1970). The observed molting frequencies and growth rates together with low rates of mortality in these experiments suggest that despite close confinement, experimental conditions did not markedly interfere with molting.

Although overall molting frequencies were consistent with previous reports, the timing of molts in each array of animal chambers was significantly nonrandom. Days when many (peaks) or few (valleys) animals molted greatly exceeded expectations. Although other studies (Reaka, 1976; Klapow, 1972) have demonstrated rhythmic synchrony in laboratory populations of other crustaceans, there is no evidence that the partial synchrony in *M. rosenbergii* is rhythmic in the same sense (Figure 3). It should be noted, however, that molting in *M. rosenbergii*, since it occurs almost always at night, does exhibit a diurnally rhythmic component and that experimental and pretreatment conditions (constant temperature and constant light–dark regimes) could have obscured even a persistent population molting rhythm. Finally, mere failure to demonstrate a significant periodicity in these data cannot rule out a significant population molting rhythm.

These data on *Macrobrachium* are consistent with the hypothesis that peaks in molting activity represent responses to an intermittent external stimulus. In all experiments the interval between successive molts was significantly correlated with animal weight. Animals that molted during peaks tended to molt sooner, based on their weights, than animals molting with few others (Table 1). This finding suggests that some factor or combination of factors acting from time to time on at least some members of each experimental group tended either to inhibit or promote molting.

There is some evidence that at least part of the effect of a molt-inducing factor accumulated in the recirculated experimental water system. When water was neither exchanged nor filtered with activated charcoal (experiment 1, days 1–32), mean molting frequency, when smoothed by an 11-day moving average, tended to rise (Figure 4). Replacing the water at day 33 reduced the smoothed molting frequency for about a week, after which it rose again. It is conceivable that the reduction in molting frequency coincident with the water exchange was a response to the presence of some physical or chemical factor in the replacement water that inhibited molting rather than to the removal of a molt-inducing factor. Care was taken to minimize this possibility by treating the replacement water and experimental water in parallel. Replacement water was drawn at the same time and from the same source as experimental water and was aerated in a container adjacent to the experimental tank until used.

Spatial autocorrelation analyses showed that animals molting within a few days of each other were likely, on the average, to be closer together than one would expect if the locations of molting animals were random. Moreover, these spatial aggregations were most pronounced during peaks in molting frequency (Figure 6). These findings suggest either that some uncontrolled environmental factor affected the timing of molting in small regions of each array of chambers or, more likely, that animals influence the probability of molting in nearby animals. Evidence suggests that any such influence is waterborne. First, the pattern of spatial aggregation corresponds to the measured exchange (Figure 5) of waterborne substances (dye) among chambers, and second, the chambers were designed with rigid, opaque walls to minimize the likelihood of visual or mechanical communication among animals.

On balance, these results strongly support the hypothesis of a molt-inducing substance. It is particularly difficult to explain the spatial aggregation results by other hypotheses. Although results similar to those reported here have not been previously reported in the literature, the concept of an exogenous chemical influence on molting is not entirely new. Some blue crab (*Callinectes sapidus*) fishermen routinely add a "peeler" (molting) crab to tanks containing conspecifics in the belief that the production of soft-shelled (recently molted) crabs is thereby enhanced. Krishnakumaran and Schneiderman (1968) reported that when strong (0.5%) ecdysterone solutions

were applied topically or by immersion, specimens of the isopod *Armadillidium vulgare* molted precociously. The latter results suggest a possible candidate for the molt-inducing substance: a polar metabolic derivative of ecdysterone released just before ecdysis (McCarthy, 1979).

Although these data indicate that molting may be partially synchronized by a waterborne signal, in light of the demonstrated effects of many variables upon the timing of ecdysis in crustaceans (Passano, 1960), it is likely that other factors also influenced the temporal pattern of molting in these experiments. For example, the fact that molting frequency was significantly correlated in the two experimental tanks used for experiment 2 would suggest that some uncontrolled factor that was not waterborne (since the two tanks had separate water supplies) influenced molt timing in both tanks. Such a factor, however, is unlikely to account for all the peaks and valleys observed, since peaks in molting frequency in the two parallel tanks used for experiment 3 were strikingly out of phase.

Although it is possible that specimens of *M. rosenbergii* are never so close to conspecifics that interanimal chemical effects on molting could be important, Ling and Merican (1961) report that its habitats include bodies of water affected by tidal action and temporary freshwater pools, where periodic reductions in water level could crowd animals together. If chemically synchronized molting does have a role, it is possible that it serves the same function that Reaka (1976) hypothesized for mantis shrimp. *M. rosenbergii* is aggressive and cannibalistic under certain conditions, and animals are most vulnerable to attack during or soon after ecdysis. The safest time to molt might be when the greatest number of potential cannibals is also molting. An animal that could detect molting in nearby conspecifics and respond appropriately could minimize its risk.

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COMPARING THE EFFECTIVENESS OF SEXUAL COMMUNICATION DISRUPTION IN THE ORIENTAL FRUIT MOTH (*Grapholitha molesta*)[1] USING DIFFERENT COMBINATIONS AND DOSAGES OF ITS PHEROMONE BLEND[2]

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Abstract—The relative efficacy of disruptant blends comprised of different combinations of the Oriental fruit moth's pheromone components was determined in field tests. Disruption was evaluated by comparing male moth catch at synthetic and female-baited traps in disruptant and non-treatment areas. Three atmospheric dosages of a 8-dodecenyl acetate (93.5% *Z*:6.5% *E*) blend, representing two successive 10-fold decreases in concentration (2.5×10^{-2} g/hectare/day to 2.5×10^{-4} g/hectare/day) were tested alone and in combination with an additional percentage of (*Z*)-8-dodecen-1-ol. Male moth orientation to traps was eliminated in plots exposed to the two highest binary acetate dosages. However, significantly more males were captured in synthetic-baited traps in the lowest acetate-alone treatment, indicating a diminution of disruption efficiency. In contrast, inclusion of (*Z*)-8-dodecen-1-ol in the disruptant blend effected essentially complete disruption of orientation at all concentrations tested. Mating success of *G. molesta* pairs confined in small cages apparently was not affected by the presence of relatively high concentrations of the binary acetate and the acetate–alcohol blends. This suggests that habituation and/or adaptation of male response, at least for comparatively "close-range" behaviors, did not occur.

Key Words—Disruption of communication, Oriental fruit moth, *Grapholitha molesta*, Lepidoptera, Tortricidae, sex pheromone, sex attractant, (*Z*)-8-dodecenyl acetate, (*E*)-8-dodecenyl acetate, (*Z*)-8-dodecen-1-ol.

[1]Lepidoptera: Tortricidae.
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INTRODUCTION

Disruption of Oriental fruit moth, *Grapholitha molesta* (Busck), communication by atmospheric permeation with pheromone has been demonstrated in several field trials (Gentry et al., 1974, 1975; Rothschild, 1975, 1979; Cardé et al., 1977). These tests generally involved the emission of (*Z*)-8-dodecenyl acetate (*Z*8–12:Ac) plus a small percentage of its geometrical isomer (*E*)-8-dodecenyl acetate (*E*8–12:Ac) from different dispensing systems. However, these two acetates alone comprise only a portion of the Oriental fruit moth's natural pheromone blend, which has since been characterized (Cardé et al., 1979 and references therein) as a 4-component mixture: *Z*8–12:Ac and *E*8–12:Ac in a 100:7 ratio, (*Z*)-8-dodecen-1-ol (*Z*8–12:OH) and dodecanol (*n*–12:OH).

Additional studies (Baker and Cardé, 1979b) have demonstrated the behavioral effect that each component contributes to orientation and close-range behavior. The two acetates alone elicited upwind flight and other behaviors such as flight initiation. However, concomitant emission of a small percentage of *Z*8–12:OH resulted in an increase in these behaviors, and especially in those behaviors occurring "late" in the sequence, such as close-range orientation and hairpencil display. Only these three components substantially affect male behavior when emitted in a blend approximating that of a *G. molesta* female; dodecanol elicits significant increases in hairpencil display only when *Z*8–12:OH is emitted at suboptimal levels.

Given the behavioral differences elicited by these various combinations of compounds, we initiated field experiments designed to compare the efficacy of disruptant blends comprised of the two acetates vs. blends containing these acetates in combination with *Z*8–12:OH.

METHODS AND MATERIALS

Experimental Design. Field tests to determine the relative effectiveness of different formulations and concentrations of disruptants were conducted during September 9–26, 1979 in an experimental orchard near Fennville, Michigan. The experiment utilized a randomized complete block design with three replicates. All blocks were located in an orchard of standard, mature apple trees planted on a 12-m spacing. These had received fungicide spray only for the previous 10 years. Two of the blocks adjoined each other, whereas the third was separated from the nearest block by 200 m of semi-dwarf apple plantings and bordered a block of peaches.

Twenty-one 0.065 hectare replicate plots were established in the test area. Each plot consisted of a 5-tree array arranged as a central trap tree surrounded by 4 trees which formed a square with a 17-m separation between adjacent corner trees. Center trees of adjoining plots were separated by at least 40 m.

Disruptant Treatments. Seven treatments were evaluated. In addition to untreated controls, three atmospheric dosages, representing two successive decade decreases in concentration of the *Z*8–12: Ac (6.5% *E*) blend were tested alone and in combination with an additional percentage of *Z*8–12: OH. The chemicals used in these tests when placed in the field were >99% pure as determined on OV-1 (100–120 mesh Gas-Chrom Q) and OV-275 (100–120 mesh Gas-Chrom R) GLC columns; the acetate mixture contained <0.2% of any 12-carbon chain-length alcohol. Chemicals were dispensed from 0.20-mm-ID hollow fibers supplied by the Controlled Release Division of Albany International Corp. These release dispensers offer long-term, zero-order release kinetics of compounds from numerous point sources.

To control precisely their number and arrangement, the fibers were stapled to 3-m wooden poles at 0.6 m intervals (from 1.2 to 3 m). Four of these poles were positioned equidistant (1.5–2.5 m) from each other in the canopy of each tree of appropriate replicate plots. This arrangement should have resulted in a relatively even atmospheric permeation of the plots.

At the highest concentration tested in *Z*8–12: Ac (6.5% *E*) plots, 50 fibers were deployed per staple point; 5 fibers per staple point were used at the intermediate level. At the lowest dosage, 8 fibers were individually stapled at a height of ca. 2 m throughout the foliage of each tree in the plot. The acetate–alcohol treatments used an identical number and distribution of acetate fibers but either 5, 1, or 1 *Z*8–12: OH fibers were stapled alongside the acetate fiber tapes at the highest, intermediate, and lowest concentrations, respectively. Laboratory determinations of meniscus recession indicated that the emission rate of the acetate mixture was 0.4×10^{-6} cm^3/day/fiber at 21° C. The $\bar{X}$ of the daily maximum temperature recorded during this experiment was 21° C; the maximum daily temperature occurs during midafternoon, corresponding closely to the time of sexual activity in *G. molesta*. A summary of the total number of fibers deployed and the estimated concentration of disruptant in different treatment areas is provided in Table 1.

Male attraction was assessed with Pherocon 1C sticky traps (Zoecon Corporation); three were deployed at a height of ca. 2 m in the canopy of the center tree in each replicate plot. One trap was baited with a 5 × 7-mm rubber septum (Arthur H. Thomas Co.) impregnated with 100 μg *Z*8–12: Ac (5% *E*) plus 1 μg *Z*8–12: OH and 300 μg *n*–12: OH. Another trap held a 2.5-cm-diam. × 3-cm-high cylindrical screen cage containing three 1 to 2-day-old virgin *G. molesta* females, while a third trap served as an unbaited control. Traps were monitored and females replaced every 1–3 days and sticky bottoms replaced as necessary to maintain trapping efficiency.

Efficacy of disruption was evaluated according to two different methods. The first involved calculation of percent disruption as the reduction in trap capture in plots exposed to disruptant treatments relative to trap catch in untreated plots. Trap data were analyzed with a 2-way ANOVA following

TABLE 1. DISRUPTION OF *Grapholitha molesta* ATTRACTION TO BAITED TRAPS USING DIFFERENT ATMOSPHERIC CONCENTRATIONS OF Z8-12:Ac (6.5% *E*) ALONE AND IN COMBINATION WITH Z8-12:OH DISPENSED FROM HOLLOW FIBER SOURCES.

Disruptant treatment	Trap bait[a]					
	Synthetic dispenser[b]			3 Virgin ♀♀		
	♂ trap catch[c]	Percent disruption[d]	Index of source location[e]	♂ trap catch[c]	Percent disruption[d]	Index of source location[e]
Z8-12:Ac (6.5% *E*)						
4000 fibers/0.065 ha (2.5×10^{-2} g/ha/day)	1a	100	3ab	0a	100	0a
400 fibers/0.065 ha (2.5×10^{-3} g/ha/day)	7a	98	18cd	0a	100	0a
40 fibers/0.065 ha (2.5×10^{-4} g/ha/day)	39b	90	57e	3a	98	12bcd
Z8-12:Ac (6.5% *E*) + Z8-12:OH						
4400 fibers/0.065 ha (2.5×10^{-2} g/ha/day)	1a	100	3ab	2a	99	3ab
480 fibers/0.065 ha (2.5×10^{-3} g/ha/day)	2a	99	6abc	1a	99	3ab
80 fibers/0.065 ha (2.5×10^{-4} g/ha/day)	18a	95	24d	8a	94	6abc
Check (no disruptant)	398c	—	88f	139c	—	57e

[a] Catch in unbaited traps is not included due to the negligible (<1%) male capture.
[b] Septa loaded with 100 μg Z8-12:Ac (5% *E*), 1 μg Z8-12:OH, and 300 μg *n*-12:OH.
[c] Values represented by the same letter are not significantly different ($P < 0.05$) according to Student-Newman-Keul's test of means transformed to $\sqrt{X + 0.5}$.
[d] Percent disruption for a specific treatment was calculated as:

$$\frac{\text{(catch in untreated areas)} - \text{(catch in disruptant area)}}{\text{catch in untreated area}} \times 100\%$$

[e] Index of source location = percentage of traps catching ≥ 1 male per trapping interval. Percentages in same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence ($P < 0.05$).

transformation to $\sqrt{X + 0.5}$. Treatment mean differences based on daily trap catch were tested for significance using the Student-Newman-Keul's multiple-range test ($P < 0.05$).

The alternative method entailed scoring traps either as empty ($X = 0$) or containing males ($X = 1$) for each trap-monitoring interval. These values were then summed over the entire test period, and results from replicates of a particular treatment were combined. The ratio of the resultant value to the

total number of observations constituted a measure of the proportion of successful male orientations over time to a specific treatment (Rothschild, 1981). We suggest the term index of source location for this coefficient.

Confined Mating of Moths. To compare the effect of different disruptant blends on the mating propensity of moths confined in small cages, moths were exposed to one of three treatments: the acetate only and the acetate–alcohol blend, both at their highest atmospheric concentration (2.5×10^{-2} g/hectare/day), and a disruptant-free control. This test was conducted from September 8 to 12 using the same experimental plots as in the previous investigation.

One-day-old virgin *G. molesta* adults came from a laboratory colony maintained on a 16:8 light–dark photoperiod regime at 25°C. Males were confined, 15 per replicate, with access to water in 15-cm-diam. × 30-cm-long cylindrical screen cages. Six replicates ($N = 90$ moths), were positioned ca. 2 m high in the shaded canopy of center trees in the experimental plots. After 24 hr, 15 females were added to each cage. Following an additional 24 hr, the females were individually removed to small plastic cups and returned to the laboratory to ascertain the proportion producing fertile ova.

RESULTS AND DISCUSSION

Disruption of Orientation to Baited Traps. Evaluation of the efficacy of disruption to pheromone-baited or female-baited traps is usually measured by comparison of the total catch in the treated and comparable check areas. However, the percent reduction in catch may not reflect the probability of a female being located by an individual male over a given observation period. For example, the percent reduction in trap catch may exceed 90%, but all sources (synthetic or female) may have been located by at least one male. Thus, the index of source location may be more indicative of the probability of mating than the percent reduction in trap catch. However, the index of source location measure will be more influenced by the sampling interval and the density of the population than the percent reduction in trap catch.

Male trap catch in each treatment plot is summarized in Table 1. In plots exposed to the two highest concentrations of the binary acetate blend, there was a virtually complete reduction of male moth orientation to traps, as measured by both percent disruption and the index of source location. However, significantly more males were caught in synthetic-baited traps located in plots treated with the lowest acetate dosage, indicating that at this level, disruption was less efficacious than at the two higher dosages. Elevation of successful orientation to traps at the lowest dosage (8 fibers/tree) of binary acetate disruptant may have been due in part to the existence of layers or windows of disruptant-free air, resulting from a less uniform dispersal of

disruptant at this concentration. Alternatively, the critical concentration of pheromone may have been below the level necessary to disrupt attraction. These results parallel those obtained in a field test conducted during 1977 (Baker and Cardé, unpublished) using a similar experimental setup; emission of Z8–12:Ac (7% *E*) at disruptant levels comparable to those reported here resulted in 100%, 99%, and 87% disruption of male orientation to synthetic-baited traps at the highest, intermediate, and lowest dosages, respectively.

In the acetate–alcohol treatment areas, male orientation to baited traps again was essentially eliminated at the two highest concentrations. However, in contrast to results obtained with the acetates alone, inclusion of Z8–12:OH in the disruptant blend resulted in a significant increase in disruption at the lowest dosage. The suppression of male captures could be attributable to a Z8–12:OH-mediated modulation of pheromone response, either resulting in a diminution of behaviors in the orientation sequence, particularly those associated with comparatively "late" orientation and precopulatory behaviors, or causing these behaviors to be displaced temporally or spatially from their position in a normal sequence.

Confined Mating of Moths. Mating success of caged pairs of males and females apparently was not affected by the presence of pheromone components. Females mated with a mean frequency of 50% in the acetate-only plot and 56% in the acetate–alcohol plot compared to 56% in the check area (not significant at the 5% level by χ^2).

Results of similar field tests with *Argyrotaenia velutinana* (Walker) (Cardé et al., 1975) and *G. molesta* (Cardé et al., 1977) also indicated no measurable reduction of mating under confined conditions. However, in both of these tests, the disruptant blend emitted did not include the pheromone components which are most important in eliciting comparatively late or close-range behaviors. Notwithstanding, our results indicated that inclusion of Z8–12:OH in the disruptant mixture did not measurably interfere with male mating initiative. In fact, on several occasions, males were observed to hairpencil toward and copulate with females shortly after they were introduced into cages exposed to the alcohol-containing blend. Thus, although preexposure to the pheromone may have altered the male response threshold, elimination of precopulatory behaviors clearly did not occur.

Several factors may have contributed to the failure of the disruptant to suppress mating. The relatively high density of moths (ca. 3.3 moth pairs/m^2 of surface area) in these cages presumably enhanced the likelihood of random intersexual encounters. Behavioral observations on the Indian meal moth, *Plodia interpunctella* (Hübner) (Sower et al., 1975) under conditions of high moth density revealed that males habituated by exposure to pheromone would nonetheless copulate with females encountered during random movement. The close proximity of individuals also may have accorded more

importance to other mating stimuli such as the tactile and visual components of precopulatory behavior known to be of importance in this species (Baker and Cardé, 1979a). Additionally, over these relatively short distances, the concentration of the female-produced pheromone plume may have been sufficiently above the background concentration of disruptant, thereby allowing successful male orientation.

CONCLUSION

Our results indicate that even very low levels of the binary acetate disruptant blend, on the order of 2×10^{-3} g/hectare/day (or a mere 200 mg/hectare over an entire season), can effect virtually complete disruption of long-range orientation in this species. Moreover, the addition of a third component, *Z*8–12:OH to the disruptant mixture potentially will achieve the same level of disruption with a reduction in the overall amount of pheromone applied. Inclusion of the *Z*8–12:OH in the blend may result in an alteration of the pheromone response, causing a truncation or displacement of certain behaviors, especially close-range ones, in the orientation sequence. Use of the complete pheromone blend may also confer some protection against evolution of resistance to pheromone management; blends composed of only a portion of the natural pheromone could allow selection for resistance by accentuating the contribution of the missing component (Cardé, 1976).

Notwithstanding, it is plausible that the *Z*8–12:Ac and *E*8–12:Ac combination could achieve effective disruption of long-range communication without causing a large proportion of males to land and engage in close-range or late behaviors, as could occur with the three-component blend. This phenomenon would be most apt to take place if the atmospheric concentration of the acetate–alcohol blend was above threshold for these behaviors (Cardé, 1981). Thus, both the two-acetate and the two-acetate plus alcohol systems should be evaluated further in a large-scale operational test where actual mating and reduction of damage are measured, although the present test suggests that the three-component blend would be the most efficacious disruptant system.

When pairs were confined under conditions that simulated very high population densities, the frequency of mating apparently was not affected by preexposure of males to the pheromone, although chance intersexual encounters may have influenced the results. The suppression of the male's ability to locate baited traps from a distance indicates that, in this species, elimination of the mating response should not be a prerequisite to successful disruption of sexual communication. Together these findings suggest that the mechanism whereby atmospheric permeation with pheromone effects mating

disruption may be by camouflaging the natural aerial pheromone plumes (Cardé, 1981) rather than by CNS habituation of pheromone response or adaptation of peripheral pheromone perception.

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ALLELOPATHIC POTENTIAL OF *Piqueria trinervia* (COMPOSITAE) AND PIQUEROLS A AND B

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Abstract—Bioassays were carried out to test extracts of roots and leaves of *Piqueria trinervia* and solutions of piquerol A and B, in order to appraise their allelopathic potential. Leaves seem to have greater concentrations of inhibitory compounds than roots. Both piquerol compounds show strong biological activity, but piquerol A is the more active.

Key Words—Allelopathy, *Piqueria trinervia*, Compositae, monoterpenes, piquerol A, piquerol B.

INTRODUCTION

The species *Piqueria trinervia* Cav., tribe Eupatorieae of the family Compositae (Tabardillo weed), is a herbaceous perennial subshrub, with a height of approximately 1 m. It is highly branched, with few opposite leaves, narrowly lanceolate and acuminate, with three very strongly marked nerves. The inflorescence is cymose corymbose. Heads have four white flowers. *Piqueria trinervia* is native of Mexico, Central America, and Haiti. It has been cultivated as an ornamental plant and horticulturists know it erroneously as *Stevia serrata*. It grows in tropical and temperate zones in Mexico. It prefers open, sunny places and is rarely found in the shade of forests. In the Valley of Mexico it is found as high as 2,500 m altitude. It blossoms abundantly in the rainy months from July to October (Paray, 1953).

One frequently finds this species as a pioneer of secondary succession that follows abandonment of agricultural fields, like a ruderal, and in open places in general.

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Paray (1953) reports that *P. trinervia* is used in popular medicine. It has antipyretic, antimalarial, and antirheumatic properties, and it is also used to combat typhus and gallbladder stones. It contains a piquerin alkaloid that seems to be a glucoside.

Piqueria is one of the genra of the Eupatorieae that has yielded the most interesting chemical substances. Bohlmann and Zdero (1968) isolated (−)-santalal, a terpenoid of low molecular weight. Bohlmann et al. (1973) found that *Piqueria trinervia* contains acetylene compounds (Domínguez, 1977).

Romo et al. (1970) isolated, among other substances, two monoterpene diastereoisomers. One of them, piquerol A, was obtained from populations of *P. trinervia* from Pedregal de San Angel, Mexico City, and the other, piquerol B, from populations from Atlacomulco, Estado de Mexico (Figure 1).

Monoterpenes are the principal terpenic inhibitors that have been isolated from higher plants (Evenari, 1949; Asplund, 1968; Anaya, 1976). Some of them are reported as allelopathics (Muller and Muller, 1964; del Moral and Muller, 1970). This fact and the tendency of *Piqueria trinervia* to grow forming more or less extended pure aggregations would suggest the possibility of an allelopathic action of this plant in preventing the growth of other species around it.

A first step in order to test an allelopathic interaction is to verify whether the compounds that the plant possesses show allelopathic potential in vitro. The present research was carried out to observe the effects of different plant extracts and piquerol A and B solutions on germination and seedling growth of different species, some of which belong to ruderal vegetation of Mexico's basin where *P. trinervia* grows.

METHODS AND MATERIALS

Seeds of the following species were used in the bioassays: *Bidens odorata* Cav., *Bidens serrulata* (Poir.) Desf., *Bromus carinatus* Hook. et Arn., *Lepidium virginicum* L., *Lopezia racemosa* Cav., *Raphanus raphanistrum L.*,

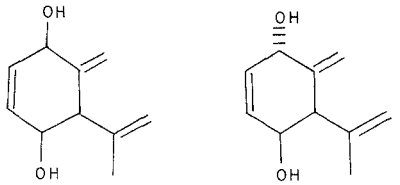

FIG. 1. Chemical structures of monoterpene diastereoisomers piquerol A and piquerol B.

that can live together with *P. trinervia*. *Mimosa pudica* L., *Mimosa sommians* H. et B., that live in tropical zones, and *Brassica campestris* L. "turnip," variety "snow ball," a cultivated plant which can live with *P. trinervia* in the wild.

Bioassays with Aqueous Extracts of Leaves and Roots. The dry material was extracted with distilled water in a 1% concentration and then filtered. Osmotic pressure was determined with a freezing-point osmometer in order to avoid the effects of this factor on germination and growth (Anaya and Rovalo, 1976). The extract was then incorporated in a solid medium (1% agar) in petri dishes, 10 cm diameter. Ten seeds were sown per treatment with three replications; germination was carried out at 28°C, with a 12 hr photoperiod, during 5 days. Afterward lengths of the radicle and stem and germination percentage were determined.

Isolation of A and B Piquerol. Dry plant material (5 kg) collected in the Pedregal de San Angel, Distrito Federal (October 1978), was extracted with chloroform (12:1) by refluxing for 4 hr. The extract was concentrated to dryness and then percolated in a silica gel column. In the fractions obtained when changing from benzene to ethyl acetate, piquerol A (400 mg) was obtained. The product was crystallized from acetone–hexane (1:9) and recrystallized from chloroform. (mp 139°C).

The product was compared to an original sample by measuring the melting point of a mixture of both substances, and by the infrared spectrum, magnetic nuclear resonance, and mass spectrometry. Piquerol B was provided by Dr. Alfonso Romo de Vivar, and this was recrystallized with acetone–hexane (1:9). IR, NMR and MS spectra found were similar to those reported previously (Romo et al., 1970).

Bioassays with Piquerol A and B. Aqueous solutions were prepared of piquerol A and B at 50, 100, 150, and 200 ppm. Osmotic pressure of the solution was measured to determine whether the level was sufficient to damage the seeds. These solutions and aqueous extracts of leaves and roots were tested on germination and growth. All the results were analyzed with an *F* test.

RESULTS

All the results are presented in Tables 1 to 3. There was always a strong growth inhibition of test seedlings by aqueous extracts of roots and leaves of *Piqueria trinervia* and especially by leaf extract that caused 50–95% inhibition. This inhibition was stronger on the radicles than on the stems.

In Table 1, results are presented of bioassays with piquerol A solutions at 50, 100, 150, and 200 ppm. Piquerol A also inhibited the roots more than the stems. Its depressive effect increased slightly and proportionally with the concentration and was obvious and strong starting with 50 ppm.

TABLE I. GERMINATION AND GROWTH OF SIX PLANTS WITH DIFFERENT TREATMENTS OF *Piqueria trinervia* AND PIQUEROL A AND B[a]

Treatments	*Bromus carinatus*			*Bidens odorata*			*Bidens serrulata*			*Lopezia racemosa*			*Lepidium virginicum*			*Raphanus raphanistrum*		
	% of germ.	% of inhibition		% of germ.	% of inhibition		% of germ.	% of inhibition		% of germ.	% of inhibition		% of germ.	% of inhibition		% of germ.	% of inhibition	
		Root	Stem		Root	Stem		Root	Stem		Root	Stem		Root	Stem		Root	Stem
Control	87	0	0	97	0	0	100	0	0	80	0	0	93	0	0	80	0	0
Extract of root	83	33**	31**	87	25**	24*	87	18**	23**	87	46**	38**	77	61**	25**	85	74**	74**
Extract of leaves	63	90**	71**	63	86**	67**	83	79**	51**	7	95**	65**	0	100**	100**	45	84**	77**
Control	95	0	0	90	0	0	90	0	0	73	0	0	75	0	0			
Piquerol A 50 ppm	83	72**	19**	67	62**	38**	87	74**	57**	87	49**	58**	60	63**	34**			
Piquerol A 100 ppm	67	92**	19**	80	82**	54**	93	87**	66**	83	72**	78**	83	91**	63**			
Piquerol A 150 ppm	42	96**	39**	83	88**	61**	87	91**	73**	77	85**	79**	30	94**	73**			
Piquerol A 200 ppm	50	97**	40**	67	90**	65**	90	94**	82**	95	88**	86**	0	100**	100**			
Control	87	0	0	80	0	0	87	0	0	90	0	0	80	0	0			
Piquerol B 50 ppm	—	—	—	87	5*	12**	—	—	—	80	1	12**	87	−4	6*			
Piquerol B 100 ppm	63	56**	−1	77	8*	32*	90	3	32**	90	−1	30**	55	−4	22**			
Piquerol B 150 ppm	63	74**	17**	80	27**	26**	77	55**	58**	100	5	43**	75	16**	32**			

[a]* 5% of significance, *F* test; ** 1% of significance, *F* test.

TABLE 2. GERMINATION AND GROWTH OF THREE PLANTS WITH TWO CONCENTRATIONS OF PIQUEROL A[a]

Treatments	*Mimosa Pudica*			*Mimosa somnians*			Turnip		
	% of germ.	% of inhibition		% of germ.	% of inhibition		% of germ.	% of inhibition	
		Root	Stem		Root	Stem		Root	Stem
Control	100	0	0	100	0	0	100	0	0
Piquerol A 100 ppm	87	45**	10*	93	63**	65**	73	60**	33**
Piquerol A 200 ppm	97	45**	37**	93	59**	59**	93	59**	40**

[a]* 5% of significance, *F* test; ** 1% of significance, *F* test.

Results of tests of piquerol A on growth of *Mimosa pudica, M. somnians*, and turnip at 100 and 200 ppm are shown in Table 2. The greatest inhibiting effect on the radicle is also manifested in this experiment, but more clearly with *M. pudica* and turnip.

Table 1 presents the effects of piquerol A and B on germination of growth of plants obtained from one of the habitats where *P. trinervia* can be found. In general, the inhibitory action of piquerol B was less than that of piquerol A. Besides, at low concentrations, piquerol B either stimulated growth or did not produce any effect. An interesting difference regarding the action of both compounds was that piquerol B inhibited stems the most, whereas A was most inhibitory to roots.

In view of the greater activity shown by piquerol A, an experiment was designed to observe the recovery percentage of some seeds and seedlings that were treated with piquerol A at 50, 100, 150 and 200 ppm concentration. Inhibited seeds and seedlings were transferred from petri dishes with piquerol A to others with 1% pure agar. Recovery was much more dramatic in seedlings and seeds that came from the highest concentration of piquerol A (Table 3).

DISCUSSION

Allelopathic potential of *Piqueria trinervia* was obviously evidenced in bioassays with extracts from leaves and roots. The leaves have a greater content of inhibiting compounds.

Piquerol A and B showed strong biological activity that, in this research, was observed in general over all species used in the bioassays.

Bioassays in allelopathic research often start by testing some extracts, leachates, concentrates, etc., on germination of seeds. Allelopathic effects generally are more obvious on roots than on stems of test seedlings (Anaya

TABLE 3. SEEDLING GROWTH RECOVERING (%) WHEN TRANSFERRED FROM DIFFERENT TREATMENTS TO PURE AGAR

Treatments	*Bidens odorata*		*Bidens serrulata*		*Lopezia racemosa*		*Lepidium virginicum*	
	Root	Stem	Root	Stem	Root	Stem	Root	Stem
Control	0	0	0	0	0	0	0	0
Piquerol A 50 ppm	20	10	34	28	0	170	32	106
Piquerol A 100 ppm	29	67	105	112	52	80	144	31
Piquerol A 150 ppm	60	44	251	121	213	189	240	74
Piquerol A 200 ppm	70	39	275	170	104	214	α	α

and del Amo, 1978). Because of this, some researchers report only the effects on the growth of roots. However, the allelopathic effects on stem growth are of great importance to an understanding of the mechanisms of biological action of active principles. In general piquerol A was more inhibitory to the growth of roots and piquerol B to the growth of stems. In two of the test seedlings piquerol B inhibited the stem and stimulated the root. Different reactions suggest different physiological and biochemical mechanisms of action: permeability of membranes, metabolic interference, blockage of auxins, inhibition of respiration or mitosis, etc. This could be important in understanding allelopathic phenomena.

Another important fact to emphasize is that piquerol A and B are further examples of the differences in biological activity between many pairs of stereoisomers, as is the case in sugars and amino acids, for instance. This behavior also can be an indication of a specific site of action that can distinguish between two diastereoisomers.

Concentration and relative proportions of piquerol A or B inside the plant appear to vary depending on environmental (soil, altitude, etc.) and biological parameters.

Nevertheless, we cannot lay aside the possibility that piquerol A and B can function also as protective agents against herbivores as secondary compounds (Siegler and Price, 1976). There exists also the possibility that these compounds have a double function, and this can be shown only through field experiments.

If the allelopathic potential of *P. trinervia* reported here proves to function efficiently in the natural environment, we could expect some ecological advantages to the plant that might vary in degree according to the biotic and abiotic conditions of the ecosystem.

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NANTUCKET PINE TIP MOTH, *Rhyacionia frustrana*[1]: Identification of Two Sex Pheromone Components[2]

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Abstract—Two compounds identified as components of the sex pheromone system of *Rhyacionia frustrana* are (*E*)-9-dodecen-1-yl acetate (I) and (*E*)-9,11-dodecadien-1-yl acetate (II), which were found in female gland extracts in the ratio of 96:4, respectively. The identifications were based on chemical and instrumental analyses, electroantennogram studies, and field trapping tests. The optimum ratio for trapping male *R. frustrana* is the range of 95:5 to 97.5:2.5 (I:II), when dispensed from rubber septa at a loading of ca. 1000 μg/lure. In addition to these two compounds, evidence was obtained for the presence of dodecan-1-ol and (*E*)-9-dodecen-1-ol in female tip extracts and in female effluvium, and for dodecan-1-yl acetate in female tip extracts.

Key Words—Sex pheromone, sex attractant, *Rhyacionia frustrana*, Nantucket pine tip moth, *Epiphyas postvittana*, light brown apple moth, (*E*)-9-dodecen-1-yl acetate, (*E*)-9,11-dodecadien-1-yl acetate, (*Z*)-9,11-dodecadien-1-yl acetate, dodecan-1-yl acetate, dodecan-1-ol, (*E*)-9-dodecen-1-ol.

INTRODUCTION

The Nantucket pine tip moth, *Rhyacionia frustrana* (Comstock), is one of a number of species in this genus that form a pest complex of ornamental and commercially grown pines (Powell and Miller, 1978). With this report,

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effective sex attractants that are also proven sex pheromones are now known for males of four of the major pest species in the *Rhyacionia* genus that attack pines in eastern North America. The other three are *R. buoliana* (Schiffermüller) (Smith et al., 1974), *R. rigidana* (Fernald) (Hill et al., 1976), and *R. subtropica* (Miller) (Roelofs et al., 1979). Sex-specific attractants, discovered through field tests, are known for two other *Rhyacionia* species, *R. neomexicana* (Dyar) (Jacobson and Jennings, 1978), and *R. zozana* (Kearfott) (Sower et al., 1979). The identification of the sex pheromone of *R. frustrana* was undertaken as part of our effort to provide effective lures for survey purposes and relatively nontoxic chemicals of potential use for control purposes.

METHODS AND MATERIALS

The glass columns used for gas chromatography (GC) were packed with one of the following materials: OV-1 or OV-101 (methyl silicone), 3% on Gas-Chrom Q (4 mm × 1.8 m); Hi-Eff (Hi-Eff 8BP, cyclohexanedimethanol succinate), 3% on Gas-Chrom Q (4 mm × 1.8 m); PDEAS (Hi-Eff 10BP, phenyldiethanolamine succinate), 10% on Chromosorb W AW-DMCS (2 mm × 1.8 m); XF-1150 (GE XF-1150, 50% cyanoethyl, methyl silicone), 10% on Chromosorb W AW-DMCS (2 mm × 1.8 m); liquid crystal MK-II, 10% on Chromosorb W HP (2 mm × 1.8 m) (Lester, 1978; Lester and Hall, 1980; the MK-II liquid crystal compound was a gift from Dr. David Hall); all supports were 100–120 mesh and all diameters are given as ID. The detectors were of the hydrogen flame ionization type and N_2 was used as the carrier gas. Mass spectra (MS) were determined with one of the following: a Finnigan 3300 dual quadrupole mass spectrometer interfaced with an OV-1 GC column (Cornell University Mass Spectrometry Center), a Perkin-Elmer Hitachi model RMU 6 interfaced with an OV-1 GC column, or a Hewlett-Packard 5985 quadrupole mass spectrometer interfaced with a capillary OV-101 GC column.

All solvents were distilled in an all-glass apparatus through a 10-plate Oldershaw column. Microreactions were carried out as described elsewhere (Hill et al., 1977; Beroza and Bierl, 1967). In addition, catalytic hydrogenations were carried out on a microscale at atmospheric pressure using methanol as the solvent.

Infested pine shoots were collected in Georgia. Insects were allowed to emerge in continuous light and were segregated by sex shortly after emergence. Previous work showed that attractancy of crude extract was unaffected by light–dark regimes or the time at which the tips were excised (Berisford, unpublished). Female abdominal tips were excised when the adults were 1 day old. For some of the work the pine shoots were sent to

Geneva where the adults were allowed to emerge at 20–25°C under a 16:8 light–dark regime. Females were collected separately from the males and were placed in an all-glass apparatus for collection of airborne materials on Porapak-Q (Hill et al., 1979).

Electroantennograms were determined as described by Roelofs (1977).

The shorthand notation used for compound structures is a modification of an abbreviated notation used for polyunsaturated acids (Holman, 1966). The geometry and position of each double bond is given first, followed by a hyphen, then the total number of carbons in the chain, followed by a full colon, and then the functional group at position 1 on the chain is given. For example: (*E*)-9-dodecen-1-yl acetate would be *E*9–12:Ac; (*E*)-9,11-dodecadien-1-yl acetate would be *E*9,11–12:Ac. Alcohol is abbreviated OH.

*E*9–12:Ac was purchased from Farchan Chemical Company and was estimated by GC to be >99% pure and free of detectable amounts of the *Z* isomer (<0.5%). A sample of 1:1 *E*/*Z*9,11–12:Ac was obtained from Dr. L. Bjostad (Bjostad et al., 1980); samples of *E*9,11–12:Ac and of *Z*9,11–12:Ac were obtained from Dr. David Hall and Dr. Brenda Nesbitt (Tropical Products Institute, London); their analysis (and ours) of each pure isomer on MK-II showed no detectable amounts (<0.5%) of the opposite isomer to be present.

Field tests were carried out in Georgia, where *R. frustrana* adult emergences occur in March, June, and August. Pherocon 1C traps [a commercial version of the Howell (1972) wing trap] were used with either filter paper (Whatman No. 1, 1 cm^2), rubber septa (red, 5 × 9 mm, A.H. Thomas Co.), or polyethylene cap (OS-6 natural polyethylene closures, Scientific Products) dispensers for the chemicals tested. Traps were placed at a height of 4–5 ft in 5 to 7-ft-high loblolly pine plantations in a randomized complete block design. For Tables 2 and 3, trap catches were transformed by $(x + 0.5)^{1/2}$, subjected to a two-way analysis of variance and ranked according to Waller and Duncan's BET test ($P \leq 0.05$).

RESULTS

Collection of a female abdominal tip extract from a nonpolar GC column in timed fractions, followed by assay of these fractions using the EAG method, consistently revealed one area of activity at the retention time of 12-carbon acetates (e.g., 7–8 min on OV-1, 161°, with 12:Ac at 7.7 min). A second, earlier, area of activity was seen with less regularity, and appeared at the retention time of 12-carbon alcohols (e.g., 3.5–4.5 min on OV-1, 161°, with 12:OH at 4.5 min). To facilitate the subsequent descriptions herein, these GC-collected and EAG-active materials are designated A (alcohols) and B (acetates), in their order of elution from the nonpolar column.

Identification of E9-12:Ac *from female* R. frustrana. Recovery of fraction B followed by injection onto a polar GC column and reassay by EAG of the collected fractions, consistently revealed only one definitive area of activity, at the retention time of *E*9-12:Ac (e.g., 5.5-7 min on Hi-Eff, 150°, with *E*9-12:Ac at 6.05 min). Material B (12-14 min from OV-1, 149°) was subjected to alkaline hydrolysis, collected from the OV-1 column at timed intervals, and assayed by EAG. The EAG activity originally seen at 12-14 min was absent, but was restored when the material collected at the retention of a 12-carbon alcohol from the saponified sample (7-8.5 min) was recovered and treated with acetyl chloride. The major GC peak in B, saponified B, and the reacetylated material coincided (±0.1 min, or closer) with *E*9-12:Ac, *E*9-12:OH, and *E*9-12:Ac, respectively, on OV-1, 149° (13.95 min for the acetate and 7.8 min for the alcohol), and on PDEAS, 149° (11.8 min for the acetate and 12.7 min for the alcohol).

An EI-MS (20 eV) of material B showed that it was monounsaturated, 12-carbon, straight-chain acetate and was identical to that of *E*9-12:Ac (*m/e* 166 for P-60; with *m/e* 226 for P not evident).

Hydrogenation of material B over Pd produced a material with retention times identical to those of 12:Ac on OV-1 and PDEAS (e.g., at 149°, these were 14.8 min and 9.95 min on the two columns, respectively, with *E*9-12:Ac at 14.35 min and 10.45 min, respectively).

Ozonolysis of B produced a compound with the same retention times on polar and nonpolar GC columns as 9-acetoxynonanal formed by ozonolysis of synthetic *E*9-12:Ac (e.g., 8.2 min and 8.15 min on OV-1, 152°, respectively, and 30.45 min and 30.35 min on PDEAS, 152°, respectively). This established that the double bond is at position 9 in the chain.

The geometry of the double bond was determined to be E from the GC retention time of B on PDEAS and XF-1150, which coincided with that of *E*9-12:Ac and not with that of *Z*9-12:Ac (e.g., on PDEAS, 149°, material B showed a peak at 11.85 min, with *E*9-12:Ac at 11.8 min and *Z*9-12:Ac at 12.5 min). In addition, on PDEAS or XF-1150, B consistently showed no peak corresponding to *Z*9-12:Ac; the *Z* isomer would have been detectable at the level of 0.5% or more of the E compound.

Material B and hydrogenated material B have different retention times from those of 10-methyl-9-undecen-1-yl acetate (10Me-9-11:Ac) and 10-methyl-undecan-1-yl acetate (10Me-11:Ac) on a polar column (e.g., on PDEAS, 152°, B elutes at 10.4 min and 10Me-9-11:Ac at 10.9 min, while hydrogenated material B is at 9.1 min and 10Me-11:Ac is at 7.6 min). These comparisons demonstrate that material B is not branched at position 10, further confirming its straight-chain structure.

E9-12:OH, 12:OH, and 12:Ac in Female Tip Extracts. Fraction A, when re-collected from a polar column in timed fractions that were assayed by

EAG, showed only one area of activity which eluted at the retention time of *E*9–12:OH (e.g., 8–9 min on PDEAS, 161°, with *E*9–12:OH at 8.85 min and 12:OH at 7.5 min).

Material A, from a female tip extract, was fractionated on XF-1150, 151°, into two materials, one enriched in 12:OH (retention time 7.0 min; fraction collected at 6.5–7.5 min, fraction C), the other in *E*9–12:OH (retention time 7.9 min; fraction collected at 7.5–8.5 min, fraction D). Each fraction was acetylated, collected from OV-1 at the retention time of 12-carbon-chain acetates, and examined on XF-1150 at 151°. The material prepared from fraction C had a peak at the retention time of 12:Ac (6.15 min), and that from fraction D had a major peak at the retention time of *E*9–12:Ac (6.95 min) and a minor peak at the retention time of 12:Ac (6.15 min; with 12:Ac at 6.15 min, *E*9–12:Ac at 6.95 min, and *Z*9–12:Ac at 7.65 min).

Material B, when examined on XF-1150, often showed a small peak with the retention time of 12:Ac (e.g., at 151°, 6.25 min, compared to 6.15 min for synthetic 12:Ac). This peak was about 12% of the *E*9–12:Ac peak.

Analysis of R. frustrana *Female Effluvium Collected on Porapak-Q.* Recorded effluvium from *R. frustrana* females was fractionated on OV-1. EAG assay of the fractions revealed two distinct areas of activity corresponding to materials A and B.

Material B was recovered and fractionated on an XF-1150 GC column. There were two EAG-active fractions, one corresponding to the retention time of *E*9–12:Ac (7.5–8.5 min at 153°, with *E*9–12:Ac at 7.9 min) and the other with a longer retention time (10–11 min). A peak (7.85 min) at the retention time of *E*9–12:Ac was evident in a GC tracing from XF-1150, 153°, of the earlier fraction.

Material A was recovered, treated with acetyl chloride, and fractionated on OV-1 to produce one EAG-active fraction at the retention time of 12-carbon acetates (9–10.5 min on OV-1, 153°, with 12:Ac at 9.9 min). On XF-1150, the acetylated product showed a major peak at the retention time of 12:Ac (6.95 min at 153°, with 12:Ac at 7.0 min), and a minor peak at the retention time of *E*9–12:Ac (7.9 min at 153°, with *E*9–12:Ac also at 7.9 min).

Initial Field Tests. Field tests were carried out to determine the efficacy of the identified compounds as trap lures for male *R. frustrana*. The compounds tested were *E*9–12:Ac, alone and in combination with 12:OH, *E*9–12:OH, and 12:Ac. All the tests failed to disclose any combination of these compounds that was as effective a lure as crude female tip extracts at the 3–5 FE level on filter paper dispensers. Polyethylene cap dispensers were somewhat superior to rubber septa dispensers when baited with *E*9–12:Ac; in typical results, rubber septa dispensers with 30 μg to 1 mg of *E*9–12:Ac caught 0–4% of the numbers caught with the crude extract (three separate tests) and polyethylene cap dispensers with 2–10 mg of *E*9–12:Ac caught 6–32% of the

numbers caught with the crude extract (five separate tests). Because of these results, a search for further sex pheromone components was undertaken.

Identification of E9,11-12:Ac from R. frustrana *Females.* GC tracings on a nonpolar column of crude female tip extracts showed, with some consistency, the presence of a small peak (material F) of slightly longer retention time than that of *E*9-12:Ac (on OV-101, 170°, retention times were 5.65 min for *E*9-12:Ac and 6.3 min for F; equivalent carbon numbers for F and 12:Ac on OV-101, 190°, were 16.22 and 15.93, respectively, compared to *n*-hydrocarbons). A conjugated, diunsaturated 12-carbon acetate could be expected at this retention time. This type of combination, i.e., monounsaturated and conjugated diunsaturated components, was known at that time to form a sex pheromone for the light brown apple moth, *Epiphyas postvittana* (Walker),[5] so we decided to investigate the possibility that this might also be the case for *R. frustrana.*

Fraction B, known to contain F, was fractionated on XF-1150. EAG assay showed two active fractions, one at the retention time of *E*9-12:Ac (6.5-7.5 min at 151°, with *E*9-12:Ac at 6.95 min) and the other (material F) at the expected retention time of a conjugated, diunsaturated 12-carbon acetate (12-13.5 min, with *Z*7,*E*9-12:Ac at 12.55 min on XF-1150, 151°).

Field tests of GC-fractionated female tip extract were conducted to determine if F was an effective lure when combined with *E*9-12:Ac. Results are presented in Table 1, and show that F is a primary sex pheromone component for *R. frustrana.* In combination with *E*9-12:Ac, it lured significantly higher numbers of male *R. frustrana* into traps than *E*9-12:Ac alone (synthetic or from a female tip extract). F plus *E*9-12:Ac was also a better lure than F plus the early GC fractions, or the untreated extract.

An EI-MS (70 eV) of material F showed that it was diunsaturated, straight-chain 12-carbon acetate and was very similar to that of *Z*7,*E*9-12:Ac (*m/e* 224 for P, and *m/e* 164 for P-60.)

The initial EAG profile of normalized values for male *R. frustrana* antennal responses to various monounsaturated acetates, alcohols, and aldehydes (unbranched 10-, 12-, and 14-carbon chains) showed the best response was to *E*9-12:Ac, with the response to *Z*7-12:Ac being less than half that to *E*9-12:Ac and also one of the poorest in the 12-carbon acetate series. At this stage of the analysis of F, a second EAG profile was determined, and the response to *Z*7-12:Ac was found to be about 87% of that to *E*9-12:Ac. This was the only major difference between the two EAG profiles. Although the EAG standard compound response profiles generally are not useful for prediction of the structures of minor sex pheromone components, it

[5]Unpublished work by one of the authors (Ada S. Hill) with Drs. T.E. Bellas, R.J. Bartell, G.H.L. Rothschild, and Ms. L.A. Lawrence; carried out at Division of Entomology, CSIRO, Canberra, Australia.

TABLE 1. TRAP CATCHES OF MALE *R. frustrana* (MAY 18, 1978)[a]

Lure		
*E*9-12:Ac (ng)	Other[b]	Average trap catch[c]
100	—	2.0f
—	5-7 min	1.0f
100	0-5 min	3.4f
100	7-15 min[a]	23.3d
—	untreated	11.4e
—	—	3.0f

[a] Replicated 5 times, except for the fourth sample, which was replicated only 3 times; dispensed on filter paper at the rate of 5 FE per trap.

[b] These samples were prepared from female tip extract by collection from XF-1150, 180° (with *E*9-12:Ac eluting at 5.6 min from XF-1150 under these conditions). The untreated sample is an aliquot of the female tip extract.

[c] Numbers followed by the same letter are not significantly different at $P \leqslant 0.03$, according to a pair-wise rank test.

was thought that the moderate EAG response to *Z*7-12:Ac might indicate that the diunsaturated *Z*7,*E*9-12:Ac was a likely candidate for the minor component F. Three of the 7,9-12:Ac isomers were available from previous work (Roelofs et al., 1974) for comparisons. On XF-1150, F showed the same retention time as *Z*7,*E*9-12:Ac (at 151°, 12.55 min for F, as compared to 12.55 min for *Z*7,*E*9-12:Ac, and 12.95 min for *E*7,*Z*9-12:Ac). However, field tests of *E*9-12:Ac in combination with *E*7,*E*9-12:Ac, *E*7,*Z*9-12:Ac, or *Z*7,*E*9-12:Ac showed that none of these materials was effective in increasing trap catches of male *R. frustrana* over those obtained with *E*9-12:Ac alone.

Ozonolysis of F produced, as the major product, a compound with the same GC retentions on nonpolar and polar columns as 9-acetoxynonanal from ozonolysis of *Z*9-12:Ac (e.g., 5.9 min and 5.85 min on OV-101, 135°, respectively, and 13.45 min for both products on XF-1150, 150°). No evidence for the presence of acetoxyaldehydes corresponding to ozonolysis products from double bonds at positions 5-8 was seen.

Examination of F on the MK-II liquid-crystal GC column showed one major peak, with the same retention time as *E*9,11-12:Ac (e.g., at 135°, 4.85 min and 4.9 min, respectively), and a small peak at the retention time of *Z*9,11-12:Ac (e.g., at 135°, 4.35 min). The amount of this small peak relative to that for *E*9,11-12:Ac was somewhat variable (5-10% of the *E*9,11-12:Ac peak).

Field Tests Establishing Efficacy of R. frustrana *Sex Pheromone Components.* The pertinent field test results are presented in Tables 2 and 3. The best synthetic lure found to date is 1000 μg of *E*9-12:Ac plus 25-50 μg of *E*9,11-12:Ac on a rubber septum. This lure was as good as 3-5 FE of the

TABLE 2. TRAP CATCHES OF MALE *R. frustrana* (APRIL 1979)

	9,11-12:Ac (μg)		
*E*9-12:Ac (μg)	*E*	*Z*	Average catch[a]
100	—	—	0.5e
1000	—	—	0.3e
100	5	—	2.4d
1000	50	—	10.3ab
100	—	5	1.3de
1000	—	50	5.2c
100	2.5	2.5	2.3d
1000	25	25	7.4bc
♀ extract, 5 FE			14.0a
Live ♀, 3 ♀			6.7c
Blank			0.6e

[a] Average number of males per trap per night; 5 replications; rubber septa. Numbers followed by the same letter are not significantly different at $P \leq 0.05$.

crude female tip extract on filter paper. In one test it was better than the live virgin females, and in the other test it was as good as the live virgin females used for lures. Addition of *Z*9,11-12:Ac in a 1:1 ratio to *E*9,11-12:Ac did not affect trap catches in either of the two tests. The lures with 100 μg of the *E*9-12:Ac component were not as effective, in general, as those with 1000 μg of *E*9-12:Ac. Reduced trap catches resulted when the *E*9,11-12:Ac-to-*E*9-12:Ac ratio was altered from the optimum of 25 and 50 μg/1000 μg, respectively.

TABLE 3. TRAP CATCHES OF MALE *R. frustrana* (JUNE 1979)

	9,11-12:Ac (μg)		
*E*9-12:Ac (μg)	*E*	*Z*	Average catch[a]
1000	25	—	4.48bc
1000	50	—	2.85cd
1000	100	—	1.62de
1000	50	50	4.48ab
300	15	—	1.37ef
—	50	—	0.31f
♀ extract, 3 FE			6.14a
Live ♀, 1 ♀			3.94bc
Blank			0.28f

[a] Average number of males per trap per night; 5 replications; rubber septa. Numbers followed by the same letter are not significantly different at $P \leq 0.05$.

DISCUSSION

The data presented here establish the *E*9–12:Ac and *E*9,11–12:Ac are produced and emitted by female *R. frustrana* moths and, when combined, will lure male *R. frustrana* moths into traps. Thus, they are sex pheromone components for the species *R. frustrana*. Whether or not other compounds are also sex pheromone components for this moth remains to be established. Two likely candidate compounds are 12:OH, already identified from *R. frustrana* female effluvium and female tip extracts, and *Z*9,11–12:Ac, the presence of which in female-produced materials has not been ruled out by the data presented here. Nonetheless, the two compounds definitively identified as primary sex pheromone components are sufficient to lure male *R. frustrana* into traps in numbers comparable to those caught using female tip extracts or live females and should thus prove useful in insect monitoring programs, insect trap-out programs, and as communication disruption agents.

With the identification of the sex pheromone of *R. frustrana*, sex pheromones for four of the major pests of pines from the lepidopteran genus *Rhyacionia* in eastern North America are now known. Cross-attraction tests between these species, using female tip extracts, have been reported (Berisford et al., 1979), in which *R. subtropica* and *R. buoliana* were found to be highly cross-attractive; *R. frustrana* was somewhat attracted to *R. subtropica* and *R. bouliana*; and the other combinations were essentially not cross-attractive. These results seem reasonable in view of the sex pheromone component structures identified for these insects, which are presented in Table 4. The most prevalent component is *E*9–12:Ac, which is utilized by three of the four species. The exception, *R. rigidana*, utilizes *E*8,*E*10–12:Ac, which is the acetate analogue of *E*8,*E*10–12:OH, the only sex pheromone component known for the codling moth, *Cydia pomonella* (Roelofs et al., 1971; McDonough and Moffitt, 1974; Beroza et al., 1974). *C. pomonella*, previously

TABLE 4. KNOWN SEX-SPECIFIC ATTRACTANTS FOR *Rhyacionia* SPECIES

Species	Status[a]	Composition (%)	
		*E*9–12:Ac	Other components
R. buoliana	P	100	—
R. subtropica	P	100	—
R. frustrana	P	95	5 (*E*9,11–12:Ac)
R. rigidana	P	—	100 (*E*8,*E*10–12:Ac)
R. neomexicana	A	100	—
R. zozana	A	100	—

[a] P = known to be the pheromone; A = known to be a sex-specific attractant from field screening tests only.

placed in the genus *Laspeyresia* (Brown, 1978), is a member of the subfamily *Olethreutinae*, as are the *Rhyacionia* species. The structural similarities between *E*8,*E*10–12:Ac and *E*9,11–12:Ac are obvious: both are straight-chain, conjugated diunsaturated 12-carbon acetates with *E* double bonds. The only difference is that the conjugated system is displaced by one carbon along the chain. The structural similarities among the sex pheromones of these species may be a manifestation of the genetic and/or evolutionary relationships among these species, and may be useful for taxonomic purposes.

The compound *E*9–12:Ac has not been reported frequently as a sex pheromone component or even a sex-specific attractant. Other species, in addition to the *Rhyacionia* species, known to use this compound are: *Eucosma sonomana* (Kearfott) (Tortricidae) (Sower et al., 1979), and *Cnephasia pumicana* (Zeller) (Tortricidae) (Biwer et al., 1977) for which it is known to be a sex pheromone component. It is known to be a sex-specific attractant for *Sitochroa chortalis* (Grote; formerly *Loxostege chortalis* Grote) (Pyralidae) (Roelofs and Comeau, 1971), *Hahncappsia neobliteralis* (Capps; formerly *Loxostege neobliteralis*) (Pyralidae) (Roelofs and Comeau, 1971), and the cembran and spruce pine forms of *Zeiraphera diniana* Guenee (Tortricidae) (Baltensweiler et al., 1978; Vrkoc et al., 1979).

The geometrical isomers *E*9,11–12:Ac and *Z*9,11–12:Ac have been reported to date as sex pheromone components for only two other species. One is *Sparganothis directana* (Walker) (Tortricidae), which uses a 35:2 mixture of the diunsaturates plus a 19:28 mixture of *E*11–14:Ac to *Z*11–14:Ac (Bjostad et al., 1980). The other is *Diparopsis castanea* (Hampson) (Noctuidae), which uses an 83:17 mixture of the two isomeric diunsaturates, plus the terminally unsaturated 11–12:Ac (Nesbitt et al., 1975).

Two other moth species are known to use a mixture of a monounsaturated and a conjugated diunsaturated straight-chain compound, both of the same chain length. One is *Epiphyas postvittana* (Walker) (Tortricidae), which is a native Australian moth (Figure 1). The ratio of mono- to diunsaturated compounds in both cases is approximately the same, about

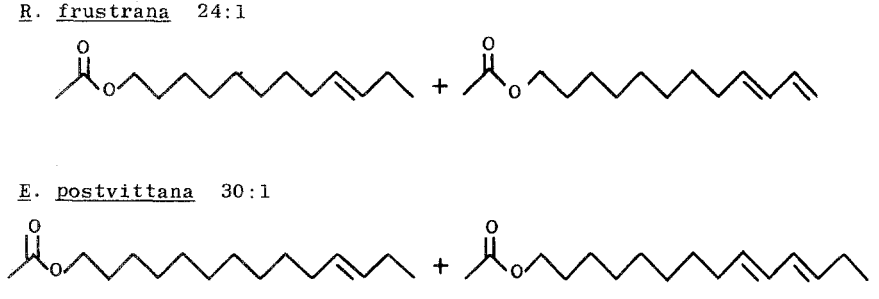

FIG. 1. Sex pheromone components of *R. frustrana* and *E. postvittana*.

25:1. Both species occur in the family Tortricidae. Another member of this family, *Hedya nubiferana* (Haworth) has been found to have *E*8,*E*10–12:Ac and *Z*8–12:Ac in a 3:2 ratio, as well as 12:Ac (Frerot et al., 1979). Roelofs (unpublished) found the *H. nubiferana* sex pheromone components to be 12:Ac, *E*8–12:Ac, *Z*8–12:Ac and *E*8,*E*10–12:Ac in a 9:5:31:55 ratio.

Further identification of sex pheromone components for *R. frustrana* and other species can be expected to help clarify taxonomic classifications, as well as genetic and/or evolutionary relationships.

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EVIDENCE FOR AN ATTRACTANT IN CUTICULAR LIPIDS OF FEMALE *Lucilia cuprina* (WIED.), AUSTRALIAN SHEEP BLOWFLY

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Abstract—Various nonpolar and weakly polar organic solvent extracts of female *Lucilia cuprina* caused more flies to be trapped in conical flasks, placed in cages of blowflies, than in untreated flasks or in flasks treated with similar extracts of male *L. cuprina*. This is attributed to an attractant present in the extracts of female flies, and since more females than males tended to be trapped, the attractant (or pheromone) possibly plays a role in the group oviposition behavior exhibited by this species. The data from experiments and gas–liquid chromatography (GLC) examinations of dichloromethane and hexane extracts of flies suggest that the attractant is a cuticular lipid.

Key Words—*Lucilia cuprina*, sheep blowfly, attractant, cuticular lipids, group oviposition.

INTRODUCTION

The blowfly, *Lucilia cuprina* (Wied.), is an important economic pest in Australia initiating most instances of cutaneous myiasis in sheep (Mackerras and Fuller, 1937; Waterhouse, 1947; Watts et al., 1976; Murray, 1978).

In relatively recent studies of its behavior, Barton Browne et al. (1969) demonstrated that *L. cuprina* females oviposit in groups due to the presence of a pheromone or pheromones in gravid (or mated, liver-fed) females. One of these pheromones appeared to be an attractant since, in the vapor phase, it induced females to group before and during egg laying. Observations indicated that one likely source of the pheromones is the cuticle. In attempts to identify components of its cuticular lipid which might be a pheromone,

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Goodrich (1970) examined the chemical composition of dichloromethane extracts of flies by several methods including GLC. Goodrich suggested that the constituents most likely to contain the pheromones responsible for the sex and ovipositing behavior of *L. cuprina* are the more volatile fatty acids, e.g., less than 14 carbons, and esters.

Pheromone-mediated group egg laying also occurs in the following insects: *Trogoderma*, *Schistocerca*, *Andrena* (sic) (Brossut, 1975), and *Musca* spp. (Bay and Pitts, 1977).

The investigations described below continue the study of the chemical-mediated behavior of this blowfly; in particular, the stimuli causing females to aggregate are considered in the light of the present tests and in view of the work of Barton Browne et al. (1969) and Goodrich (1970).

METHODS AND MATERIALS

Blowflies. Approximately 1000 adults per cage of the Ciba-Geigy Combined Field Strain emerged from pupae over a 3-day period in rectangular cages (35 × 33 × 44 cm), temperature: 24–27°C, relative humidity: 65–80%, 15 hr light–9 hr dark. Sugar and water were provided ad libitum, and on each of the two days following removal of the pupae, ovine liver was supplied as a source of protein.

The age of insects used in experiments was 8–10 days, and the percentage of females which were gravid approached 90%. The sex ratio was generally around 1:1.

Single Solvent Extraction of Blowflies. Flies were sexed 3–4 days prior to extraction, and the males and females held in separate cages. The males and females were extracted in separate 500-ml flasks by swirling with Univar A/R grade pentane (1.5 min) or dichloromethane (3 min) at a ratio of 25 ml solvent per 100 females or per 137 males. Different numbers of males and females were extracted as an adjustment for their differing cuticular surface areas. Each extract was filtered through a Quickfit sintered-glass filter, stored at −16° to −18°C, and its concentration estimated by driving off the solvent from 2.0-ml samples overnight at 40–45°C, weighing the residues, and calculating the mean residue weight.

Double Solvent Extraction. Flies were sexed on the day of extraction. The males and females were extracted in separate 500-ml flasks by swirling with a mixture of Univar A/R grade hexane and methanol (2:3, v/v, respectively), first at a ratio of 15 ml solvent per 100 females or per 137 males (1.5 min), then, at a ratio of 10 ml solvent per as above (0.5 min). The two volumes were combined, and after storage at −16° to −18°C (3 days), the hexane was separated from the methanol using a separating funnel. Each of

the hexane and methanol fractions was evaporated to dryness in a Büchi Rotavapor at 55–60° C, the residue weighed, redissolved in fresh solvent, filtered, and the concentration adjusted to 2.0 mg/ml.

Collection of Airborne Volatiles. Flies were sexed, and the females were placed in two cages, approx. 1000/cage, which were sealed in a double-walled plastic bag fitted with an inlet and outlet tube. Two cages containing males were treated similarly. The inlet tube from each bag was connected to a cylinder of compressed medical air, and each outlet to a glass 100-ml chamber (13 cm long × 3 cm diam.) filled with gas chromatography supports (27.5 g), namely, Porapak Q 50–80 mesh, Gas chrom W 80–100 mesh, and Gas Chrom P 80–100 mesh, in the following respective proportions by volume: 50%, 30% and 20%. The supports had been washed in hexane (1× 300 ml, 1× 200 ml) and purged at 150° C (24 hr) with N_2 at a flow rate of 100 ml/min.

A total of about 1000 liters of air, at an average rate of 240 ml/min, was passed through each collecting chamber over a 3-day period. At the end of the period, each lot of packing was extracted with hexane (1× 250 ml for 1 hr, 1× 150 ml for 1 hr) by mixing in a 500-ml flask at 50° C. The two volumes were combined, filtered, stored at −16° to −18°, and the concentration estimated by driving off the solvents and weighing the residues as described above.

GLC. The hexane fractions from double-solvent extracts of the Ciba-Geigy strain flies as well as of a strain of Canberra *L. cuprina* were examined in a Hewlett Packard 5830A GC fitted with a 18850A terminal and 7671A automatic sampler. The lipids in 1.0-μl samples of concentration 10 mg/ml were separated in OV-101 supported on Gas Chrom P 80–100 mesh or in OV-17 supported on Gas Chrom A 80–100 mesh. The supports were loaded with 3% liquid phase by weight, and were packed in Pyrex columns (2 m long, 2 mm ID, 6 mm OD). Operating conditions were: injection port, 300° C; flame-ionization detector, 300° C; initial oven temperature, 60° C (2 min); final oven temperature, 280° C (6 min); rate of temperature increase, 10° C/min; and N_2 carrier rate, 30 ml/min. Eicosane was used as an internal standard.

Estimating Attraction. The assay method is based on a capture technique in which insects are lured into a trap by an attractant. Such a technique is often employed when trapping blowflies in the field.

In the following laboratory tests, 50-ml Quickfit conical flasks, fitted with a plastic cone in the neck of each flask to reduce the rate of escape of trapped flies, were used as traps for blowflies in cages. The potential attractants were the residues from extracts of flies. The residues were obtained by treating each flask with a measured volume of extract and driving off the solvent either by incubating at 40–45° C (6–12 hr) or in a Büchi rotary evaporator at 55–60° C. Unless stated otherwise, each flask contained 5 mg residue or approx. 100 fly equivalents. Control (or blank) flasks were treated with a small volume of solvent which was driven off as described above.

The flasks were placed into cages, 7–12 cm from the edges, containing insects supplied with sugar and water placed at the center of the base. Each rectangular cage contained approx. 240 flies in equal sex ratio, and when cylindrical cages (43 cm high, 51 cm diam.) were employed, each cylindrical cage contained approx. 325 flies in equal sex ratio. Four flasks were placed in a square array in a rectangular cage (experiments 1 and 2), or six flasks were placed in a hexagonal array in a cylindrical cage (experiments 3–5). After two days, the flasks were removed from the cages, and the flies caught in each flask were sexed and counted.

The experiments were conducted in an insectary, temperature 26–28° C; relative humidity, 45–75%; 15 hr light–9 hr dark; light intensity, 1.6 lux, unless stated otherwise.

Design and Analysis of Experiments. In each rectangular cage, the four flasks consisted of duplicates of each of two different treatments, and the duplicates were placed diagonally opposite each other. In each cylindrical cage, the six flasks consisted of duplicates of each of three different treatments, and the duplicates were placed at random. In the tables summarizing the results of experiments, each figure is the sum of the numbers of males or females trapped in a pair of duplicates.

When an experiment was conducted on more than one occasion, fresh test materials and assay flies were used on each occasion.

A split-plot analysis of variance was performed on the square roots of the counts of males or females caught in each flask (Snedecor, 1956).

RESULTS AND DISCUSSION

Chemical Nature of Dipteran (Sex) Pheromones. Recent investigations have shown that sex pheromones in Diptera are components of the cuticular lipid which are saturated or unsaturated hydrocarbons (Mayer et al., 1972; Girard and Budris, 1975; Kostelc et al., 1975; Langley et al., 1975; Uebel et al., 1975, 1976, 1977, 1978a,b; Richter et al., 1976; Lang, 1977; Sonnet et al. 1977; Carlson et al., 1978; Linley and Carlson, 1978), but oxygen-containing lipids such as ketones and esters may also have an effect (Uebel et al., 1978c). Moreover, some sex pheromones may act as aggregation pheromones, as in *Musca domestica* (Carlson and Beroza, 1973; Richter et al., 1976; Ghizdavu, 1978).

Thus, when testing for the presence of pheromones in the extracts of *L. cuprina*, it seemed reasonable to concentrate on the nonpolar fraction.

Since Goodrich (1970) used dichloromethane extracts, such extracts were tested for attractiveness to blowflies in the first two experiments described below. However, all types of lipid tend to dissolve in this solvent which is of intermediate polarity. As it was desirable to test the nonpolar

lipids, e.g., hydrocarbons, separately from the polar ones, e.g., short-chain fatty acids, a mixture of a nonpolar solvent (hexane) and a polar solvent (methanol) was also used to extract flies. The hexane and methanol fractions were separated and treated as described above. The nonpolar fractions, i.e., the hexane-soluble lipids, were also tested for attractiveness in the first two experiments.

Experiment 1. Both the dichloromethane extract and hexane fraction from females caused larger numbers of flies to be caught in flasks (Table 1, Pt. 1, $F = 10.23$, $P < 0.05$; Pt. 2, $F = 13.74$, $P < 0.05$). From the present trials, this was due to more flies entering these flasks than the control flasks, rather than to a differential rate of escape; the effect of the odor was short-range and did not extend beyond the entrance to a flask, i.e., around 10 cm or less from the source. This suggests that there is an attractant in the extracts of females and that it is relatively weak or short-range. Furthermore, significantly more females than males were attracted to the residues from a dichloromethane extract ($F = 16.36$, $P < 0.01$), and a comparable result is apparent for the hexane fraction but the difference between sexes was not significant ($F = 0.51$, $P > 0.10$).

The methanol fraction from the hexane-methanol extract used above was also tested, and it was unattractive (extract v blank, $F = 0.01$, $P > 0.10$). The attractant thus appears to be confined to the nonpolar fraction.

Experiment 2. Both the dichloromethane extract and hexane fraction

TABLE 1. SUMMARY OF RESULTS OF EXPERIMENT 1, PARTS 1 AND 2[a]

	Numbers of flies trapped (sums of pairs of flasks)							
	Pt. 1, dichloromethane				Pt. 2, hexane fraction			
	Extract		Blank		Extract		Blank	
Cage	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
1	47	30	16	21	25	15	6	7
2	62	41	5	13	17	17	8	7
[b]	109	71	21	34	42	32	14	14
[c]	180		55		74		28	

[a] Pt. 1. The numbers of flies attracted to flasks containing a dichloromethane extract of female *L. cuprina* of solvent alone. Pt. 2. The numbers of flies attracted to flasks containing the hexane fraction from a hexane-methanol extract of female *L. cuprina* or solvent alone.
[b] Totals by sex of fly.
[c] Totals by extract.

TABLE 2. SUMMARY OF RESULTS OF EXPERIMENT 2, PARTS 1 AND 2[a]

	Numbers of flies trapped (sums of pairs of flasks)							
	Pt. 1, dichloromethane				Pt. 2, hexane fraction			
	Extract		Blank		Extract		Blank	
Cage	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
1	23	14	106	15	11	8	11	16
2	6	10	65	8	7	14	13	20
[b]	29	24	171	23	18	22	24	36
[c]	53		194		40		60	

[a] Pt. 1. The numbers of flies attracted to flasks containing a dichloromethane extract of male *L. cuprina* or solvent alone. Pt. 2. The numbers of flies attracted to flasks containing the hexane fraction from a hexane–methanol extract of male *L. cuprina* or solvent alone.
[b] Totals by sex of fly.
[c] Totals by extract.

from males were unattractive, judging from the relative numbers of flies trapped; indeed, the residues from the former repelled females (Table 2, Pt. 1, $F = 6.67$, $P < 0.05$), and a comparable result is evident for the latter but the contrast was not significant (Table 2, Pt. 2, $F = 0.07$, $P > 0.10$).

Considering experiments 1 and 2, the odors from extracts of females caused more flies, particularly females, to be trapped, while the odors from the corresponding extracts of males caused fewer flies to be trapped in flasks. These results are consistent with an attractant being present in the female extracts, and since more females than males were attracted, it is possibly one of the stimuli inducing gravid females to aggregate.

Since the attractant appeared to be confined to the non-polar fraction from females, pentane extracts of females and of males were employed in the following two trials to test for attraction to the pentane-soluble lipids. The extracts were presented to caged blowflies at different light intensities on days 1 and 2 of experiment 3, allowing observations to be made on the influence of light intensity on the numbers of insects caught in the various treatment groups. In view of the outcome of experiment 3, only a pentane extract of females was examined for any dose–response effect in experiment 4.

Experiment 3. The female extract induced more flies, particularly females, to enter flasks than the blank, although not significantly so; the blank, in turn, trapped more insects than the male extract (Table 3, $F = 3.48$, $P < 0.05$). The female extract was significantly more attractive than that of the male ($F = 6.42$, $P < 0.05$); but despite a higher overall response by females ($F = 31.36$, $P < 0.01$), there was not a significantly greater attraction

of this sex to the female extract (extract-sex, $F = 1.78$, $P > 0.10$). The higher overall response of females was partially owing to a far larger number of this sex being trapped on day 2 (days, $F = 3.97$, $0.05 < P < 0.10$), which was due to their increased activity at the higher light intensity.

These data provide further evidence for the presence of an attractant in the nonpolar fraction from female extracts that tends to attract more females than males.

Experiment 4. There was a significant regression of the numbers of females trapped on the weight of residue (Table 4, $F = 8.59$, $P < 0.01$), indicating that an increased dose of residue led to a larger number of females

TABLE 3. SUMMARY OF RESULTS OF EXPERIMENT 3[a]

		Numbers of flies trapped (sums of pairs of flasks)					
		Pentane blank		Female pentane extract		Male pentane extract	
Day	Cage	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
1	1	29	17	9	10	21	10
	2	28	7	25	12	12	14
	3	1	3	41	15	17	14
	4	31	12	29	16	17	11
	[b]	89	39	104	53	67	49
	[c]	128		157		116	
2	1	10	14	91	16	6	9
	2	8	13	88	12	20	8
	3	64	16	31	14	48	6
	4	58	13	38	11	57	8
	[d]	140	56	248	53	131	31
	[e]	196		301		162	
	[f]	229	95	352	106	198	80
	[g]	324		458		278	

[a] The number of flies attracted to flasks containing solvent alone or a pentane extract of male or female *L. cuprina* at a light intensity of 1.6 lux, day 1; and 3.2 lux, day 2.
[b] Totals by sex of fly, day 1.
[c] Totals by extract, day 1.
[d] Totals by sex of fly, day 2.
[e] Totals by extract, day 2.
[f] Totals by sex of fly, days 1 + 2.
[g] Totals by extract, days 1 + 2.

TABLE 4. SUMMARY OF RESULTS OF EXPERIMENT 4[a]

		Numbers of flies trapped (sums of pairs of flasks)					
		0 mg		5 mg		20 mg	
Day	Cage	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
1	1	29	17	26	14	55	22
	2	5	18	32	19	47	22
2	1	4	12	46	18	34	11
	2	18	16	20	12	64	20
	[b]	56	63	124	63	200	75
	[c]	119		187		275	

[a]The numbers of flies attracted to flasks containing solvent alone, or 5 mg or 20 mg residue from a pentane extract of female *L. cuprina*.
[b]Totals by sex of fly.
[c]Totals by weight of residue.

responding. On performing such an analysis on the numbers of males trapped, there was no significant dose–response relationship. More females than males were attracted to the extract ($F = 12.95$, $P < 0.01$), and the higher dose was almost significantly different from the lower dose of residue ($F = 4.24$, $0.05 < P < 0.10$). These results are attributable to larger numbers of females responding to the attractant as its concentration in the air near the opening of each flask increases, while the males remained relatively unresponsive to the attractant even when it was at comparatively high concentrations.

Experiment 5. Greenblatt et al. (1977) obtained the main component of the sex pheromones of *Trogoderma* spp., 14-methyl-8-hexadecenal, from their air-space volatiles, and the aldehyde was not detected in extracts of beetles. They suggested that a response to extracts of beetles is probably due to the corresponding alcohol and ester (see also Yinon et al., 1970). The following experiment was conducted to test whether there are components of the attractant in the air-space of *L. cuprina*, which may not have been obtained by extracting flies.

The data below suggest that the air-space of female blowflies is a poor source of the attractant or that the method of trapping fly odors was inappropriate, as the numbers of insects caught in the various treatment groups are virtually equal (Table 5). In the analysis, there were no sources of variation even approaching significance. The lack of a significant contrast is not surprising in view of the following information.

In Table 6, each figure is the mean of three observations, except for the three figures in parentheses which are single observations. The position of

eicosane relative to the other constituents was the reference point when identifying the hydrocarbons present in each peak using Goodrich's data (1970).

The GC profiles for the Ciba-Geigy strain and a Canberra strain are similar, as are the profiles for males and females in the smaller lipids (13–24 carbons), but relatively large sex differences are apparent among the concentrations of some 25 to 31-carbon lipids. When these profiles were compared with those obtained by Goodrich for the hydrocarbons, it was clear that lipids other than hydrocarbons (for example, esters) are present in the hexane fractions. Substantial quantities of esters and fatty acids were found in the dichloromethane extracts of flies (Goodrich, 1970).

The differences between the GC profiles presumably account for the contrasting effects of the hexane fractions and the pentane extracts of males and females on female behavior. The largest difference between the male and female extracts occurs at a peak with the same retention time as branched saturated hydrocarbons with a 26-carbon backbone; the respective concentrations of such lipids in males and females are 18–19% and 38% (Table 6). The main component of the attractant is most likely to be among these lipids in the female fraction.

The main component of the attractant may therefore be of relatively low volatility and, since substances of low volatility may not have been effectively collected in the gas chromatography supports used in the last experiment (see Byrne et al., 1975), this suggests that the lack of a differential response to the extracts was due to a low concentration of the attractant in the extracts of GC supports.

TABLE 5. SUMMARY OF RESULTS OF EXPERIMENT 5[a]

	Numbers of flies trapped (sums of pairs of flasks)					
	Blank		Female extract		Male extract	
Cage	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
1	37	24	23	25	31	24
2	22	30	35	23	29	28
[b]	59	54	58	48	60	52
[c]	113		106		112	

[a] The numbers of flies attracted to flasks containing solvent alone, or the hexane extracts of GC supports in which airborne volatiles from male or female *L. cuprina* had been collected.
[b] Totals by sex of fly.
[c] Totals by extract.

TABLE 6. PERCENTAGE CONCENTRATIONS OF CONSTITUENTS OF HEXANE FRACTIONS FROM MALE AND FEMALE *L. cuprina*, FOR CIBA-GEIGY STRAIN AND CANBERRA STRAIN[a]

Number of carbons	Female		Male	
	Ciba-Geigy	Canberra	Ciba-Geigy	Canberra
13	0.13	0.07	0.50	0.26
14	0.75	0.66	1.16	1.14
15	0.18	0.16	0.14	0.25
16	2.54	1.67	1.98	2.30
17	0.09	0.11	0.19	0.14
18	0.94	0.91	1.14	2.42
19	0.31	0.19	0.46	0.39
	0.42	0.23	0.57	0.55
	0.35	0.24	0.47	0.48
20	0.19	0.14	0.23	0.26
	0.58	0.34	0.66	0.71
21	0.06	0.06	0.11	0.14
22	0.43	0.13	0.46	0.27
23	0.62	0.27	0.90	0.38
24	?	(0.08)	(0.07)	?
25	4.34	4.32	1.44	1.19
	2.96	3.54	1.60	1.56
26	1.03	0.99	0.74	0.57
	38.32	37.96	19.30	18.12
27	2.70	1.81	6.26	4.81
	3.51	3.87	8.28	10.28
28	—	—	—	—
	28.72	29.40	27.45	27.52
29	1.63	1.79	5.19	5.30
	2.35	2.94	7.56	8.71
30	—	—	—	—
31	6.77	6.97	13.06	11.61
>31	(0.08)	1.10	?	0.71
	100.00	99.95	99.92	100.07

[a] Mean of three observations; numbers in parentheses indicate single observations.

Another factor which may have affected both experimental results and GC profiles is the transfer of cuticular lipid from one sex to the other. Males and females spent at least four days in the same cage, and during this time cuticular material may have been transferred between sexes, as is known to occur in *Stomoxys calcitrans* (Harris et al., 1976). Such cross-contamination would tend to decrease sex differences, and thus would bias the results of an experiment toward not finding a difference. Despite this possible effect, differences between the cuticular lipids of the sexes were demonstrated above.

DISCUSSION

The typical Dipteran (sex) pheromone consists of hydrocarbons of around 20–30 carbons and is effective over a short-range only (see the literature quoted above). However, nonhydrocarbons of about 20 or more carbons may also play a role (Uebel et al., 1978c). Thus, from the above GC data, the attractant may have a molecular size comparable to that of other pheromones. The attractant is clearly not a sex pheromone nor an oviposition stimulant, since more females than males responded to the female extracts in the present trials. Also, when oviposition occurred in flasks, it appeared to depend on the numbers of flies trapped, rather than on the extract that the flask contained. It is suggested that the attractant (or pheromone) is one of the stimuli inducing gravid female blowflies to aggregate before and during oviposition; it may hence be regarded as a type of aggregation pheromone. Ghizdavu (1978) has noted previously that substances acting as sex pheromones can also act as aggregation pheromones depending on the age and sex of the insects.

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NASONOV PHEROMONE OF THE HONEY BEE, *Apis Mellifera* L. (HYMENOPTERA, APIDAE). PART III. Regulation of Pheromone Composition and Production

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Abstract—GC and GC-MS analyses of the multicomponent Nasonov pheromone of the honey bee, and of the air above insects releasing the pheromone, show that constant composition is maintained during release, despite differing volatilities of the components. The regulating mechanism may involve a specific enzyme process, detected in excised Nasonov glands, which converts the major component geraniol into the more volatile (*E*)-citral. Analysis of honey bees of known ages and at different times of year shows that maximum secretion occurs when foraging is most likely.

Key Words—Honey bees, *Apis mellifera*, pheromone, Nasonov pheromone, enzymic oxidation, terpenoids.

INTRODUCTION

The secretion from the Nasonov gland of the worker honey bee comprises seven terpenoids: (*Z*)-citral, (*E*)-citral, nerol, geraniol, nerolic acid, geranic acid, and (*E,E*)-farnesol in established proportions (Pickett et al., 1980). Reconstituted mixtures, with proportions naturally present in the secretion, show full attraction to foragers only if all seven components are present (Williams et al., 1981a). In this bioassay using foragers, in electron antennography (Williams et al., 1981b), and in a bioassay involving cluster formation (Ferguson et al., 1979), (*E*)-citral, one of the most volatile but least abundant components, was most active. During release of the pheromone, the proportion of (*E*)-citral would be expected to fall unless supply could be maintained and, since Shearer and Boch (1966) reported that citral was

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formed, possibly from geraniol, during aging of the Nasonov secretion, Blum (1971) suggested that terpenes might be transformed by enzymic oxidation in the gland. Here, we present firm evidence for this suggestion, and also investigate the variation in amounts of pheromonal secretion in honey bees of known ages and at different times of the year to extend work by Boch and Shearer (1963).

METHODS AND MATERIALS

Methods are described in the order in which they appear under Results and Discussion. After comparing the composition of pheromone released naturally from glands, and from synthetic mixtures, changes in the composition of pheromone in contact with excised Nasonov glands were investigated. The apparent presence in glands of a regulatory mechanism involving enzymic oxidation was confirmed and further characterization attempted, by standard biochemical procedures and by comparison with the course of chemical oxidation in the air.

Composition of Nasonov Secretion after Pheromone Release. Worker honey bees (ca. 500) were removed from their colony and placed on the floor inside a cage $2 \times 2 \times 2$ m in a glass house. Many released pheromone for several minutes from the Nasonov gland. Such insects were selected and the secretion from the Nasonov groove of the live insect analyzed by capillary column gas chromatography with a flame-ionization detector (GC–FID) after direct extraction (Pickett et al., 1980). Seven replicate analyses gave the mean ratio of (*E*)-citral to geraniol.

Analysis of Nasonov Pheromone Released into Air (Methods A and B). In method A, honey bees (50), captured as they left their hive to forage, were placed in the separatory funnel (D1/62, Quickfit) of the glass apparatus (Figure 1). Traps (head, MF 28/3/250; trap, MF 24/3/8; Quickfit) were cooled with solid CO_2, the ground-glass joints sealed with PTFE sleeves (FVS/3 and FVS/2 Fi-vac, Fisons), and ground-glass taps sealed with

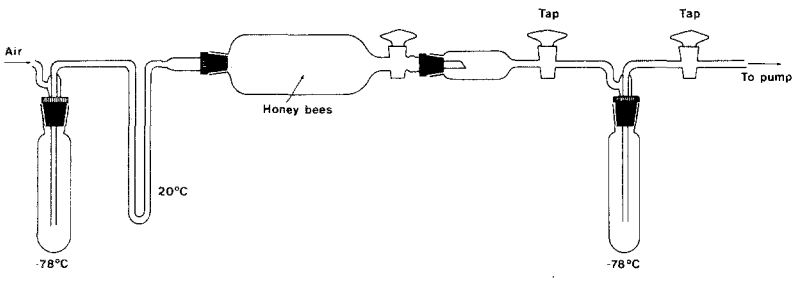

FIG. 1. Apparatus for trapping Nasonov pheromone.

Apiezon grease. Taps were opened and air was drawn through the apparatus by a mechanical pump (5 ml/sec) fitted to a water bubbler. The first trap removed water and other volatile components from the air which was then warmed to 20° C before reaching the honey bees and stimulating them to release Nasonov pheromone. Pheromone and other volatiles from the honey bees, including water, condensed in the second trap. After ca. 1 hr the second trap was warmed to 20° C, the condensate extracted with pentane (2 × 2 + 1 ml), and concentrated (0.7 ml) under N_2.

Concentrated condensates were analyzed by capillary column GC (Ucon 5100 and heat-treated Carbowax 20M, glass, 50 m × 0.25 mm) coupled with mass spectrometry (MS) (VG Micromass 70-70F) at 40–140°, 2° C/min (Pickett et al., 1980). Components were identified from mass spectra and peak enhancement with authentic compounds (Pickett et al., 1980). The mean ratio between areas of peaks from (*E*)-citral and geraniol was determined from two condensates.

In method B, the needle tip of a 10-μl glass syringe (10A-FN-SP, Scientific Glass Engineering) containing pentane (5 μl) was held close (ca. 10 mm) to the Nasonov gland of a honey bee, releasing pheromone. The plunger was slowly depressed to maintain a droplet of pentane at the needle tip until approximately 0.4 μl remained. This was withdrawn into the syringe and three such samples analyzed by capillary column GC-MS (single ion monitoring at *m* /*z* 69 at maximum sensitivity, Pickett et al., 1980). Peaks were identified by retention times only.

Analysis of Air above Synthetic Pheromone Mixture. Synthetic pheromone components were mixed, without solvent, in the natural proportions (Pickett et al., 1980). A portion (100 μl) was placed in a 50-ml tube and a sample taken from the air halfway down the tube by method B. The sample was analyzed by capillary column GC-MS (Pickett et al., 1980) and peaks identified from retention times and mass spectra. The procedure was repeated and the mean ratio between areas of peaks from (*E*)-citral and geraniol determined.

Analysis of Synthetic Pheromone Mixture after Exposure to Air. Portions (1 ml) of synthetic pheromone components (3000 honey bee equivalents, Pickett et al., 1980) in paraffin oil (5 ml) were placed in four 90-mm-diameter glass petri dishes exposed to the air. Two dishes were extracted immediately with hexane (5 ml) and analyzed by packed column GC-FID (Pickett et al., 1980) in duplicate, recording the areas of the peaks from (*E*)-citral and geraniol. The remaining two dishes were analyzed similarly after a 60-min exposure.

Quantitative analysis of Geraniol and (E)-Citral in Contact with Excised Glands. Solutions of geraniol and (*E*)-citral (5–70 ng/μl and 3–40 ng/μl, respectively) in hexane containing octadecane (90 ng/μl) as internal standard were analyzed in duplicate by packed-column (2.5% Carbowax 20 M, glass, 6

ft × ¼ in, Chromosorb G-HP 80–100#) GC-FID at 150° for 20 min, 10°/min to 200°C (Pickett et al., 1980) and areas normalized against those of octadecane. From means of the duplicate analyses, regression analysis gave calibration equations for the two components.

Nasonov glands (20) from honey bees captured as they left their hives to forage were excised (Pickett et al., 1980), crushed with the solution of octadecane in hexane (200 μl), and stored at 4° C. Samples of the hexane phase were periodically analyzed in duplicate by GC and absolute amounts of geraniol and (*E*)-citral calculated from the calibration equations.

The following modifications were used in similar experiments: (1) 0.02 M phosphate buffer (100 μl; pH 7) added to crushed glands; (2) 12 M urea in buffer (100 μl; pH 7) added to crushed glands; (3) ether replaced hexane; (4) hexane phase separated from honey bee tissue by gravity filtration through paper; and (5) nerol at 0.1 μg/μl added to solution of octadecane in hexane

Relative Proportions of Geraniol, (E)-Cital, Nerolic Acid, and Geranic Acid during Contact with Excised Glands. Nasonov glands (160), excised from foragers (Pickett et al., 1980), were placed in a tube with hexane (5 ml) and a solution (3 ml) containing nonanoic acid (20 μl) as internal standard in hexane (150 ml) at 4°C. Samples (1.2 ml) of the hexane phase were periodically removed, filtered, and a portion (1 ml) treated with diazomethane (Schlenk and Gellerman, 1960). After concentration to 100 μl (N_2 stream), duplicates (0.2 μl) were analyzed by capillary column (Ucon 5100) GC–FID (Pickett et al., 1980) at 100° C. Areas of GC peaks were normalized against those of methyl nonanoate and means calculated from duplicates to give the relative GC peak areas for geraniol, (*E*)-citral, nerolic and geranic acids (as methyl esters).

After 44 hr, the nonanoic acid solution (1.7 ml) and a solution (2.8 ml) of geraniol (20 μl) in hexane (150 ml) were added. After 73 hr, more nonanoic acid solution (1.7 ml) and a solution (2.8 ml) containing geraniol (200 μl) in hexane (150 ml) were added. GC analyses were continued periodically after each addition.

Conversion of Geraniol by Homogenized Nasonov Glands. Excised Nasonov glands of foragers (20) were homogenized, using a glass rod and tube, with 0.02 M phosphate buffer (200 μl; pH 7) and centrifuged at 2700 $g_{av.r.}$ for 5 min. The supernatant was retained for testing and solids resuspended in more buffer (200 μl); after centrifuging, the solids were suspended in fresh buffer (200 μl) for testing. A solution (1 ml) of geraniol (200 μl) in hexane (150 ml) was added to the first supernatant and to the final suspension of the solid residue and the mixtures stored at 25° C.

The hexane phases were analyzed immediately and after 3 and 6 days. A sample (100 μl) was removed and added to a solution (1 ml) of nonanoic acid (20 μl) in hexane (150 ml). The solution was treated with diazomethane

(Schlenk and Gellerman, 1960) and concentrated to 100 μl in N_2. Duplicates (0.2 μl) were analyzed by capillary column GC-FID as above.

The effects of the following variations in procedure were also examined. (1) adding Triton X-100 detergent (0.1, 1.0, and 5.0%) to the buffer used; (2) after homogenising the glands with buffer (5× the usual scale), ultrasonication (60 W, 20 kHz, 15 min, ice cooling) was applied, followed by ultracentrifugation at 150,000 $g_{av.r.}$ at 4° C; (3) replacement of geraniol by nerol or decan-1-ol; and (4) addition of sodium cyanide (10^{-3} M) to the buffer used to make the final suspension of glandular residue.

Aerial Oxidation of Geraniol and (E)-Citral. Geraniol or (*E*)-citral (10 μl, 56 μmol) in hexane (1 ml) was sealed in a glass ampoule under air (40 ml, 360 μmol O_2). The ampoule containing geraniol was stored in sunlight for 7 days and that containing (*E*)-citral in darkness at 4° C for 3 months. The contents were then analyzed by packed-column GC-FID and capillary column (Ucon 5100) GC-MS, 40°–150° at 4° C/min (Pickett et al., 1980). Major components were identified from mass spectra of authentic compounds (Pickett et al., 1980) and by peak enhancement on two GC columns unless otherwise stated. Yields were calculated from peak area in GC-FID.

Variation in Amounts of Geraniol and (E,E)-Farnesol in Nasonov Glands of Honey Bees of Different Ages and at Different Times of Year. Bees of known ages were obtained by incubating combs of sealed brood at 35° C during August. On emergence, the bees were painted distinctively on the thorax and introduced to a colony. At intervals during the next nine months, 10 bees of each age, given in Figure 6, were removed from the colony and the amounts of geraniol and (*E,E*)-farnesol in individual excised glands determined by GC (Pickett et al., 1980).

RESULTS AND DISCUSSION

The proportion of (*E*)-citral to geraniol in the Nasonov secretion immediately after release of the pheromone was 1.3 ± 0.2 (SE) parts to 100 [(*Z*)-citral, 0.8 ± 0.2, nerol, 0.6 ± 0.1]; this did not differ significantly from the proportion in glands that had not recently released pheromone (1.1 ± 0.2 to 100, see Pickett et al., 1980). Nasonov pheromone trapped from air above enclosed honey bees (Figure 1) contained 5 parts of (*E*)-citral to 100 of geraniol. Figure 2 is a chromatogram of the entrapped pheromone; peaks A to D are from (*Z*)-citral, (*E*)-citral, nerol, and geraniol, repectively. (*E*)-Citral in air collected above individual honey bees releasing pheromone (method B) could not be measured accurately, but geraniol was confirmed as major component of the released pheromone.

The ratio of (*E*)-citral to geraniol in air above the synthetic pheromonal

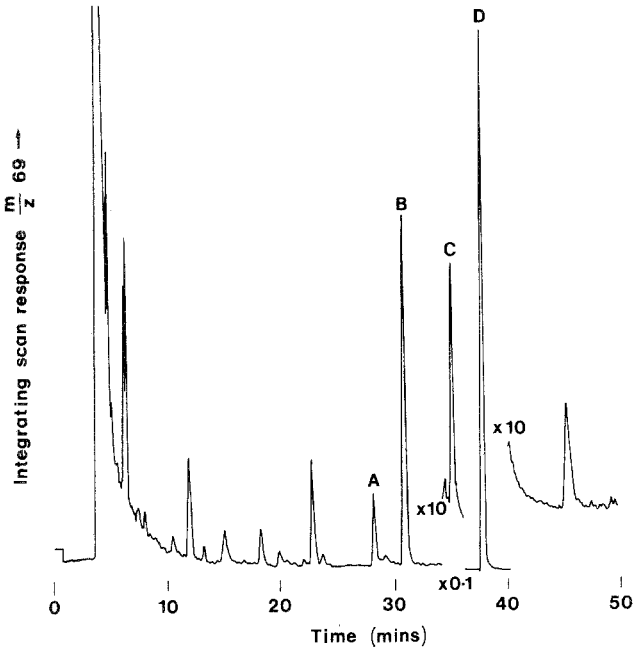

FIG. 2. GC-MS of trapped Nasonov pheromone (heat-treated Carbowax 20M capillary column, 50 m × 0.25 mm, glass).

mixture, immediately after exposure, was 5 parts to 100, the same as for the natural pheromone released into air. In the liquid phase of the synthetic pheromone mixture, the proportion of (*E*)-citral to geraniol fell, as expected, from 1.1:100 to 0.4:100 on exposure to air for 60 min. This indicated a mechanism for forming (*E*)-citral during release of the pheromone to maintain the proportion of (*E*)-citral to geraniol constant during release.

Nasonov glands stored in hexane gave a level of (*E*)-citral which rose to a maximum after 10 hr (Figure 3) whereas the geraniol level fell steadily, suggesting that endogenous geraniol is converted into (*E*)-citral by the gland. In support, conversion was observed at a similar rate in the presence of phosphate buffer but stopped when this was replaced by a 12 M solution of the protein denaturant urea (Mahler and Cordes, 1971), when ether replaced hexane, or when the hexane layer was separated from glandular tissue. Nerol added to the hexane was not converted (i.e., $<1\%$) and the GC peaks from (*E,E*)-farnesol remained unchanged throughout each experiment. Therefore, a specific enzymic process in the Nasonov gland appeared to be involved in converting geraniol to (*E*)-citral.

The combined amounts of geraniol and (*E*)-citral diminished as conver sion of geraniol proceeded, indicating that (*E*)-citral was in turn converted

into another compound. Relative proportions of other pehromone components, including the terpenoid acids (as methyl esters) were therefore determined during storage of excised Nasonov glands (Figure 4, only proportions of components where changes occurred during storage are given). As the proportion of geraniol fell, the proportion of geranic acid rose greatly and that of nerolic acid slightly. The procedure used for this study was less accurate for (*E*)-citral the level of which had already begun to rise before the experiment. After geraniol was completely converted, a fourfold excess (Figure 4) and a 40-fold excess (Figure 5) over the endogenous amount of geraniol added to the same glandular preparation resulted again in conversion with accompanying increase in proportions of the other compounds.

Thus, Nasonov glands converted the major Nasonov component, geraniol, into (*E*)-citral which in turn gave geranic acid in the preparations employed. This conversion is not surprising as it involves oxidation of an alcohol through the aldehyde to the acid, with the rest of the molecule unchanged. The slight increase in nerolic acid implies some isomerization of the *E* to the less sterically favored *Z* form.

Further characterization of the proposed enzyme system would require its isolation. With this objective, extraction of enzymes into buffer was attempted. However, the supernatant from tissue homogenized with buffer did not convert geraniol except when high proportions of the detergent Triton X-100 were present (Table 1). After 3 days, formation of geranic acid (estimated as methyl ester) corresponded to 28 ± 6% of converted geraniol

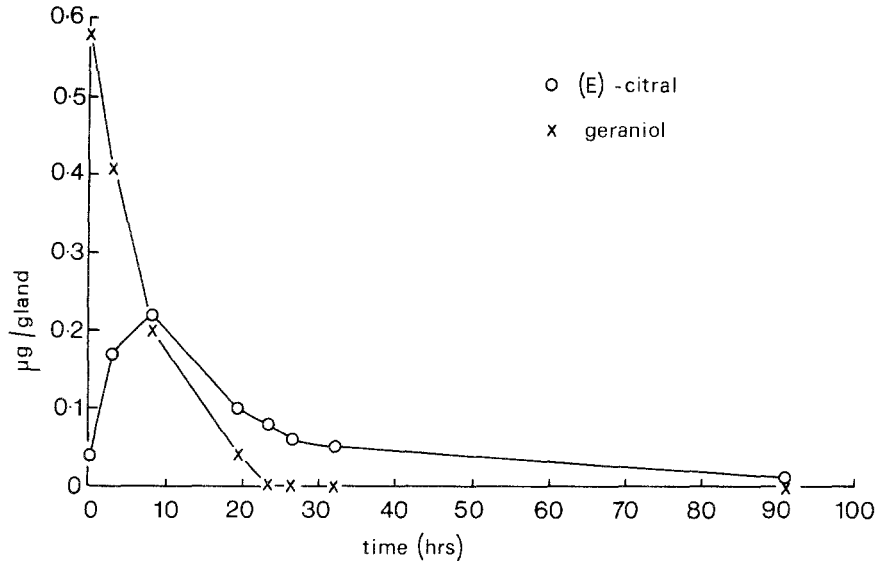

FIG. 3. Conversion of endogenous geraniol by excised Nasonov glands.

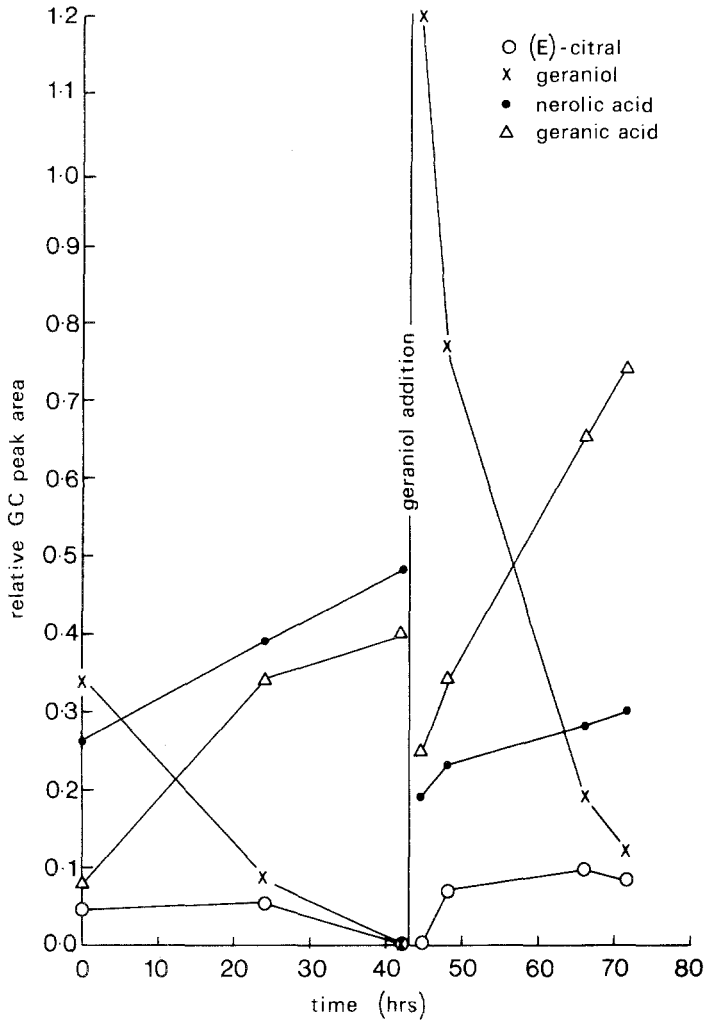

FIG. 4. Conversion of endogenous and exogenous (fourfold excess) geraniol by excised Nasonov glands.

and after 6 days, 35 ± 5%. The long reaction times in these experiments resulted from the large excess of geraniol (i.e., ca. 40 times the endogenous level) which facilitated the chemical analyses. Ultrasonication of glands in buffer resulted in complete loss of activity both in the pellet and supernatant after ultracentrifugation. These studies indicated that the Nasonov gland enzymes responsible for converting geraniol are normally bound to insoluble material, probably glandular membranes.

The extracted solid residue from the homogenization procedure was used to investigate further the specificity of the conversion process. Nerol and decan-1-ol, the reduced straight-carbon-chain analog of geraniol, were both tested and formation of nerolic or decanoic acid (as methyl esters) investigated. The acids could not be detected (i.e., $<1\%$ conversion), confirming the specificity of the process and suggesting that a mixed-function oxidase system was not involved. That metalloenzymes were not involved was corroborated by a further experiment where CN^- ions, even at a concentration of 1 mM, did not diminish conversion of geraniol to geranic acid.

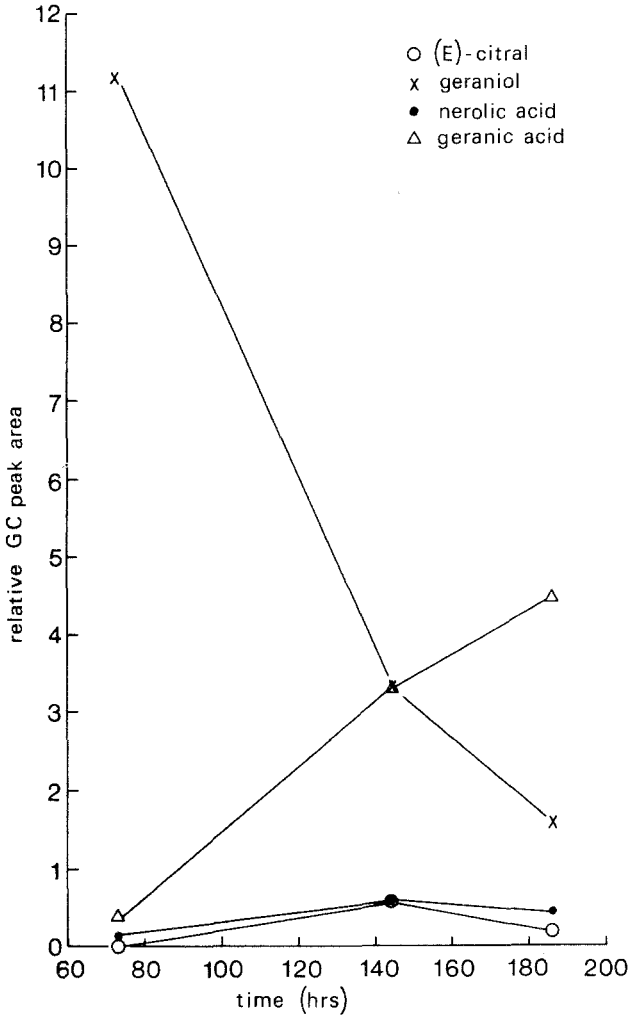

FIG. 5. Conversion of exogenous (40-fold excess) geraniol by excised Nasonov glands.

TABLE 1. CONVERSION OF GERANIOL (ca. 40 TIMES ENDOGENOUS LEVEL) BY NASONOV GLANDS AFTER HOMOGENIZATION

Preparations	% geraniol converted	
	3rd day	6th day
Buffer only		
Supernatant	0	0
Residue	29	61
0.1% Triton		
Supernatant	0	0
Residue	23	63
1.0% Triton		
Supernatant	16	27
Residue	27	55
5.0% Triton		
Supernatant	6	19
Residue	20	40

Products from aerial oxidation of geraniol and (*E*)-citral were examined to investigate differences between the enzymic and the chemical processes. The latter were expected to give additional compounds, including epoxides from oxidation at the double bonds, by analogy with aerial oxidation of other terpenoids (Pickett et al., 1977). Appreciable oxidation only occurred when geraniol was stored with air in sunlight (through glass). Principal volatile products were (*Z*)-citral, retention time (Ucon 5100 capillary column) 25.7 min (6%); (*E*)-citral, retention time 27.0 min (7%); recovered geraniol, retention time 30.0 min (56%), and other compounds: retention time 26.5 min (3%); retention time 37.4 min (4%); retention time 38.4 min (10%), and retention time 40.4 min (2%). The compound retention time 40.4 min was tentatively identified by MS alone as geraniol 6,7-epoxide *m/z* 170 (M^+) 152 ($M^+ - 18$), 41, 59, 81, 85, 43, 71, 39, 27. Compounds with peaks at retention time 37.4 min and 36.4 min also had parent ions at *m/z* 170 and were probably related epoxides. Aerial oxidation of (*E*)-citral proceeded in darkness, and the major volatile products were: recovered (*E*)-citral (40%); geranic acid, retention time 58 min (13%), and a compound, retention time 36.4 min (23%) tentatively identified by MS only as (*E*)-citral 6,7-epoxide, *m/z* 168 (M^+), 97, 27, 43, 57, 29, 71, 55, 39. Although aerial oxidation of (*E*)-citral gave fewer products than did geraniol, probably because double bonds did not isomerize without sunlight, chemical oxidation by aerial oxygen gave major products in addition to those from enzymic oxidation. Even traces of these other products were not detected after enzymic oxidation, although the enzyme system is necessarily aerobic.

Production of Nasonov pheromone varied with the age of the honey bee and with time of year. Newly emerged honey bees had little geraniol (mean $0.09 \pm 0.01\,\mu g/10$ bees) and no detectable (*E*,*E*)-farnesol (Figure 6). Observations at the hive entrance showed that no marked bees foraged before they were 7 days old. The amounts of both components increased rapidly with age to a maximum at 28 days old. Maximum recovery from an individual honey bee (35 days old) was 5.8 μg geraniol and 1.6 μg (*E*,*E*)-farnesol. In cold periods, such as those at the beginning of October and during winter, the levels of geraniol and (*E*,*E*)-farnesol were low and foraging ceased. In spring, levels increased as foraging resumed but then fell, presumably due to physiological aging, shortly before the bees died.

Thus Nasonov pheromone production is closely related to the physiological needs of the honey bee; it increases when young and overwintered bees begin to forage but diminishes in winter when foraging ceases. This confirms and extends work of Boch and Shearer (1963).

We conclude therefore that the honey bee controls the ratio of (*E*)-citral, the more volatile component, to geraniol during release of the Nasonov pheromone. The mechanism is most likely a highly specific enzyme system, found in excised glands, to convert geraniol into (*E*)-citral. (*E*)-Citral is further converted into geranic acid. This latter process may be an artifact of the preparation but more likely represents the mechanism by which geranic acid is produced. The small quantity of nerolic acid from this process could have resulted from isomerase activity which would be necessary if (*Z*)-citral, nerol, and nerolic acid were produced from geraniol. In addition the pheromone secretion is only produced when needed.

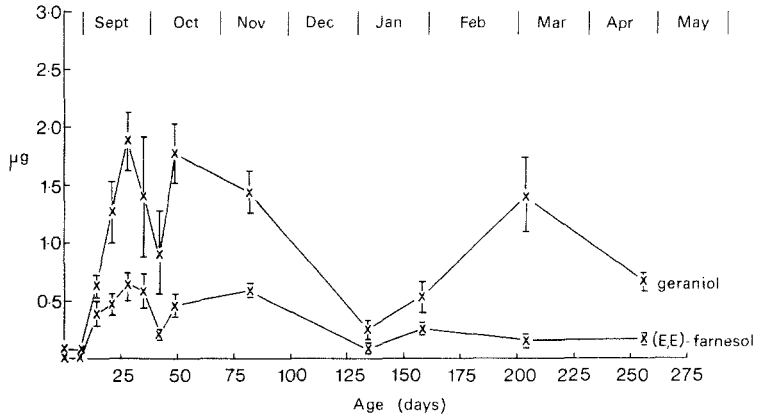

FIG. 6. Variation in mean amounts of geraniol and (*E*,*E*)-farnesol in the Nasonov secretion (μg of component per honey bee $\pm$SE).

Many other insects have multicomponent pheromones and, where components differ in volatility, mechanisms similar to those in the honey bee studied here may operate to maintain pheromone composition. The formation of pheromone components in exocrine secretions, from chemical precursors rather than by synthesis from water-soluble biochemicals such as organic pyrophosphates, may also be a common phenomenon.

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YEAST AND CORN HYDROLYSATES AND OTHER NUTRITIOUS MATERIALS AS ATTRACTANTS FOR ONION AND SEED FLIES[1,2]

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Abstract—The attractancy of enzymatic yeast hydrolysate, a corn hydrolysate, brewers' yeast, honey, ammonia, *n*-dipropyl disulfide, and several combinations of these treatments was tested in an onion field containing onion flies, *Hylemya antiqua* (Meigen) and seedcorn flies, *H. platura* (Meigen). Enzymatic yeast hydrolysate (concentrated powder) proved to be highly attractive to both fly species; females were more responsive than males. Brewers' yeast and corn hydrolysate were intermediately attractive, while all other treatments were only slightly or not at all attractive. Although no steps were taken to prolong release of volatiles, some of the nutritious materials remained highly attractive even after 11 days. The yeast products may, at certain times, be even more attractive than specific host volatiles. However, their activity was not constant over time and may be influenced by the stage of reproductive development of female flies.

Key Words—Onion fly, *Hylemya antiqua*, seedcorn fly, *Hylemya platura*, Diptera, Anthomyiidae, yeast hydrolysates, brewers' yeast, food attractants.

INTRODUCTION

Investigations into the chemical stimuli which mediate host finding by the onion fly, *Hylemya antiqua* (Dindonis and Miller, 1980, 1981a–c), have shown that flies of both sexes are attracted by damaged and decomposing

[1]Diptera: Anthomyiidae
[2]Paper No. 9511 of the Michigan State University Agricultural Experiment Station.

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onions as well as high release rates (mg/hr) of certain synthetic constituents of onions. Moreover, upon arriving at sources of damaged and decomposing onions, onion flies were observed to feed for extended periods (Dindonis and Miller, 1981a).

The question arose as to how various food attractants which were not host-related might compare to specific host odors as potential baits for *H. antiqua* monitoring traps. We report here results of field tests on the comparative attractiveness of various foodstuffs to *H. antiqua* as well as to the seedcorn fly, *H. platura*, which coinhabited the onion field under study.

METHODS AND MATERIALS

Experimental Design. Materials to be evaluated for attractiveness to flies were placed under acetate cone traps (Dindonis and Miller, 1980) deployed along the edges of an *H. antiqua*-infested commercial onion field in Stockbridge, Michigan. Either 30% aqueous ethylene glycol or 0.5% Triton X solutions was used as an ensnarement medium in the traps. A linearly arranged randomized complete-block design (see Dindonis and Miller, 1980 for justification) with a 3- to 4-m intertrap spacing was used with four replicates in each experiment.

Experiment 1. The treatments, dispensed in 60-mm plastic petri dishes covered with 30 mesh/cm cotton fabric, were: (1) EYH, enzymatic yeast hydrolysate (NBC, Cleveland, Ohio) (10 g of solid concentrate); (2) BY, brewers' yeast (Bio-Serv, Inc., Frenchtown, New Jersey) (10 g solid concentrate); (3) MB, Miller 3787 Insecticide Bait (10 ml of liquid concentrate) which is an acid-catalyzed hydrolysis product derived from corn (Miller Chemical and Fertilizer Corporation, Hanover, Pennsylvania); (4) H, honey (Michigan Harvest Honey, Lowell, Michigan) (10 ml concentrate liquid); (5–8) EYHS, BYS, MBS, and HS which were 10 ml of 10% solutions of each respective substance above in 5% aqueous sucrose; (9) *n*-Pr_2S_2, *n*-dipropyl disulfide (>99% pure by GLC) (Eastman Organic Chemicals, Rochester, New York) dispensed from a saturated size 3 BEEM™ polyethylene enclosure (Dindonis and Miller, 1981b); (10) C_1, a control, 10 ml of 5% aqueous sucrose solution; and (11) C_2, a control consisting of an empty dispenser dish.

EYH, BY, and H were selected because they elicited significant aggregation of *H. antiqua* at feeding dishes in laboratory studies (Niemczyk, 1965; McLeod, 1964); moreover, yeast products are known to be attractive to other fly species, e.g., the Caribbean fruit fly, *Anastrepha suspensa* (Lopez et al., 1971), and the apple maggot fly, *Rhagoletis pomonella* (Neilson, 1960). Likewise, corn and other plant hydrolysates have been known to attract insects. The sucrose solutions of each of the treatments were meant to: (1) lower the release rate of volatiles from each substance, and (2) promote

microbial activity, e.g., ethanol release by the live yeasts. *n*-Pr_2S_2 was included because it could be released at a steady rate (Dindonis and Miller, 1980b), and hence this treatment (as well as the controls) served as a reference point for fly activity over time.

Experiment 1, was conducted July 30 to August 10, 1979. Flies were collected after 1, 2, 3, 4, 7, and 11 days and traps were rerandomized within blocks on day 3.

Experiment 2. If microorganisms were to colonize the proteinaceous foodstuffs, one of the likely end products of amino acid catabolism would be ammonia, which is itself attractive to certain flies, e.g., the apple maggot fly (Hodson, 1943; Oatman, 1964; Moore, 1969). In a second experiment, 5% household ammonia (NH_3) was evaluated for attractancy to *H. antiqua* and *H. platura*. Also, trap catches elicited by the combinations of EYH + *n*-Pr_2S_2 and *n*-Pr_2S_2 + NH_3 were compared to those of each material tested separately. Combinations were achieved by placing separate dispensers for each material under one trap. Experimental procedures were similar to those of experiment 1. This test was conducted August 17–24, 1979, during which time baits were replenished as necessary.

RESULTS

Experiment 1. The average catches of onion and seedcorn flies over the 11 days of this test are presented in Table 1. Overall, the patterns in catch were very similar for the two species. The most highly attractive material for females of both fly species was EYH; catches were more than double those of any other treatments and were 17 and 27 times the control catches for *H. antiqua* and *H. platura* females, respectively. BY, MB, and the diluted yeast products (BYS and EYHS) were all similarly attractive to onion fly females, producing catches ca. 6 times the control. In this test, H, MBS, HS, and surprisingly, *n*-Pr_2S_2, did not catch significantly more female *H. antiqua* than the control 5% sucrose solution. Likewise, BY, BYS, EYHS, as well as MB and MBS, were intermediately to slightly attractive to *H. platura* females while H, HS, and *n*-Pr_2S_2 were not attractive.

The materials most attractive to onion fly males were BYS and BY, followed by EYH. However, these treatments did not, on the average, elicit stronger responses from males than females (Table 1). Few *H. platura* males were caught; however, the yeast products and MB did catch numbers distinguishable from controls.

Judging from catches by the controls and unattractive treatments, the male–female ratios for *H. antiqua* and *H. platura* in this field were ca. 1 : 1 and 6 : 1, respectively. EYH was clearly more attractive to the females than males, while EYHS and MB also caught significantly more *H. antiqua* females than

TABLE 1. ONION AND SEEDCORN FLY RESPONSES TO VARIOUS NUTRITIOUS MATERIALS IN THE FIELD

Treatments[a]	Mean number of flies caught per treatment[b]							
	Hylemya antiqua				*Hylemya platura*			
	♀	♂	♀ + ♀	♀/♂	♀	♂	♀ + ♂	♀/♂
1. Enzymatic yeast hydrolysate (EYH)	191a	27bc	218a	7.1a	54a	5a	59a	10.8[c]
2. Brewers' yeast (BY)	74b	38ab	112b	2.0bcd	22b	3ab	25b	7.3
3. Miller insecticide bait (MB) (corn hydrolysate)	57c	19cde	76c	3.0b	11c	2b	13c	5.5
4. Honey (H)	12cd	10ef	23e	1.2d	4d	1bc	5d	4.0
5. Dilution of 1 (EYHS)	60b	21cd	81c	2.9bc	24b	2b	26b	12.0
6. Dilution of 2 (BYS)	81b	45a	126b	1.8cd	21b	3ab	24b	7.0
7. Dilution of 3 (MBS)	30c	14def	44d	2.1bcd	10c	0.5bc	11c	20.0
8. Dilution of 4 (HS)	15cd	12def	27de	1.3d	2d	0.3c	2d	6.7
9. *n*-Dipropyl disulfide (*n*-Pr_2S_2)	23c	8f	31de	2.9b	3d	0.5bc	4d	6.0
10. 5% sucrose solution (control 1)	13cd	8ef	21e	1.6d	3d	0.5bc	4d	6.0
11. Empty dispenser (control 2)	11d	11def	22e	1.0d	2d	0.3c	2d	6.7

[a]See Methods section for a complete description of treatments.
[b]Means followed by the same letters within columns are not statistically different at the 5% level as determined by a planned F test for mean separation of data transformed to $(X + 0.5)^{1/2}$ for trap catches and log $(X + 0.5)$ for sex ratios.
[c]The large number of zero catches for *H. platura* males precluded accurate statistical analysis of sex ratios.

males. Although the low catches of male *H. platura* precluded accurate analysis of sex ratios for this species, there was a trend toward elevated female catches for EYH, EYHS, and possibly MBS.

The patterns in female *H. antiqua* catch over time are presented in Figure 1. On day 1, both EYH and BY were highly attractive. By day 2, the attractiveness of BY relative to the other treatments was greatly reduced while the relative attractiveness of BYS began to increase significantly, peaking at day 3 for both females and males. At this time, yeasts were active and ethanol could be detected by the human nose.

The catch of onion fly females by EYH declined over the first 4 days but then stabilized. It cannot be concluded that this decline represented reduced attractiveness only; as judged from catches by the controls and unattractive treatments, fly activity also declined initially and subsequently rose slightly.

At all times, EYH caught significantly more flies than any other treatment, and after 11 days, it was still catching at the rate of 10 times the control.

For *H. platura*, attractive materials maintained activity over the 11 days with only slight declines. There were no clear increases in the attractiveness of any treatments with respect to the others.

Experiment 2. Five percent NH_3 proved to be unattractive to *H. antiqua* females and males (Table 2). In this experiment, *n*-Pr_2S_2 was significantly attractive to *H. antiqua* females but not males. Addition of NH_3 to *n*-Pr_2S_2 did not increase catch significantly for females but did so for males. During experiment 2, EYH was not significantly more attractive to female *H. antiqua* than *n*-Pr_2S_2; trap catches by EYH were only 8 times the control rather than 17 as in experiment 1. Also, the sex ratio for the EYH catch did not highly favor females; however, fewer females than males were caught in the control traps. Addition of *n*-Pr_2S_2 to EYH did not increase catch over EYH alone.

For *H. platura*, NH_3 proved to be significantly repellent. However, this effect was not apparent when *n*-Pr_2S_2 and NH_3 were combined. EYH trap

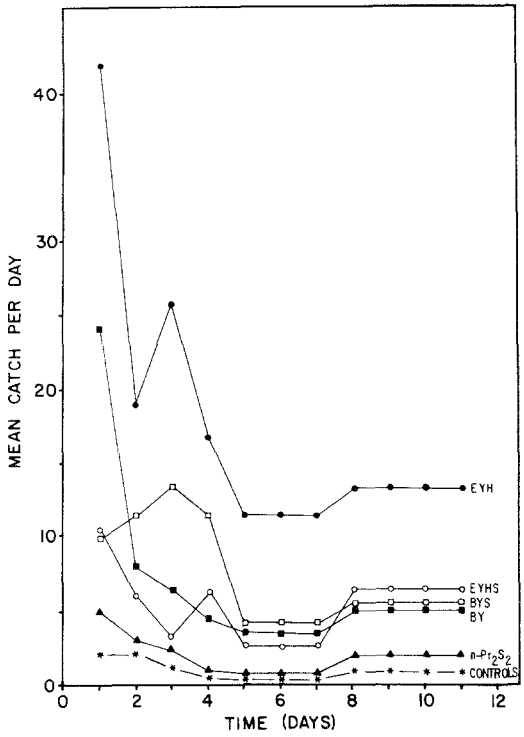

FIG. 1. Patterns in female onion fly trap catch as elicited by various nutritious materials over time. Points between 5 and 7 and 8 and 11 days are averages for those periods.

TABLE 2. ONION AND SEEDCORN FLY RESPONSES TO AMMONIA, *n*-DIPROPYL DISULFIDE, ENZYMATIC YEAST HYDROLYSATE AND THEIR COMBINATIONS IN THE FIELD

Treatments	Mean number of flies caught per treatment[a]			
	Hylemya antiqua			*Hylemya platura*[b]
	♀	♂	♀ / ♂	♀
1. Ammonia (NH_3)	4c	7c	0.6bc	0.3e
2. *n*-Dipropyl disulfide (*n*-Pr_2S_2)	28b	20b	1.4a	3d
3. 1 + 2	37ab	36a	1.0ab	4cd
4. Enzymatic yeast hydrolysate (EYH)	41ab	33ab	1.2a	24a
5. 4 + 2	53a	32ab	1.7a	11b
6. Unbaited	5c	12bc	0.4c	4cd

[a] Means followed by the same letters within columns are not statistically different at the 5% level as determined by a planned *F* test for mean separation of data transformed to $(X + 0.5)^{1/2}$ for trap catches and to log $(X + 0.5)$ for sex ratios.

[b] A total of four male *H. platura* were caught, precluding both statistical analyses for males and establishment of sex ratios.

catch was only 8 times the control compared to 27 in experiment 1. Addition of *n*-Pr_2S_2 to EYH caused a significant reduction in catch for *H. platura*.

DISCUSSION

Several of the nutritious materials included in these experiments yielded large catches of onion and seedcorn flies in the field; hence, they do offer potential as baits for monitoring traps. Preliminary observations in the field suggest that volatiles from the nutritious materials elicited fly behaviors very similar to those elicited by chopped onions, i.e., a series of positive taxes (most likely anemotaxes) punctuated by periods of resting on the substrate (Dindonis and Miller, 1981a). Hence, volatiles from these materials such as EYH probably serve as long-range attractants in the field as well as arrestants and short-range attractants as documented by the laboratory cage experiments of Niemczyk (1965).

The reason for onion fly responsiveness to these materials is most certainly nutritional. In the lab, vitellogenesis cannot occur without an exogenous source of protein (McLeod, 1964). Furthermore, certain protein sources (micronutrients might also be involved) are clearly superior to others in promoting oogenesis. For *H. antiqua*, brewers' yeast and yeast hydrolysate (apparently not enzymatic) were superior to soya flour or a mixture of

brewers' yeast and soya flour (McLeod, 1964). Moreover, in the lab, the foodstuffs most adaptive for egg production seemed to elicit the largest aggregations in feeding dishes and the most prolonged feeding (McLeod, 1964; Niemczyk, 1963).

In the field, female onion flies must likewise secure exogenous protein. The strong selection pressure to do so should, over time, lead to the development of finely tuned physiologic mechanisms for efficient finding of optimal protein sources.

The pattern in attractiveness of the various nutritious materials that emerged from the present field experiments with *H. antiqua* is essentially consistent with the pattern in feeding preferences found in the lab (Niemczyk, 1965). In both cases EYH was the most stimulatory material, especially for females. Materials such as BY and acid corn hydrolysates promoted significantly less feeding in the lab and less long-range orientation in the field. While honey appeared to be a fairly good short-range "attractant" in the lab, it was totally inactive in our field experiment. Although more tests need to be conducted to determine which materials are most effective at promoting oogenesis (EYH should be compared to BY), it appears that *H. antiqua* is most attracted to the odors from protein sources that would be maximally adaptive reproductively.

The work of Niemczyk (1965) would further suggest that the attractiveness of EYH to *H. antiqua* females might vary with time. In the lab, newly eclosed flies do not aggregate on EYH dishes; however, by ca. day 3, strong aggregation on this material is noticed and it lasts until ca. day 12, peaking at day 8. Males are fairly uniformly responsive to EYH. Hence, as age structure within a fly population changes, responsiveness to EYH could rise or fall. Possibly, this happened between experiments 1 and 2 of this study, for identical EYH sources were comparatively less attractive to females during experiment 2.

The variability in attractiveness of *n*-Pr_2S_2 is harder to explain. In all our previous experiments (Dindonis and Miller, 1981c, and references therein), one capsule filled with this compound was significantly attractive to both male and female onion flies. The field in which the present experiments were conducted contained large numbers of damaged and maggot-infested onions, especially during experiment 1. Possibly, a high background of host volatiles masked the *n*-Pr_2S_2 plumes in a manner equivalent to the disruption of pheromone communication by the atmospheric permeation technique.

Since certain foodstuffs like EYH seem to be as attractive to *H. antiqua* (if not more so) as any of the host cues so far investigated, the chemistry of their volatiles should be explored. Perhaps, as suggested for the case of green lacewing, *Chrysopa carnea*, attraction to protein hydrolysates (Hagen et al., 1976; van Emden and Hagen, 1976), the active constituents, may be volatile degradation products of certain amino acids like tryptophan. Syn-

thetic EYH volatiles should be useful tools in monitoring *H. antiqua* and *H. platura* as well as other insect populations. In the interim, the powdered concentrates may be useful baits.

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ATTRACTANT LURES FOR MALES OF THE PEA MOTH, *Cydia nigricana* (F.) CONTAINING (*E*)-10-DODECEN-1-YL ACETATE AND (*E,E*)-8,10-DODECADIEN-1-YL ACETATE

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Abstract—Lures containing different amounts of (*E*)-10-dodecen-1-yl acetate and (*E,E*)-8,10-dodecadien-1-yl acetate were formulated in natural rubber stoppers or polyethylene vials and tested in traps in the field to establish relative attractiveness to males of the pea moth, *Cydia nigricana* (F.), dose–response relationships, and the effects of weathering. Initially, both formulations of (*E,E*)-8,10-dodecadien-1-yl acetate were much more attractive than those of (*E*)-10-dodecen-1-yl acetate, but their activity diminished considerably after only 5 days in the field because an inhibitor was formed, and polyethylene formulations were not attractive after one week. However, lures containing (*E*)-10-dodecen-1-yl acetate, with or without antioxidants, in both rubber and polyethylene were still attractive after three months' exposure in the field and are therefore suitable for practical monitoring of pea moth populations.

Key Words—Pea moth, *Cydia nigricana* (F.), Lepidoptera, Olethreutidae, sex attractant, (*E*)-10-dodecen-1-yl acetate, (*E,E*)-8,10-dodecadien-1-yl acetate, lure, formulation, antioxidant, monitoring.

INTRODUCTION

The pea moth, *Cydia nigricana* (F.) is a major pest of peas in the main pea growing areas of Britain and other parts of Europe. In the past, populations of this insect have been difficult to monitor effectively, the best method being to search for eggs on the foliage of the crop (Gould and Legowski, 1964). This laborious task required skilled observers but yielded unreliable information because only a small proportion of the crops at risk could be examined.

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Nevertheless, egg counts were used with some success as a basis for regional insecticide spray warnings issued by the Agricultural Development and Advisory Service and the Processors and Growers Research Organisation. The discovery that (*E,E*)-8,10-dodecadien-1-yl acetate (*E,E*8,10–12:Ac), which has been identified in the female sex pheromone (Greenway and Wall, 1980), and (*E*)-10-dodecen-1-yl acetate (*E*10–12:Ac) are potent sex attractants for the male pea moth (Wall et al., 1976) provided an alternative method, and the presence of economically significant populations can now be detected easily and conveniently by trapping moths with lures containing *E*10–12:Ac (Macaulay, 1977). This technique is now the basis for the rational application and timing of pesticides to control pea moth in Britain and some other parts of Europe.

For reliable monitoring data, lures must be formulated to provide a stable source of attraction during the flight period of the insect. Various substrates have been investigated as formulations for controlling the release of lepidopteran attractants (Fitzgerald et al., 1973, and references therein); polyethylene (Glass et al., 1970) and natural rubber (Maitlen et al., 1976) have usually proved effective. The present experiments were designed to investigate the relative potencies of the two pea moth sex attractants in such formulations to provide reliable long-lasting attractive lures for monitoring moth populations and for other studies on pea moth behavior. The antioxidants, BHT (Doolittle et al., 1976), UOP88, and UOP688 (Wolf et al., 1972) were also tested in combination with *E*10–12:Ac since they had been found to preserve the activity of certain insect attractant formulations.

METHODS AND MATERIALS

Lures. The attractants were *E*10–12:Ac (99.6% pure by GLC) from Farchan Division, Story Chemical Corporation, Willoughby, Ohio, and *E,E*8,10–12:Ac (99.1% pure), synthesized from the parent alcohol (Chemical Samples Co., Columbus, Ohio). The antioxidants were *N,N'*-dioctyl-*p*-phenylenediamine (UOP88), *N*-phenyl-*N'*-octyl-*p*-phenylenediamine (UOP688) (UOP Chemical Co., East Rutherford, New Jersey) and 2,6-di-*tert*-butyl-4-methylphenol (BHT). Lures were prepared by applying the required amount of attractant with or without antioxidant in redistilled dichloromethane (100 μl) either to the cup of the rubber stopper (natural rubber serum stopples, 5000/9602, Perkin-Elmer Ltd., Beaconsfield, Bucks.) or inside the polyethylene vial (XLON XT1530 specimen tubes, 9.5 × 38 mm, Scientific Supplies, London). The solvent was allowed to evaporate while the attractant was absorbed, the hinged lids of the polyethylene vials were closed and the lures stored in screw-cap glass bottles in the dark at −15° C until required or treated as described below.

Field Tests. In experiments [at Arches Hall Farm, Ware, Herts. (G.R.TL395196); Grange Farm, Hexton, Beds. (G.R.TL106317); and Manor Farm, Buckworth, Huntingdon (G.R.TL151763)] in peas or in wheat fields after peas, traps were set out in rows (replicates) and treatments were assigned to traps randomly within each row; the distance between traps within replicates was never less than 20 m. All treatments were replicated at least three times and replicates were done either concurrently or sequentially in the same field. Most lures were tested in the field over a short period (8 days or less), so that the amount of attractant in each lure would not be depleted substantially and the relative rates of release would be constant throughout the test periods.

Lures were suspended on aluminium wire within triangular aluminium traps (Lewis and Macaulay, 1976), the removable inserts coated with Tangletrap (Tanglefoot Company, Grand Rapids, Michigan) and the traps erected on stands at crop height in wheat or on the ground in peas. Traps were examined at regular intervals (often daily) in the morning before the flight period, captured moths were counted and removed and the sticky inserts replaced if necessary.

Experiment 1. Lures containing *E*10–12:Ac formulated in rubber stoppers (1, 10, 10^2, and 10^3 μg) and in polyethylene vials (1, 10, 10^2, 10^3, and 10^4 μg) were compared with those containing *E,E*8,10–12:Ac in rubber (10^{-1}, 1, 10, and 10^2 μg) and polyethylene (10^{-1}, 1, 10, 10^2, and 10^3 μg) in wheat after peas. Trapped moths were counted each day for 5 days.

Experiment 2. Lures containing 3×10^2, 10^3, 3×10^3 and 10^4 μg of *E*10–12:Ac in rubber were tested in wheat after peas with five replicates of variable duration (2–8 days).

Experiment 3. Six of the most effective formulations from experiment 1 (*E*10–12:Ac lures of 10^2 and 10^3 μg in rubber, 10^3 and 10^4 μg in polyethylene; *E,E*8,10–12:Ac lures of 10^2 μg in rubber and 10^3 μg in polyethylene) were exposed in traps in the field for periods of 0, 1, 2, or 3 weeks during June (range of average temperatures 13.0–19.3° C) and their relative attractiveness assessed in pea crops for 8 days (*E*10–12:Ac lures) or 1 day (*E,E*8,10–12:Ac) in separate experiments.

Experiment 4. Rubber and polyethylene lures containing *E*10–12:Ac (10^3 μg) alone or with 2×10^2 μg of an antioxidant (BHT, UOP88, or UOP688) were exposed in traps in the field for periods of 0, 1, 2, or 3 months between March and June (range of average temperatures 3.9–13.6° C). Similar lures were placed in glass bottles on a south-facing windowsill in a laboratory for three months. Lures were field tested in replicates in wheat after peas lasting for 4–17 days each, depending on weather conditions.

An analysis of variance was done for each experiment after log transformation of data.

Analyzing Lures. The residual content of *E* 10–12: Ac or *E,E* 8,10–12: Ac in lures was determined by extraction with redistilled hexane (10 ml) containing nonadecane (10^2 μg/ml) as an internal standard and subsequent GLC according to Greenway et al. (1980).

RESULTS AND DISCUSSION

Attractiveness of Fresh Lures. When *E* 10–12: Ac and *E,E* 8,10–12: Ac were field tested at doses between 10^{-1} and 10^4 μg, initially (day 1, Figure 1), *E,E* 8,10–12: Ac was much more attractive than *E* 10–12: Ac over the whole of the dose range in both rubber and polyethylene formulations ($P < 0.01$). Both formulations were equally effective for both attractants except that 10^{-1} μg of *E,E* 8,10–12: Ac was more attractive in rubber than polyethylene ($P < 0.02$). With *E,E* 8,10–12: Ac there was no significant effect of dose between 1 and 10^2 μg, but below this there was a decrease in activity, whereas *E* 10–12: Ac showed a significant dose–response relationship [similar to that

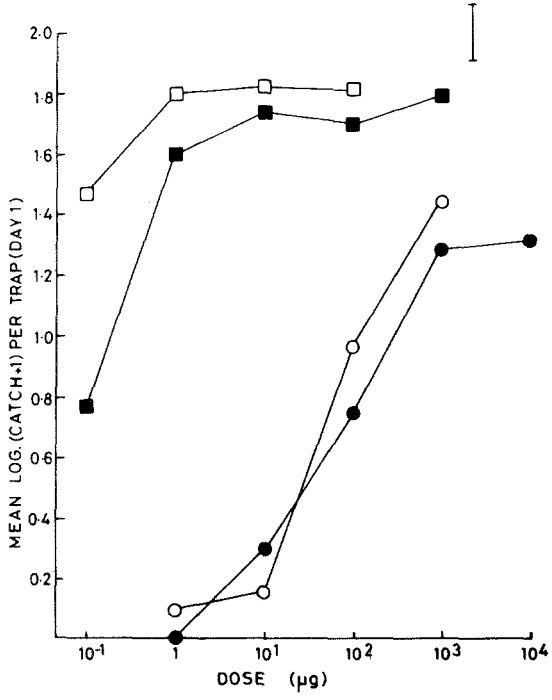

FIG. 1. Catches of male *C. nigricana* in traps containing rubber or polyethylene lures with different doses of *E* 10–12: Ac and *E,E* 8,10–12: Ac (day 1). ○, *E* 10–12: Ac in rubber; ●, *E* 10–12: Ac in polyethylene; □, *E,E* 8,10–12: Ac in rubber; ■, *E,E* 8,10–12: Ac in polyethylene. Bar = standard error of difference between means.

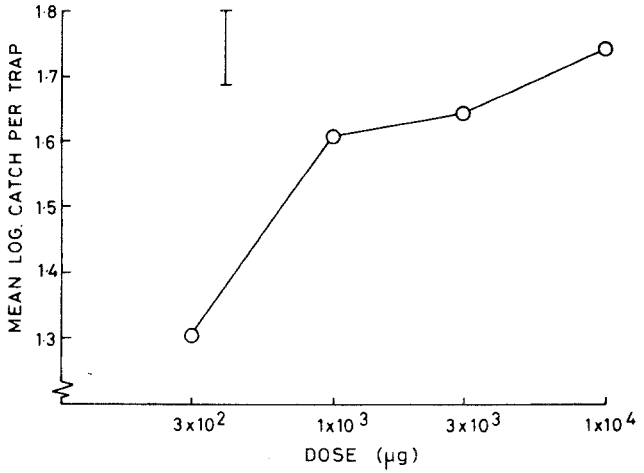

FIG. 2. Catches of male *C. nigricana* in traps containing rubber lures with different doses of *E*10–12:Ac. Bar = standard error of difference between means.

found for *Adoxophyes orana* (Fischer von Röslerstamm) by Minks and Voerman, 1973] between 1 and 10^3 μg, leveling off thereafter up to 10^4 μg (Figure 1). The data in Figure 2, obtained later when it had become clear that *E*10–12:Ac in rubber would provide the most suitable lures for monitoring pea moth, confirm a significant effect of dose up to 10^3 μg ($P < 0.05$) but no further increase in attractiveness up to 10^4 μg.

The responses to both attractants reached plateaus at 1 μg for *E,E*8,10–12:Ac and 10^3 μg for *E*10–12:Ac, and this maximum response was greater to *E,E*8,10–12:Ac than to *E*10–12:Ac. There was no sharp optimum response to dose as found for *Trichoplusia ni* (Hübner) (Gaston et al., 1971), nor the continuing increase of catch with dose as in *A. orana* (Minks and Voerman, 1973).

Attractiveness of Weathered Lures. After 5 days in the field (Figure 3), the dose–response and formulation effects with *E*10–12:Ac were very similar to those on the first day (Figure 1), but for *E,E*8,10–12:Ac the pattern of catches had changed dramatically especially for the polyethylene formulations. Both formulations of *E,E*8,10–12:Ac had declined in activity so that, although the rubber lures were still more attractive than both *E*10–12:Ac formulations, their relative superiority was less than on the first day; this is indicated by a significant chemical–day interaction ($P < 0.05$) for the rubber formulations, which results from the decline in activity of *E,E*8,10–12:Ac compared with no change in the activity of *E*10–12:Ac. The decrease in attractiveness was very marked with the polyethylene formulations, some of which lures were actually less attractive than equivalent *E*10–12:Ac lures. Thus, by day 5 the two formulations of *E,E*8,10–12:Ac differed markedly

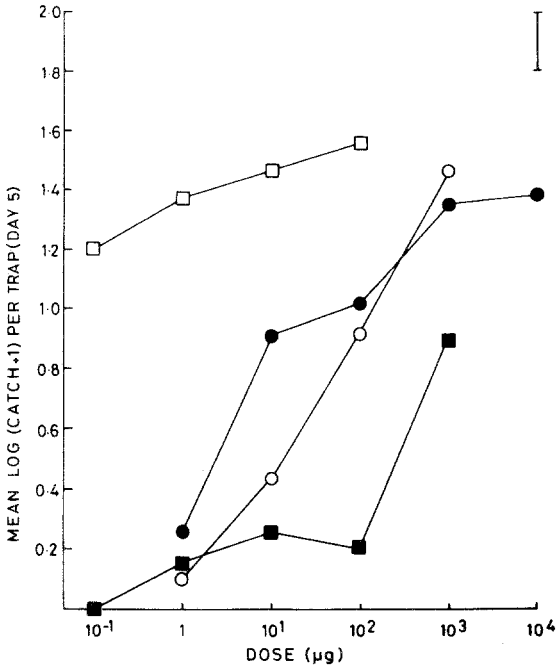

FIG. 3. Catches of male *C. nigricana* in traps containing rubber or polyethylene lures with different doses of *E*10–12:Ac and *E,E*8,10–12:Ac (day 5). ○, *E*10–12:Ac in rubber; ●, *E*10–12:Ac in polyethylene; □, *E,E*8,10–12:Ac in rubber; ■, *E,E*8,10–12:Ac in polyethylene. Bar = standard error of difference between means.

as a result of a differential loss of attractiveness, ($P < 0.001$) while exposure of *E*10–12:Ac lures for this period had not resulted in a reduction of their attractiveness (see also Figures 5 and 6). These data are consistent with earlier results (Wall et al., 1976), which showed that rubber *E*10–12:Ac lures were more attractive over 14 days than those containing an equal dose of *E,E*8,10–12:Ac, although the latter were much more attractive initially.

Figure 4 shows the effects of weathering on two selected lures containing *E,E*8,10–12:Ac for up to three weeks. These results confirm and extend those in Figure 3; there was a substantial decline in the activity of *E,E*8,10–12:Ac in both formulations ($P < 0.01$). Overall, 10^2 μg in rubber was considerably more effective than 10^3 μg in polyethylene ($P < 0.01$) due to the different weathering properties of the two formulations which have similar activities when fresh (see also Figure 1). After one week, the polyethylene lure was not attractive at all, whereas the rubber lure was still attractive even after three weeks.

Analyses of residual *E,E*8,10–12:Ac in both the rubber and polyethylene lures showed that although there had been substantial losses of attractant

during the 3-week period of weathering, some remained in both formulations (62% in rubber and 19% in polyethylene). Such amounts of the attractant should have resulted in lures of activity equal to fresh lures since this requires only 10^{-1} μg on rubber and 1 μg in polyethylene (Figure 1), so that the lures should have maintained constant attractiveness and not declined rapidly. Thus, the results in Figures 3 and 4, the lure analyses, and other unpublished data suggest the formation of an inhibitory compound(s) from decomposition of part of the *E,E*8,10–12: Ac in the lures. This was confirmed by showing that when weathered polyethylene *E,E*8,10–12: Ac lures were placed in traps with fresh lures they decreased catches to a mean of 0.5 moths per trap compared to 24.3 moths. These results agree with those of other workers who have found that under similar conditions pheromone conjugated dienes are more susceptible to oxidative degradation (C. Descoins, personal communication) than related monoenes and that this decomposition is more rapid in polyethylene than in rubber formulations (Maitlen et al., 1976). However, such decomposition products in attractant lures are presumably inactive since only McLeod and Starrett (1978) have produced evidence for the formation of an inhibitor(s) in lures for *Ostrinia nubilalis* (Hübner). The relative stability of the rubber formulation (compared to polyethylene) may be due to the

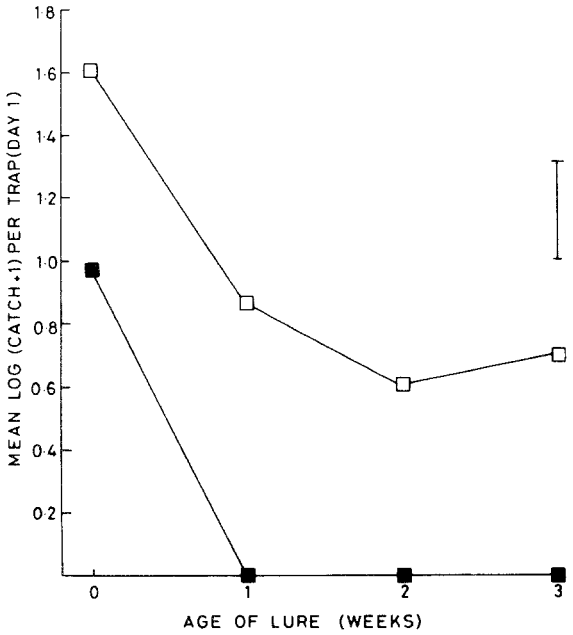

FIG. 4. Effect of age of lure on catches of male *C. nigricana* in traps containing *E,E*8,10–12: Ac. □, 10^2 μg, *E,E*8,10–12: Ac in rubber; ■, 10^3 μg *E,E*8,10–12: Ac in polyethylene. Bar = standard error of difference between means.

presence of antioxidants in the rubber, greater penetration of sunlight, or singlet oxygen (Davidson, 1979) into the polyethylene.

Thus, although *E,E*8,10–12: Ac is a very powerful attractant for the pea moth, inhibitor formation precludes its use for population monitoring at present where a long-lasting lure is required.

In contrast to *E,E*8,10–12: Ac, all the *E*10–12: Ac lures tested, irrespective of dose or formulation, maintained their initial attractiveness even after weathering for 3 weeks (Figure 5). The rubber lures were less variable than polyethylene lures, and 10^3 μg in rubber was the most effective lure with good initial activity which was maintained for 3 weeks. As expected (Figures 1, 2, and 3) (Wall and Greenway, 1981), 10^2 μg in rubber was less attractive than 10^3 μg in rubber over 3 weeks ($P < 0.01$).

Figure 6 shows the attractiveness of rubber and polyethylene *E*10–12: Ac lures weathered for up to 3 months with and without antioxidants. All the

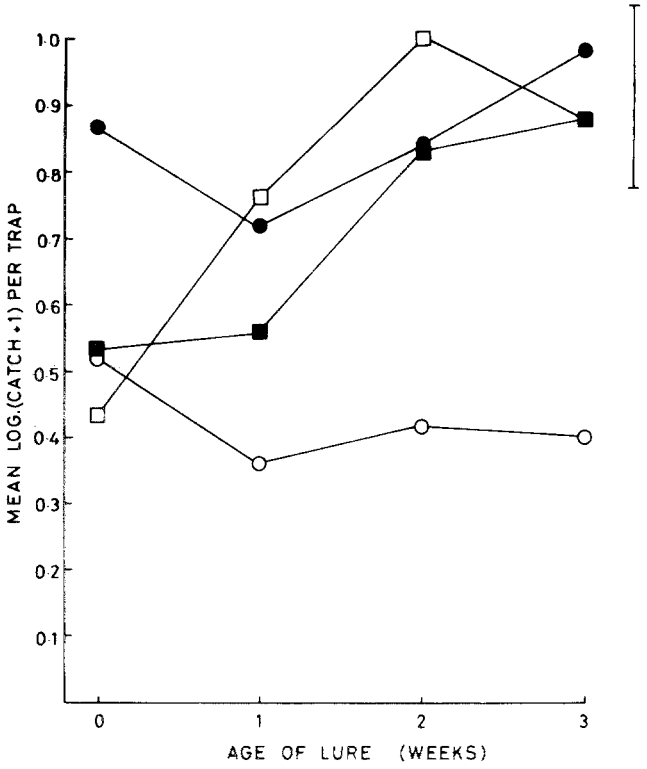

FIG. 5. Effect of age of lure on catches of male *C. nigricana* in traps containing *E*10–12: Ac. ○, 10^2 μg *E*10–12: Ac in rubber; ●, 10^3 μg *E*10–12: Ac in rubber; □, 10^3 μg *E*10–12: Ac in polyethylene; ■, 10^4 μg *E*10–12: Ac in polyethylene. Bar = standard error of difference between means.

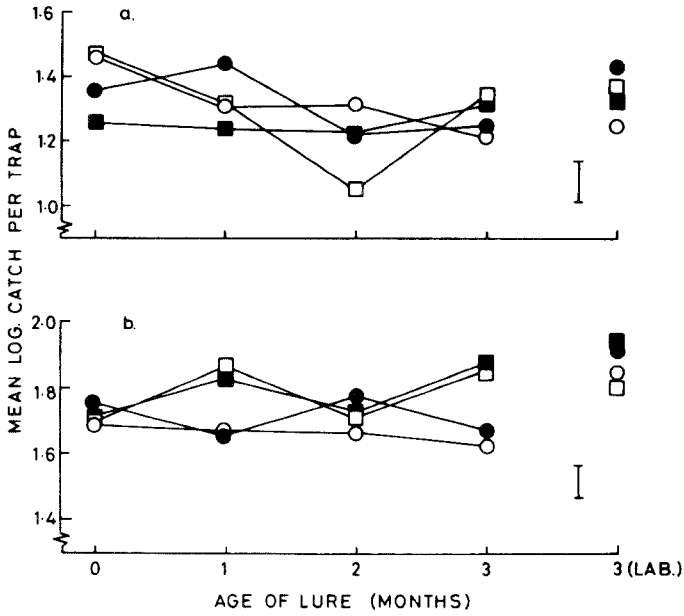

FIG. 6. Effects of antioxidants and age of lure on catches of male *C. nigricana* in traps containing 10^3 μg *E*10–12:Ac (a) in rubber and (b) in polyethylene. ○, *E*10–12:Ac alone; ●, *E*10–12:Ac + BHT; □, *E*10–12:Ac + UOP88; ■, *E*10–12:Ac + UOP688. LAB = stored in laboratory in light at room temperature. Bar = standard error of difference between means.

rubber lures retained activity during this period (Figure 6a), and none of the antioxidants had any effect on the attractiveness of either fresh or weathered lures ($P > 0.2$). Storage in the laboratory in "unfavorable conditions" (room temperature and exposure to air and sunlight) did not affect the lures. Comparision of field-weathered lures of different ages, irrespective of antioxidant treatment, showed a decline in activity during the 3-month period as the attractant was lost by evaporation, and for *E*10–12:Ac alone there was a linear decline with approximately 60% attraction remaining after 3 months. Analysis of these lures showed that a mean of 630 μg and 870 μg *E*10–12:Ac remained after 3 months in the field and in the laboratory, respectively.

Polyethylene lures differed from the rubber formulations in that *E*10–12:Ac alone was less attractive overall than the combination of all lures containing antioxidants ($P < 0.05$) but no single treatment was more attractive than *E*10–12:Ac (Figure 6b). There was no decline in the attractiveness of lures after 3 months in the field, and those stored in the laboratory were no less attractive than fresh lures. Lures contained a mean of 370 μg or 860 μg of *E*10–12:Ac after 3 months in the field or laboratory, respectively.

CONCLUSIONS

Field experiments with lures for male pea moth have shown that *E,E*8,10-12:Ac when fresh is much more attractive than *E*10-12:Ac in both rubber and polyethylene formulations. However, *E,E*8,10-12:Ac lures lose activity rapidly, especially in polyethylene, through the formation of an inhibitor(s); consequently, they would not provide a reliable source of attraction for monitoring pea moth populations over a period comparable with the flight season. Such lures might become useful for monitoring low populations of pea moth if a stable formulation could be produced.

On the other hand, rubber and polyethylene lures containing 10^3 μg of *E*10-12:Ac, although less attractive than those containing *E,E*8,10-12:Ac, retain much of their initial biological activity after 3 months in the field, even without antioxidants. For practical monitoring purposes, *E*10-12:Ac also has the advantage that traps containing it do not catch so many moths that the sticky surface becomes rapidly clogged with dead insects thus badly affecting trapping efficiency.

Rubber lures containing 10^3 μg of *E*10-12:Ac do lose some attractiveness over a 3-month weathering period, so that more detailed work on rates of release of *E*10-12:Ac from attractant formulations is necessary to produce a lure with constant attractiveness throughout the flight season of this species. This period could be as long as 3 months under British summer conditions. Our results indicate that for *E*10-12:Ac in rubber it may be possible to select an initial dose above 10^3 μg which, while not more attractive initially, will maintain constant attraction to the end of the flight season.

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AIR PERMEATION TESTS WITH (*Z,Z*)-3,13-OCTADECADIEN-1-OL ACETATE FOR REDUCTION IN TRAP CATCH OF PEACHTREE[1] AND LESSER PEACHTREE BORER MOTHS[2,3]

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Abstract—Air permeation trials were conducted to determine whether trap catches of the peachtree borer (PTB) *Synanthedon exitiosa* (Say) and lesser peachtree borer (LPTB) *S. pictipes* (Grote and Robinson) could be reduced through application of the synthetic pheromone of the PTB, (*Z,Z*)-3,13-octadecadien-1-ol acetate (*Z,Z*-ODDA), at 92% isomeric purity. The pheromone was released into the air of peach orchard test plots with hanging laminated plastic dispensers or hollow fiber dispensers in the trees and by aerial dispersal of microcapsules containing a solution of the lure. Results showed that all formulations of *Z,Z*-ODDA reduced trap catches of both PTB and LPTB in treated plots relative to catches in traps in untreated plots. In one air permeation experiment, three types of laminated dispensers and one type of hollow fiber dispenser each reduced trap catch of PTB by 93–100% and of LPTB by 75–95%. In a separate test, two formulations of microencapsulated *Z,Z*-ODDA did not reduce trap catch of male PTB as effectively as the laminated dispenser; results for LPTB were similar for both the dispensers and microcapsules. Catches of male PTB moths in traps baited with laminated and or fiber pheromone dispensers containing

[1] *Synanthedon exitiosa* (Say).
[2] *Synanthedon pictipes* (Grote and Robinson).
[3] Mention of a commercial or proprietory product does not constitute an endorsement of this product by the USDA.
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ODDA (94:6% *Z,Z*:*E,Z*) were not significantly different. LPTB male moths were also captured in these traps, the catches being dependent upon the initial pheromone loading and the type of dispensers.

Key Words—Peachtree borer, lesser peachtree borer, *Synanthedon exitiosa*, *S. pictipes*, Lepidoptera, Sessiidae, *Z,Z*-3,13-octadecadien-1-ol acetate sex pheromone, traps, air permeation, trap catch reduction.

INTRODUCTION

Female sex pheromones of *Synanthedon exitiosa* (Say), the peachtree borer (PTB), and of *S. pictipes* (Grote and Robinson), the lesser peachtree borer (LPTB), were identified by Tumlinson et al. (1974) as the *Z,Z* and *E,Z* isomers, respectively, of 3,13-octadecadien-1-ol acetate (ODDA). Field tests of traps baited with these compounds and with blends of other isomers established that trap catches of male PTB and LPTB are influenced by the isomeric purity of the synthetic pheromone. Commercial *Z,Z*-ODDA containing 1–6% of each of the other three isomers is far more attractive to male PTB than the pure *Z,Z* compound (Barry et al., 1978). Karandinos et al. (1977), McLaughlin et al. (1977), and Tumlinson et al. (1974) reported variation in LPTB catch according to purity of the *E,Z* attractant. Nevertheless, in experiments aimed at mating reduction rather than survey of populations, McLaughlin et al. (1976) used both the *Z,Z* and the *E,Z* isomer in comparative experiments to disrupt the communication of both PTB and LPTB and found that either isomer (at 92% isomeric purity) was equally effective against both species.

Air permeation experiments were therefore conducted in Georgia in 1976 and 1977 to determine whether trap catches of male PTB and LPTB could be reduced through applications of the lure. Three controlled-released devices were tested in an effort to establish which type would disperse an effective level of lure over a reasonable period: aerially dispersed microcapsules containing a solution of *Z,Z*-ODDA and plastic laminated dispensers or hollow fiber dispensers of lure attached to peach trees.

METHODS AND MATERIALS

Air Permeation Test 1 (1976). In the first test in 1976, four peach orchards located on the grounds of the Southeastern Fruit and Tree Nut Research Laboratory in Byron, Georgia, were exposed to Hercon (Herculite Products, Inc., New York, New York) plastic dispensers containing *Z,Z*-ODDA; two orchards of similar size were used as controls. Each 0.8-hectare test orchard contained 196 two- or three-year-old peach trees. On March 12, one dispenser was hung on each tree at a height of 1 m in four exposed

(treated) orchards. The orchards were retreated with new dispensers on June 21, and the test was continued for another 17 weeks. Each dispenser, 2.54-cm^2, was made from 75-μm-thick vinyl film (total dispenser thickness, 175 μm) and initially contained 20 mg of lure. Control (untreated) and treated plots were each monitored with eight Pherocon 1C traps (Zoecon Corp. Palo Alto, California). Four of these traps were baited with a #64 rubber band (Keener Rubber, Inc., 721 Commerce Court, Alliance, Ohio) containing 0.5 mg of *Z,Z*-ODDA (94% *Z,Z* + 6% *E,Z*) to trap PTB (Yonce et al., 1976); the other four were similarly baited with *E,Z*-ODDA (100% *E,Z*) to trap LPTB. Traps were checked each week, and the captured insects were removed. Baits were replaced about every 6 weeks, and traps and sticky surfaces were replaced once every 2 weeks. The reduction in trap catch of each insect in the treated orchards as compared to that in the untreated control orchards was the criterion used to assess the degree of communication disruption.

Air Permeation Test 2 (1977). The test plan was modified for the 1977 experiments. A 30.3-hectare commercial orchard near Fort Valley, Georgia, was subdivided to provide five treatment plots (0.14 hectare each) each containing 36 trees. The distance between plots was 49 m. One plot was reserved as the control (untreated) and the other four were treated with different types of dispensers containing *Z,Z*-ODDA. The isomeric purity of the *Z,Z*-ODDA (Chem Samp Co., Columbus, Ohio) was 94% (<3% of each of the other three geometric isomers). The Hercon dispensers in treatment A were made of 112.5 μm vinyl film and contained 28 mg of lure. In treatment B, the thickness of the vinyl layers of the laminate was the same as in treatment A but the lure content was increased to 39 mg. Each dispenser in treatment C was made from acrylic plastic film (75 μm) and contained 38 mg of *Z,Z*-ODDA. The dispensers in treatment D were hollow plastic fibers (Conrel Co., Albany International, Norwood, Massachusetts) containing a total of 11 mg of lure in 50 fibers per dispenser. On June 24, one dispenser was placed on every tree in each treated plot at a height of 0.9 m. Each week for 15 weeks, the dispensers were removed and transferred to another of the five plots so that each treatment, including the control, was at each location three times. The LPTB and PTB males were monitored daily with pheromone-baited traps in a manner similar to that described for test 1 in 1976; however, only 1 trap for each insect was placed in each plot. Again, the reduction in male captures in treated plots vs. control plot was used in computations of the percentage reduction in effective mating communication.

Air Permeation Test 3 (1977). In a second 1977 disruption test, peach trees near Byron, Georgia, were treated with microencapsulated (National Cash Register Co., Appleton Papers Inc., Kettering, Ohio) *Z,Z*-ODDA. Two capsule formulations differing in *Z,Z*-ODDA content (10% or 4% in three parts xylene and one part amyl acetate solvents) were tested. The gelatin-walled capsules were 50–250 μm in diameter and were plastic coated; 1% RA-

1645 (Monsanto Corp., Indian Orchard, Massachusetts) was added as an adhesive. The aqueous slurry of the microcapsules was applied at the rate of 19.7 g of *Z,Z*-ODDA per hectare to 0.8-hectare plots with a back-pack sprayer on March 25. The trees were re-treated on July 11. A reference plot was treated with hollow fiber dispensers at the rate of 1 per tree as described in test 2. Each plot, including an untreated control area, was monitored with four traps baited with *Z,Z*-ODDA to trap PTB and four traps with *E,Z*-ODDA to trap LPTB. Trap catches were recorded twice weekly for 32 weeks.

Trapping Test (1977). The attractancy of the four types of laminated and fiber dispensers used in test 2 was evaluated at Fort Valley, Georgia, from August 3 to October 7, which covered the peak flight of both PTB and LPTB. Pherocon 1C traps, each baited with one type of dispenser, were arranged in a randomized complete block with four replicates of each dispenser type. Rubber bands (#64) baited with 0.5 mg of *Z,Z*-ODDA served as reference dispensers and were replaced every 2 weeks. The traps were placed 50 m apart and hung at a height of 0.9 m. Counts of LPTB and PTB were made twice weekly from these traps that were baited with the PTB pheromone (*Z,Z*-ODDA). The sticky bottoms of the traps were replaced once every 2 weeks. Data were subjected to analyses of variance and means were separated by Duncan's multiple-range test.

Residual Lure Analysis. The lure content of each formulation was determined by gas chromatographic analysis of a hexane–acetone extraction of the lure obtained by soaking the sample in solvent for 2 days at room temperature. A 1.8-m-long column, 3 mm in diameter, packed with 3% OV-1 on Gas Chrom Q (100/120 mesh) was used for analysis; a solution of *Z,Z*-ODDA served as the external standard. The four isomers of ODDA were not separated under these conditions.

RESULTS

Air Permeation Test 1 (1976). The laminated dispensers containing *Z,Z*-ODDA caused a 96% reduction in trap catch of male PTB over a 31-week period (Table 1). The population level of this insect was quite low with a mean catch per trap per week of 1.1. On the other hand, the population of LPTB was considerably higher, with an eightfold greater trap catch in the untreated plots; the trap catch of this insect was reduced by 95% in the treated plots. Based on residual lure content of the dispensers, 1 mg per dispenser (of the 20 mg originally present) remained after 27 weeks of field aging; therefore, a total of about 3.7 g of *Z,Z*-ODDA was released from the 196 dispensers into each treated plot during the 31-week test.

Air Permeation Test 2 (1977). In the 1977 air permeation tests, three different types of laminated dispensers and 1 hollow fiber dispenser were evaluated for effectiveness in reduction of trap catch of both PTB and LPTB

TABLE 1. RESULTS OF AIR PERMEATION TESTS USING PEACHTREE BORER PHEROMONE (*Z,Z*-ODDA): REDUCTION IN TRAP CATCH OF MALE PEACHTREE BORERS AND LESSER PEACHTREE BORERS IN PLOTS TREATED WITH LAMINATED PLASTIC OR HOLLOW FIBER DISPENSERS AND MICROCAPSULES

Test and dispenser type	Initial *Z,Z*-ODDA per dispenser (mg)	$t_{1/2}$ (days)[a]	*Z,Z*-ODDA per hectare (mg)	Test period (weeks)	Air permeation tests			
					PTB		LPTB	
					Mean catch/ trap/week	Reduction (%)	Mean catch/ trap/week[c]	Reduction (%)
Test 1, 1976								
Vinyl laminate	20	NM[b]	4,942	31	0.04	96	0.45	95
Control	—	—	—	31	1.1	—	9.05	—
Test 2, 1977								
Treatment A Vinyl laminate	28	13	6,919	15	0	100	17.87a	80
B Vinyl laminate	39	19	9,637	15	0.07	98	19.20a	78
C Acrylic laminate	38	24	9,390	15	0.13	96	12.26a	86
D Hollow fibers	11	50	2,718	15	0.20	93	22.27a	75
E Control	—		—	15	3.07	—	87.67b	—
Test 3, 1977								
Microcapsules 10%	NM	NM	19,768	32	0.19	57	4.06	64
Microcapsules 4%	NM	NM	19,766	32	0.19	57	2.16	81
Hollow fibers	11	50	2,718	32	0.03		3.56	69
Control	—	—	—	32	0.44	—	11.41	—

[a] $t_{1/2}$ (days) refers to half-life.
[b] NM = not measured.
[c] Means followed by the same letter are not statistically different at the 95% level of confidence according to Duncan's multiple-range test.

(Table 1). All four of these formulations gave similar reductions of PTB (93–100%) and LPTB (75–86%) catches. In all air permeation tests to date (McLaughlin et al., 1976; McLaughlin, 1979), disruption of the LPTB has required more pheromone than disruption of the PTB. The fiber dispenser was the least effective treatment for both insects; however, it was initially loaded with about one third less pheromone than the laminated dispensers.

Analysis of the residual lure contents of the dispensers indicated that more than 99% of the *Z,Z*-ODDA in the three laminated dispensers was released during the 15 weeks of the test. However, only 50% of the applied *Z,Z*-ODDA was evaporated into the plot from the fiber dispensers. Residual analyses showed that evaporation from the fibers ceased after about 120 days in the field even though about 50% of the original *Z,Z*-ODDA remained. Assuming first-order release rates for all dispensers, we calculated that half-lives ranged from 13 to 24 days for the Hercon dispensers, and the half-life of the fibers was roughly 50 days (Table 1).

Air Permeation Test 3 (1977). In the third air permeation test, the two formulations of microencapsulated *Z,Z*-ODDA were evaluated vs. the hollow fiber dispensers as described above (Table 1). The PTB catch was reduced 57% with the two microcapsule formulations vs. 93% with the hollow fiber dispensers. So few insects (14) were caught in the control that the evaluation may not be meaningful. Against the higher population of LPTB, the 4% microcapsule treatment reduced trap catch 81% vs. 64% for the 10% formulation and 69% for the hollow fibers. For a given quantity of *Z,Z*-ODDA, the 4% treatment yields many more point sources than the 10% formulation; this could account for increased disruption efficiency.

Although we did not directly compare the best laminated dispenser vs. the 4% microcapsules formulation in the field, we conclude from their performance in the insect populations at the time of each test that the acrylic laminate dispenser not only will give the greater reduction in trap catch of LPTB but also is effective at the higher population level that existed during this test.

Trapping Test 1977. The three types of laminates and the hollow fibers were evaluated in a separate trapping test to measure their effectiveness as baits. We thought it reasonable that the most attractive dispenser might be also the best for air permeation tests to achieve mating disruption. In addition to the laminated and fiber dispensers, rubber bands (#64) baited with 0.5 mg of *Z,Z*-ODDA served as the standard dispensers since these had been used in past years; while these dispensers utilize a relatively low amount of lure, they must be replaced every 2 weeks.

In Table 2, the comparative results of the four varieties of dispensers plus the standard rubber band are given. Catches of male PTB moths in traps baited with the laminated and fiber pheromone dispensers containing 28–39 and 11 mg, respectively, of *Z,Z*-ODDA (94:6 *Z,Z*:*E,Z*) were not significantly

TABLE 2. RESULTS OF TRAPPING TEST WITH LAMINATED PLASTIC AND HOLLOW FIBER DISPENSERS IMPREGNATED WITH PEACHTREE BORER PHEROMONE (*Z,Z*-ODDA) AUGUST 3 TO OCTOBER 7, 1977

Dispenser type	Initial mg *Z,Z*-ODDA per dispenser	Mean no. per trap[a]	
		PTB	LPTB
Vinyl laminate	28	82.3a	70.8b
	39	87.8a	149.0a
Acrylic laminate	38	81.8a	178.0a
Hollow fibers	11	76.0a	9.3c
Rubber band (standard)[b]	0.5	68.2a	4.0c
Control	—	0.5b	0c

[a]Means followed by the same letter are not statistically different at the 95% level of confidence according to Duncan's multiple-range test.
[b]Replaced every 2 weeks with freshly baited rubber band; other dispensers used throughout test.

different. LPTB male moths were also captured in these traps; the laminated dispensers were more effective than those of hollow fibers despite the difference in lure content.

This indicates that attraction of LPTB to *E,Z*-ODDA is not strongly inhibited by the presence of the *Z,Z* isomer as originally reported by Tumlinson et al. (1974). The laminated dispensers with 38–39 mg of the 94:6 mixture contained 2.3 mg of the *E,Z* isomer and caught the most LPTB males.

DISCUSSION

The population levels of PTB and LPTB were so different that any conclusion on the relative amounts of pheromone required to disrupt communication within these two species was not possible in any of the air permeation tests. However, the data do show that LPTB were attracted to baits loaded with the mixture of 94% *Z,Z*, 6% *E,Z*, and the numbers caught were in approximate proportion with the initial application rate and the release rate of the dispensers.

The trap results and those of the air permeation tests indicated to us that the acrylic dispenser should be used in both air permeation and trapping tests. The thickness of the acrylic layers can be increased to extend its useful lifetime. The laminate-type dispensers have also been effective in air permeation tests with the Oriental fruit moth (Gentry et al., 1979) and might be loaded with more than one compound to simultaneously disrupt mating communication in these and other orchard pests.

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SEVEN-WEEK PERSISTENCE OF AN OVIPOSITION-DETERRENT PHEROMONE

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Abstract—Cabbage leaves sprayed with a water solution of the oviposition-deterrent pheromone of *Pieris brassicae* remain deterrent to ovipositing females for at least 14 days. Under laboratory conditions, the pheromone, when dried on a glass surface, retains activity for a period of at least 7 weeks. After 7 days under vacuum conditions, some pheromone is still present, indicating a low volatility and/or high stability of (an active fraction of) the pheromone. After 125 eggs are slowly rinsed with 300 ml water, they still release detectable quantities of the pheromone.

Key Words—Oviposition, deterrent pheromone, *Pieris brassicae*, Lepidoptera, Pieridae.

INTRODUCTION

Pheromones conveying messages to conspecifics often have a short news value. The physicochemical properties of such pheromones fullfil, among other requisites, the temporal aspects of the message (Wilson and Bossert, 1963). Thus, the alarm pheromone of aphids dissipates within 30–60 min (Nault et al., 1973), and the substance which signals to nectar collecting bees that a flower has been visited recently also disappears within about 10 min (Frankie and Vinson, 1977). On the other hand, trail pheromones in social Hymenoptera may last up to 3 days (Jander and Jander, 1979), and territorial flags in this insect group remain active for at least 12 days (Hölldobler and Wilson, 1978).

Oviposition-deterrent pheromones, which promote an even spatial distribution of eggs in many insect species (Prokopy, 1980), may also be

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expected to remain active for days rather than minutes. The volatile oviposition deterrent indicating the occupancy of a *Melandrium* flower by a conspecific egg of the moth *Hadena bicruris* lasts for only one day. This suffices, however, since the ovipositing females also show a clear preference for 1-day-old flowers over those of 2 days old (Brantjes, 1976). The marking pheromone produced by the apple maggot fly *Rhagoletis pomonella* lasts for at least 4 days (Prokopy, 1972). The solitary larvae of *H. bicruris* and *R. pomonella* protect their limited food supply by cannibalistic behavior or some other unknown mechanism to younger conspecifics (Prokopy, 1980). Likewise, the oviposition-deterrent pheromone of the sorghum shoot fly, *Atherigona soccata*, remains active for at least 5 days (Raina, 1980).

Pieris brassicae L. (Lepidoptera: Pieridae) butterflies produce batches of 20–100 eggs, to which during the process of egg-laying an oviposition-deterrent pheromone is added (Rothschild and Schoonhoven, 1977). Since, under natural conditions, egg incubation periods may vary between 5 and 15 days and the gregarious larvae in this case do not kill conspecifics, a higher degree of persistence of the oviposition-deterrent pheromone than observed in other insects seems important. This paper describes the minimum period during which the oviposition deterrent pheromone of *P. brassicae* remains biologically active. Since a study on persistence or decay of a pheromone requires some idea of the quantities involved, we also report some preliminary data on quantities of pheromone present in *P. brassicae* eggs.

METHODS AND MATERIALS

P. brassicae females were obtained from a lab culture on cabbage plants, started by Drs. W.A.L. David and B.O.C. Gardiner in 1953. Tests involving 8–12 females, 3–5 days old, were conducted in cages 70 × 90 × 70 cm. under artificial or natural light conditions. Pheromone solutions were prepared by carefully shaking 125 or 250 eggs (3–27 hr after oviposition) with 1 ml water for 5 min in a small test tube. The egg wash was painted with a soft brush or sprayed with a perfume vaporizer onto both sides of a cabbage leaf about 30 min before the start of an experiment. A control leaf, carefully matched for equal size and age and taken from the same plant, was treated with distilled water. The experimental and control leaves, with their petioles in water, were simultaneously offered at a 45° angle to the butterflies for 4–6 hr. Every 30 min the leaves were switched to alleviate position effects. All experiments were conducted under room conditions (20–28° C).

To determine pheromone persistence, 1 ml of egg wash was put into a 3-cm-diam Petri dish and air dried. It was stored uncovered, but protected from falling dust under room conditions or at reduced pressure (15 mm Hg) in a desiccator. After the storage period, the remaining pheromone was dissolved in 1 ml water and applied to the experimental cabbage leaf to be tested.

To determine the quantity of pheromone present, either 125 eggs were shaken with 1 ml water for 5 min, repeating this procedure 8–10 times and using fresh water every time, or 125 eggs were put on a filter paper in a funnel and rinsed with 300 ml water in 30 min or 1000 ml in 90 min. After being rinsed in either of the two ways, the eggs were shaken with 1 ml water for 5 min. The latter wash was tested.

RESULTS

Egg wash applied to leaves of intact cabbage plants renders these leaves almost completely deterrent to ovipositing females for a period of 3 days (Table 1). Thereafter, deterrency diminishes somewhat, although marked effects remain discernable for 14 days or longer.

Egg wash (1 ml from 250 eggs), air dried in a Petri dish, retains high deterrency for a least 7 weeks (Table 2). It also retains high activity for at least 7 days after exposure to low air-pressure conditions (Table 3).

An approximate estimation of the amount of the pheromone present in an egg batch can be obtained by rinsing eggs with various amounts of water. After shaking 125 eggs 8–10 times with 1 ml water or rinsing 125 eggs with 300 ml water, a deterrent effect of the next rinse wash can still be observed (Table 4). When the eggs are rinsed with 1000 ml water, the concentration of the inhibiting factor becomes too low to be detectable in our tests (Table 4).

TABLE 1. PHEROMONE ACTIVITY ON LEAVES OF INTACT CABBAGE PLANTS AFTER VARIOUS PHEROMONE EXPOSURE PERIODS TO ROOM CONDITIONS[a]

Exposure period (days)	*N*	Control leaf	Treated leaf
0	2	5/95[b]	0/0[b]
1	1	3/84	4/4
2	1	3/59	0/0
3	1	7/232	0/0
4	1	6/200	3/70
6	1	7/230	2/38
6*	1	7/98	1/2
8	1	8/250	2/40
8	1	19/386	0/0
12	1	12/390	3/80
14	1	6/160	2/34

[a] All experimental leaves were sprayed with 1 ml water wash from 125 eggs except those marked *, which were sprayed with 1 ml wash from 250 eggs. The pheromone-treated leaves remained attached to the plants until they were tested, 0–14 days after spraying. *N* = number of replicates.
[b] Number of egg batches/total number of eggs.

TABLE 2. PHEROMONE ACTIVITY AFTER VARIOUS EXPOSURE PERIODS TO ROOM CONDITIONS IN OPEN PETRI DISHES[a]

Exposure period (days)	N	Control leaf	Treated leaf
14	1	7/190[b]	1/4[b]
40	3	17/585	2/22
49	2	13/406	3/5

[a] 1 ml from 250 eggs.
[b] Number of egg batches/total number of eggs.

DISCUSSION

Behavioral evidence (Rothschild and Schoonhoven, 1977) as well as electrophysiological results (Behan and Schoonhoven, 1978) indicate that *P. brassicae* females detect conspecific eggs via olfactory as well as contact chemoreceptors. Either the same compound stimulates both receptor types or the pheromone is composed of volatile and nonvolatile factors. The evidence presented here indicates the presence of one (or more) highly stable component(s) with low vapor pressure, since activity is maintained when exposed for 7 weeks to air in Petri dishes or for 1 week under vacuum conditions. When applied to plant leaf surfaces, the pheromone is still relatively durable, although after 3 days its activity decreases somewhat, presumably owing to biological factors such as leaf growth. Even under these circumstances, however, an oviposition inhibiting effect is still detectable after 14 days.

The stability of the oviposition-deterring pheromone of *P. brassicae* seems unusually high compared to examples cited in the literature (see

TABLE 3. PHEROMONE ACTIVITY AFTER EXPOSING AIR-DRIED EGG WASH (1 ml from 250 eggs) FOR VARIOUS PERIODS TO LOW AIR PRESSURE AT ROOM-TEMPERATURE

Exposure period (days)	N	Control leaf	Treated leaf
3	1	6/91[a]	0/0[a]
5	2	15/355	2/29
6	1	7/160	6/6
7	1	5/181	0/0

[a] Number of egg batches/total number of eggs.

TABLE 4. ACTIVITY OF EGG WASH (1 ml of 125 eggs) AFTER EGGS RECEIVED VARIOUS PRETREATMENTS

Pretreatment	*N*	Control leaf	Treated leaf
Eggs washed			
8× with 1 ml water	2	24/460[a]	5/87[a]
10× with 1 ml water	1	8/140	0/0
Eggs rinsed			
With 300 ml water	5	42/1280	10/241
With 1000 ml water	2	11/530	8/330

[a] Number of egg batches/total number of eggs.

Introduction). Conceivably, however, the amount of pheromone present in the wash of 125 eggs is very large and some evaporation or degradation of the compound would then escape our attention. Some preliminary experiments suggest that the amount present in an egg batch is considerable when tested with the pheromone distributed evenly over a whole leaf surface. A rinse with 300 ml water still leaves enough pheromone to be demonstrated in our oviposition test. Apparently the eggs release the pheromone only gradually.

It is concluded that the oviposition-inhibiting pheromone of *P. brassicae* shows a high degree of stability. The apparent stability may, to a certain extent, be somewhat exaggerated due to the probably high concentration of the compound tested. Nonetheless, it remains an example of a highly persistent insect pheromone.

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ANTIBIOTICS IN MICROBIAL ECOLOGY
Isolation and Structure Assignment of Several New Antibacterial Compounds from the Insect-Symbiotic Bacteria *Xenorhabdus* spp.

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Abstract—Nine strains of the terrestrial bacterial genus *Xenorhabdus*, all isolated as symbionts of nematodes, were examined for their abilities to produce substances with antibiotic activites when grown in pure culture. All nine produced measurable antibiotic activities against one or more of the test strains utilized. The inhibition patterns indicated that different compounds were being produced by the various bacteria. Two of the species that showed particularly strong inhibition patterns were studied in detail. The inhibitory compounds were purified and identified. Strain R produced a mixture of active substances, the major components of which were hydroxyl- and acetoxyl-bearing indole derivatives, presumably produced via tryptophan. Strain Hb, on the other hand, produced only two antibiotics, 4-ethyl- and 4-isopropyl-3,5-dihydroxy-*trans*-stilbenes, which are presumed to arise via polyketide pathways.

Key Words—Bioluminescent bacteria, symbiosis, *Xenorhabdus* spp., antibacterials, 3-(2′-acetoxy-3′-keto-4′-methylpentyl)-indole, 3-(2′-hydroxy-3′-keto-4′-methylpentyl)-indole, 3-(2′-acetoxy-3′-keto-4′-methylhexyl)-indole, 3-(2′-hydroxy-3′-keto-4′-methylhexyl)-indole, 3,5-dihydroxy-4-ethyl-*trans*-stilbene, 3,5-dihydroxy-4-isopropyl-*trans*-stilbene.

INTRODUCTION

The role that inhibitory compounds play in microbial ecology is not well understood, although it is apparent that many organisms have the capacity to produce one or more chemicals with antibiotic activities. These compounds have often been found to be of great use to humans, while their purposes in the

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environment, with regard to naturally occurring inhibitions of microbes, have remained mysterious. Part of the problem in assigning roles to the production of such compounds is that the places where they might be effective are not obvious; presumably the producing organism must be in high concentration and in an environment where it is of some advantage to inhibit invading microbes.

One such environment has been described in the remarkable symbiosis–parasitism exhibited by members of the bacterial genus *Xenorhabdus* (Thomas and Poinar, 1979). These bacteria live as symbionts in the guts of parasitic nematodes. The symbionts are carried here until the nematode finds a suitable host, which may be any of a wide variety of insects, including fleas, beetles, bees, or others; in laboratory experiments caterpillars are commonly used. The nematode, after entering the animal's gut tract via the mouth, penetrates the gut wall, thus entering the hemocoel. In response to factors in the hemolymph, the nematode then releases the bacterial symbionts, which grow in the hemolymph, kill the insect, and allow the nematode to complete its life cycle in the hemolymph. Without the bacteria, the nematode can kill the insect but cannot complete its life cycle. This cycle, which has been described by several authors (Khan and Brooks, 1977; Poinar et al., 1977), is outlined in Figure 1.

One of the curious features of the cycle is that the infected insects do not putrefy, as usually occurs after infection and degradation of organic substrates by bacteria. One explanation for the lack of putrefaction is that the bacteria that are degrading the parasitized animal produce substances with antibacterial activities, and these substances keep putrefying bacteria from invading, thus preserving the environment for the life cycle of the nematode. We report here the presence of inhibitory compounds in a variety of the nematode bacterial isolates tested and the chemical characterization of the active compounds from two different isolates.

METHODS AND MATERIALS

Bacteria. Bacteria were obtained from Dr. G. Poinar (University of California, Berkeley) and Dr. Williams Brooks (North Carolina State University). The strains, designations, and sources are listed in Table 1. The bacterial strains used as indicators were obtained from our own culture collection. Bacteria from nematodes were maintained on nutrient agar, or SWCLS medium. This consisted of 25% sea water, 3 g glycerol, 5 g bactopeptone, 3 g yeast extract per liter of 25% sea water. Solid medium contained 12 g of agar per liter.

Sensitivity Tests. For screening of antibiotic activities the strains were cross-streaked on agar plates; inhibition was seen at the interfaces of the

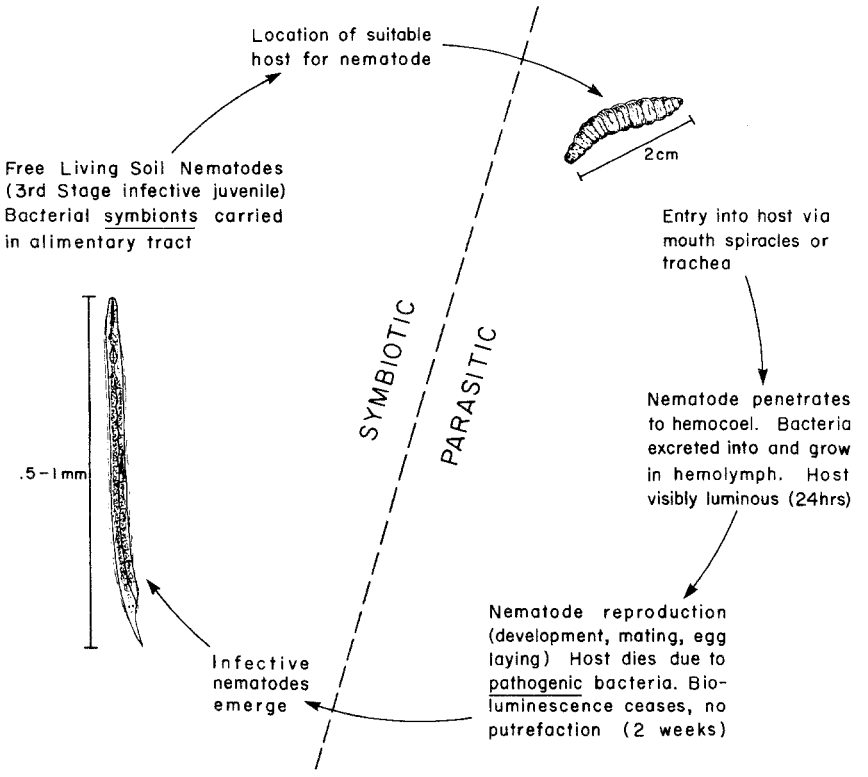


FIG. 1. Involvement of luminous bacteria in the nematode/insect life cycle.

cultures. Cells to be assayed for antibiotic production were also grown to stationary phase in liquid medium, the cells removed by centrifugation and filtration, and the growth medium tested. For these tests, a filter paper disk was first saturated with the growth medium, then placed, after it was allowed to dry in the air at room temperature, on a freshly streaked lawn of sensitive bacteria. This latter method was used for all of the purification procedures to assay the biological activity of the various fractions.

Antibiotic Extraction and Purification. Fifteen liters of cell-free media from strains R and Hb were extracted three times with 3-liter portions of ethyl acetate. For each strain the extracts were combined and reduced in vacuo to 100 ml. The extracts were then dried over anhyd. magnesium sulfate, filtered and further evaporated to yield a viscous residue. Strain R yielded 500 mg, and strain Hb 550 mg of extract. In each case the oily residue was applied to a column of silica gel (Grace grade 62, 60–200 mesh) (2.5 × 60 cm) packed using isooctane. Subsequent elution of the column with isooctane, methylene chloride, and ethyl acetate mixtures led to the collection of 30 fractions. For strain R antibiotic activity was detected in fractions eluted with 10% methylene chloride in isooctane and with fractions eluted with 20% methylene

TABLE 1. ANTIBACTERIAL ACTIVITIES[a] OF *Xenorhabdus*[b] STRAINS

Strains from nematodes[d]	Test strains[c]										
	H98	B392	PL721	B398	NZ11D	B378	B332	MJ-1	B404	B334	B333
R	+	−	−	+	+	+	−	+	+	−	−
Hb	+	−	+	+	+	−	−	+	+	−	−
NL	+	−	±	+	+	−	−	±	+	−	−
AT	+	−	+	+	+	−	−	+	+	±	−
B1	+	−	+	+	+	+	−	+	+	+	−
B2	+	−	+	+	+	+	−	+	+	−	−
A11	+	−	+	+	+	−	+	+	+	−	−
Hm	−	−	+	+	+	−	−	+	+	−	−
DN	−	−	−	+	+	−	−	−	+	+	−

[a] These activities determined by both cross-streaking and disk inhibition assays.
[b] This is a new genus of nematode symbionts (Thomas and Poinar, 1979).
[c] These are luminous bacteria from our collection. *Vibrio* (*Beneckea*) spp. (H98, B392, B378, B332, B334, B398, MJ-1; *Photobacterium* spp. NZ11D, PL721). Strains are described in Reichelt and Baumann (1973) and Hastings and Nealson (1977).
[d] Some of the bacterial isolates from nematodes were kindly supplied by Dr. G. Poinar. AT, R, A11, and DN are isolates of *X. nematophilus*, with AT indicating the ATCC type species # 19601. Hb and Hm are strains of *X. luminescens*, while NL is a spontaneous nonluminous isolate of Hb. B1 and B2 were supplied by Dr. William Brooks. B1 was designated as *Achromabacter nematophilus* while B2 was designated NC-19 (Khan and Brooks, 1977).

chloride in isooctane. For strain Hb antibiotic activity was detected in fractions eluted with 20% methylene chloride in isooctane.

Subsequent purification of antibiotic column chromatography fractions was accomplished by high-pressure liquid chromatography (HPLC) on a 16 mm × 50 cm preparative silica gel column. For the purification of all compounds, mixtures of ethyl acetate in isooctane gave good results.

Instrumental Analyses. Infrared spectra were recorded on a Perkin Elmer model 137 spectrophotometer. Ultraviolet spectra were recorded on a Beckman Acta M-VI spectrophotometer. Optical rotations were obtained using a 10-cm microcell on a Perkin Elmer model 141 polarimeter and low resolution mass spectra were recorded on a Hewlett-Packard 5930A instrument. High resolution mass measurements were obtained through the Department of Chemistry, University of California, Los Angeles. Nuclear magnetic resonance spectra were recorded on a Varian HR 220 spectrometer with Nicolet FT system (^{1}H) and on a Varian CFT-20 spectrometer (^{13}C). All solvents were distilled in glass prior to use.

RESULTS

When the nematode symbionts were examined by either cross-streaking or filter paper methods, it was seen that many had the ability to produce compounds with antibiotic activity (Table 1). The various strains differed

both with respect to the number and types of sensitive strains, suggesting that there were several different compounds involved; only two of the isolates, Hb and NL, showed identical patterns of inhibition of the test strains.

Two of the more active strains, R and Hb, were chosen for further study, and the antibiotic components, excreted into the culture medium, were isolated, purified (as described above), and structurally defined.

Strain R Antibiotics. The antibiotic metabolites from strain R were found to consist of a mixture of the homologous indole derivatives I–IV (Figure 2). Each compound inhibited the test strains used and showed the following spectral features:

Compound I, 3-(2′-hydroxy-3′-keto-4′-methylpentyl)-indole, was isolated as 4% of the extract, and showed $[\alpha]_D = 65.4°$ (c 1.2, $CHCl_3$). The mass spectral fragmentation showed $M^+ m/e = 231$ for $C_{14}H_{17}NO_3$. [^{1}H]NMR ($CDCl_3$): δ8.08, 1H, bs; 7.63, 1H, d, $J = 7$ Hz; 7.36, 1H, d, $J = 7.5$ Hz; 7.16, 2H, m; 7.10, 1H, s; 4.62, 1H, dd, $J = 7{,}5$ Hz; 3.33, 1H, dd, $J = 15{,}4$ Hz; 3.05, 1H, dd, $J = 15{,}7$ Hz; 2.90, 1H, m; 1.10, 3H, d, $J = 7$ Hz; 0.98, 3H, d, J = 7 Hz. [^{13}C]NMR (benzene-d_6): 190.2, 136.1, 128.0, 121.2, 120.1, 117.7, 117.0, 109.5, 109.2, 73.4, 34.4, 28.2, 17.2, 15.4.

Compound II, 3-(2′-acetoxy-3′-keto-4′-methylpentyl)-indole, was isolated as the major antibiotic produced (20% extract) and showed $[\alpha]_D = 39.5°$ (c 1.0, $CHCl_3$). The mass spectrum showed $M^+ m/e = 273$ which analyzed for $C_{16}H_{19}NO_3$, addition of C_2H_2O to compound I. [^{1}H]NMR ($CDCl_3$): δ8.28, 1H, bs; 7.63, 1H, d, $J = 8$ Hz; 7.33, 1H, d, $J = 8$ Hz; 7.16, 2H, m; 7.0, 1H, s; 5.47, 1H, dd, $J = 7{,}5$ Hz; 3.23, 2H, m; 2.72, 1H, m; 2.05, 3H, s; 1.09, 3H, d, $J = 7$ Hz; 0.98, 3H, d, $J = 7$ Hz. [^{13}C]NMR (acetone-d_6): 183.0(s), 170.5(s), 137.0(s), 128.0(s), 124.0(d), 122.4(d), 120.0(d), 119.3(d), 112.4(d), 110.4(s), 79.2(d), 38.0(d), 27.0(t), 21.0(q), 19.1(q), 17.8(q). UV (MeOH): λ_{max} 289, 279, 273 nm, $\epsilon = 5900$, 7050, 6700. IR ($CHCl_3$): 3600, 1728 cm^{-1} (broad).

Compound III, 3-(2′-hydroxy-3′-keto-4′-methylhexyl)-indole, was isolated as 3% of the organic extract and showed $[\alpha]_D = 112.5°$ (c 0.8, $CHCl_3$). [^{1}H]NMR ($CDCl_3$): δ8.08, 1H, bs; 7.63, 1H, d, $J = 7$ Hz; 7.36, 1H, d, $J = 7.5$ Hz; 7.16, 2H, m; 7.10, 1H, s; 4.57, 1H, dd, $J = 7{,}5$ Hz; 3.32, 1H, dd, $J = 15{,}4$ Hz; 3.03, 1H, dd, $J = 15{,}7$ Hz; 2.76, 1H, m; 1.66, 1H, m; 1.45, 1H, m; 0.87, 3H, t, $J = 7$ Hz. [^{13}C]NMR (benzene-d_6): 190.2, 136.1, 128.0, 121.0, 120.2, 117.7, 117.9, 109.5, 109.0, 73.7, 42.2, 29.0, 26.8, 15.2, 11.4.

I, R = H
II, R = Ac

III, R = H
IV, R = Ac

FIG. 2. Molecular structures of antibiotics isolated from *Xenorhabdus* sp. (strain R).

Compound IV, 3-(2′-acetoxy-3′-keto-4′-methylhexyl)-indole, of the extract showed $[\alpha]_D = 58.2°$ (c 1.0, $CHCl_3$): [^{1}H]NMR ($CDCl_3$); δ8.29, 1H, bs; 7.53, 1H, d, $J = 7$ Hz; 7.32, 1H, d, $J = 8$ Hz; 7.16, 2H, m; 7.0, 1H, s; 5.46, 1H, dd, $J = 7.5, 5$ Hz; 3.24, 2H, m; 2.61, 1H, m; 2.04, 3H, s; 1.90, 1H, m; 1.70, 1H, m; 0.98, 3H, d, $J = 6$ Hz; 0.87, 3H, m. [^{13}C]NMR (benzene-d_6): 192.4(s), 170.0(s), 136.5(s), 128.0(s), 123.3(d), 122.3(d), 119.7(d), 119.0(d), 111.8(d), 110.2(s), 78.0(d), 44.4(d), 26.8(t), 26.5(t), 20.3(q), 15.2(q), 11.4(q). UV (MeOH): λ_{max} 289, 280, 273 nm, $\epsilon = 6200, 6990, 6560$. IR ($CHCl_3$): 3600, 1725 cm^{-1}.

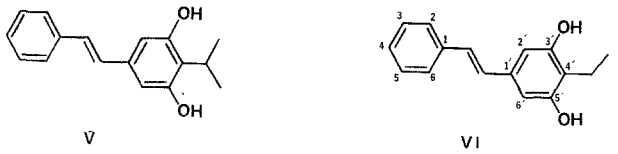
Strain Hb Antibiotics. The antibiotic metabolites from strain Hb were found to be the homologous *trans*-stilbene derivatives V and VI (Figure 3). The following spectral features were exhibited by these compounds:

Compound V, 3,5-dihydroxy-4-isopropyl-*trans*-stilbene, isolated as 20% of the extract, was analyzed for $C_{17}H_{18}O_2$ by high-resolution mass spectrometry (obs. $M^+ m/e = 254.1288$; calc. 254.1302). [^{1}H]NMR ($CDCl_3$): δ7.46, 2H, d, $J = 7$ Hz; 7.25, 3H, m; 7.00, 1H, d, $J = 16$ Hz; 6.90, 1H, d, $J = 16$ Hz; 6.50, 2H, s; 4.90, 2H, bs (D_2O exc.); 3.45, 1H, heptet, $J = 7$ Hz; 1.30, 6H, d, $J = 7$ Hz. [^{13}C]NMR (MeOH-d_4): 2× 158.4(s), 139.8(s), 137.7(s), 130.7(d), 2× 130.4(d), 2× 129.1(d), 2× 128.1(d), 122.9(s), 2× 109.5(d), 26.4(d), 2× 21.8(q). UV (MeOH) of the diacetate derivative: λ_{max} 283 nm, $\epsilon = 29{,}500$. IR ($CHCl_3$): 3700, 3500, 1620, 1590 cm^{-1}.

Compound VI, 3,5-dihydroxy-4-ethyl-*trans*-stilbene, isolated as 6% of the extract, was analyzed for $C_{16}H_{15}O_2$ by low-resolution mass spectrometry ($M^+ m/e$ = obs. 240). [^{1}H]NMR ($CDCl_3$): δ7.46, 2H, d, $J = 7$ Hz; 7.25, 3H, m; 7.00, 1H, d, $J = 16$ Hz; 6.90, 1H, d, $J = 16$ Hz; 6.70, 2H, s; 4.90, 2H, bs (D_2O, exc.); 2.60, 2H, q, $J = 7$ Hz; 1.35, 3H, t, $J = 7$ Hz. UV (MeOH); λ_{max} 288 nm, $\epsilon = 16{,}500$. IR($CHCl_3$): 3700, 3500, 1620, 1590 cm^{-1}.

Transformations of Compound VI. As part of the structure elucidation of the ethylated stilbene VI, the metabolite was acetylated (Ac_2O/py/25°) and the bisacetate was hydrogenated (Pt/Et_2O). The derivatives obtained had the following spectral features:

3,5-Diacetoxy-4-ethyl-*trans*-stilbene: [^{1}H]NMR ($CDCl_3$): δ 7.50, 2H, d, $J = 6$ Hz; 7.35, 3H, m; 7.15, 2H, s; 7.10, 2H, d, $J = 2$ Hz; 2.50, 2H, q, $J = 7$ Hz; 2.40, 6H, s; 1.20, 3H, t, $J = 7$ Hz.



V VI

FIG. 3. Molecular structures of antiobiotics isolated from *Xenorhabdus* sp. (strain Hb).

1-Phenyl-2-(3′,5′-diacetoxy-4′-ethylphenyl)ethane: MS, low resolution, $M^+ m/e = 326$ for $C_{20}H_{22}O_4$; UV (MeOH) λ_{max} 256, 220 nm; $\epsilon = 550, 4300$; [^{1}H]NMR ($CDCl_3$): δ 7.20, 5H, m; 6.70, 2H, s; 2.90, 4H, s; 2.40, 2H, q, $J = 7$ Hz; 2.30, 6H, s; 1.10, 3H, t, $J = 7$ Hz.

DISCUSSION

The structure elucidations of compounds I–VI followed readily from interpretation of their spectral data, including comparisons with suitable spectral standards. The indoles I–IV showed UV and NMR spectra virtually superimposable with indole itself (Scott, 1964), and the substitution pattern on indole and the composition of the side chains were easily confirmed by proton spin-decoupling NMR experiments. In each case the methylene protons at the C-1′ position were related to the C-2′ methine proton, and the C-4′ methine proton could be related to the adjacent methyl groups (compounds I, II) or adjacent methyl and ethyl substituents (compounds III, IV). The alcohols I and III were converted to II and IV, the major antibiotics, upon treatment with acetic anhydride in pyridine. Although chiral metabolites, no attempt was made to determine the absolute stereochemistries at C-2′.

Assignment of the structures for IV and V based upon spectral analysis posed several interesting problems. The molecular formulae were readily established by mass spectrometry; however, due to the twofold symmetry of these derivatives, both the ^{13}C and ^{1}H NMR spectra were not initially readily interpretable. The ^{13}C spectrum of V, for example, exhibited only 12-carbon resonances for the 17-carbon atom structure, indicating the degenerate methyl groups of the isopropyl substituent, and the degenerate carbons C-2,6, C-3,5, C-2′,6′, and C-3′,5′ on the aromatic nuclei. Once the symmetry of these compounds was established, the data quickly allowed the formulation of structures V and VI. The Hb antibiotics were assigned as *trans*-stilbene derivatives based upon the 16-Hz olefin coupling constants, and alternative structures with the hydroxyl groups substituted at C-2′ and C-6′ were considered. While this alternative could not be excluded based upon ^{1}H NMR data, calculations of the predicted ^{13}C bands clearly eliminated this alternative (Wehrli and Wirthin, 1978).

As part of the confirmation of structures V and VI, the ethyl derivative VI was acetylated and the bisacetate obtained was catalytically hydrogenated. One mole of H_2 was smoothly taken up, and the product, 1-phenyl-2-(3′,5′-diacetoxy-4′-ethylphenyl)-ethane, was purified by HPLC. The ^{1}H NMR features of this compound, and its classical benzenoid UV absorption at 256 and 220 nm (Pasto and Johnson, 1969), confirmed the structure as proposed.

The antibiotics isolated in this study from the two strains of *Xenorhabdus*

are, curiously, of quite dissimilar structure types. Compounds I–IV would appear to be degradation products of tryptophan, a nutrient in the culture medium. The stilbenes V and VI, however, are not clearly derived from simple precursors, nor are stilbenes considered common isolates from bacteria. Rather, groups of stilbenes have been isolated from terrestrial plants (Devon and Scott, 1972), and these compounds are thought to be derived via polyketide (fatty acid) biosynthesis (Geissman and Crout, 1969). The different patterns of inhibition noted in those strains not chemically investigated would suggest further variations in antibiotic composition. The pathways for biosynthesis of the antibiotics described here have not been investigated, but it should be a fairly straightforward problem to explore using mutant strains and defined media.

It has been noted that the infected insects in the life cycle shown in Figure 1 do not putrefy, and it has been speculated that the bacteria may prevent putrefaction by the production of inhibitory substances. While this has not been proven, the observation that inhibiting substances are present in all the media assayed, and the successful isolation of discrete antibiotics from the two strains studied, strongly supports these speculations. It should also be noted that both *X. luminescens* and *X. nematophilus* have been recently reported to contain defective phages that may be responsible for lysis of *Bacillus cereus* (and possibly other bacteria) (Poinar *et al.*, 1980a). Thus, several antibacterial mechanisms may be operating in this system.

The final point in question is whether any of these mechanisms operate under natural conditions. Are these compounds, or the defective phages discussed above, effective agents in preventing putrefaction in the infected insects? Perhaps this system is one of the best for the study of natural activities and uses of compounds with antibacterial activities.

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CHEMICAL BASIS OF DIFFERENTIAL FEEDING BEHAVIOR OF THE LARCH SAWFLY, *Pristiphora erichsonii* (HARTIG)[1]

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Abstract—The machanisms by which the larvae of the larch sawfly, *Pristiphora erichsonii* (Hartig), are prevented from feeding on the single needles of the new shoot of tamarack, *Larix laricina* (DuRoi) K. Koch, were studied. As a result of extensive purification attempts, five deterrent chemicals were isolated and identified. They are: abietic, dehydroabietic, 12-methoxyabietic, sandaracopimaric, and isopimaric acid. These chemicals, particularly the first two, are abundant in mid-July to August in the single needles and, apparently, provide the basis for the deterrency against the larval feeding.

Key Words—Larch, tamarack, larch sawfly, *Pristiphora erichsonii*, abietic acid, dehydroabietic acid, 12-methoxyabietic acid, sandaracopimaric acid, isopimaric acid, *Larix laricina*.

INTRODUCTION

The mechanisms by which defoliating insect populations are affected by plant chemistry and by chemically associated defenses have been subjected to intensive investigation, and a number of hypotheses have been offered in

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explanation (Feeny, 1976; Rhoades and Cates, 1976). In general, the research on which these hypotheses were formulated was conducted on English oak (*Quercus rubor* L.), cresote (*Larvea*), and a number of herbaceous plants including crucifers, milkweeds, and legumes. Little if any published information is known relating to the mechanisms governing the behavior of defoliators of coniferous trees.

Our research on the sawfly defoliators, *Neodiprion rugifrons* Midd. and *N. swainei* Midd., two monophagous species associated with *Pinus banksiana* Lamb., revealed that the rejection of early current season foliage by feeding larvae in favor of needles 1+ years old was related to the presence of compound A [13-keto-8(14)-podocarpen-18-oic acid] and compound B (dehydroabietic acid) in juvenile needles (Ikeda et al., 1976). As current needles matured and compound A declined in content, late-feeding, first-generation *N. swainei* larvae accepted even the current season needles. When the level of feeding inhibitors approached that of 1+-year-old needles, second generation *N. rugifrons* larvae accepted current year needles. Compound B had a less clear influence.

The larch sawfly *Pristiphora erichsonii* (Hartig) is monophagous on *Larix* spp., and its larvae demonstrate the unique feeding behavior of rejecting single needles on long shoots in favor of tufted needles on short shoots. Female sawflies oviposit in late May to early June in the long shoots of *Larix laricina* (DuRoi) K. Koch., a deciduous conifer that is widely distributed throughout northeast North America, and northwest to the interior of Alaska. The long shoots produce single needles; short shoots on older wood produce groups or tufts of 15–45 needles each. Eclosing larvae initially take a few bites from the single needles then move to the tufted needles where they complete development. They move as a colony and may consume all tufted needles—yet they will not again feed on the single needles. The overriding mechanism governing this preferential feeding habit is a chemical factor(s) present in the single needles (Wagner et al., 1979). We report here on the chemical basis and identity of the materials governing the feeding behavior of the larch sawfly larvae.

METHODS AND MATERIALS

Biological Materials. Larch sawfly larvae and tamarack foliage were collected from several locations in Wisconsin counties of Columbia, Dane, Langlade, Marquette, Portage, and Waupaca. Larvae were collected in instars 1–3 and reared exclusively on tufted tamarack foliage. Collection techniques and rearing procedures followed guidelines of Wagner (1977) and Heron and Drouin (1969).

For large-scale isolation of the biologically active chemical factors, single

needles were collected at Columbia County in June 1978. Quantitative analyses of biologically active components in single and tufted needles were conducted on needles collected in Columbia County on July 17, August 14, and October 1.

Bioassay. A 7 to 10-cm long, 1-year-old twig with tufted needles was used for the bioassay. The center tufts on the twig were removed until two fascicles approximately equal in size and orientation remained at each end. The twig was rinsed with distilled water and dried at room temperature for about 30 min. Needles at one end were topically treated by pipetting several drops of the test material plus solvent at the base and allowing it to drip down to the needle tip. Needles on the other end were treated in the same manner with solvent only. The twig was dried at room temperature for an additional 30 min before testing. The treated twig was suspended horizontally at about 5 cm height by a pin, through the center of the twig and into a rubber stopper so that the needles did not touch the surface. Five 3rd, 4th, or early 5th instar larvae were placed on the chemically treated test needles at each end of the twig, and the behavior of the larvae was observed in a fluorescent-tube lighted incubator at 20° C. The floor and four inner sides of the incubator were covered with moss-green blotter paper. At least four bioassay replications for each fraction were made per test. The number of larvae on both ends was counted at hourly intervals for 4 hr. The percent of larvae on the solvent end vs. total larvae was employed to gauge biological activity. The test fraction was recognized to have deterrent activity when more than 70% of the larvae moved away from the extract-treated end and settled on the solvent-treated end.

Chromatographic Materials. The following materials were used for column and thin-layer chromatography (TLC): silica gel 100 (230 mesh, E. Merck Company), silica gel H (E. Merck Company), and charcoal (American Norit Company) for column chromatography; silica gel HF-254, 0.25 mm and 0.5 mm thickness, for analytical and preparative TLC, respectively. In the latter case the coated plates were activated at 130° C for 2–3 hr, before use. In some cases $AgNO_3$ (6%) -impregnated silica gel was used for column chromatography. A typical procedure was as follows: 50% aqueous solution of $AgNO_3$ was added to silica gel. After evaporation of water under reduced pressure, gel suspended in solvent was poured into a column that was covered with aluminum foil. Throughout the experiment the column was maintained in the dark. TLC plates with silica gel–phosphate buffer solution (pTLC) were prepared in the following manner: 20 g of silica gel H were suspended in 24 ml of phosphate buffer solution (pH 6.0, I = 0.05) and 24 ml of distilled water to make the slurry that was used to coat glass plates (20 × 20 cm). The plates were oven-dried at 130° C for 2–3 hr. Precoated TLC plates (silica gel 60 F-254, E. Merck Company, 0.2 mm thickness) also were used for quantitative analysis.

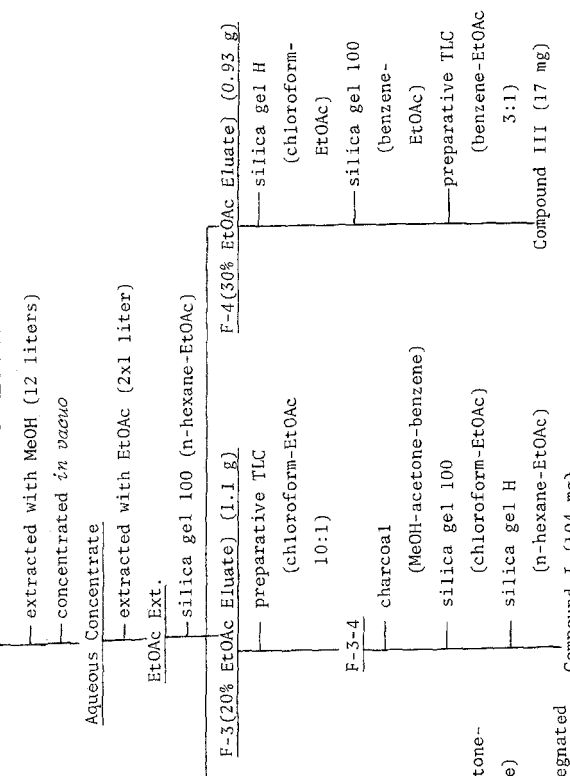


FIG. 1. Extraction and isolation procedure of active principles. The identities of compound *I*, *II*, *III* and mixture *IV* are shown in Figure 2.

In gas chromatography (GLC), a Varian Aerograph 2400-instrument was used. The column (2 mm ID, 135 cm length) packed with 10% OV-3 Chromosorb W AW-DMCS was used, and the chromatography was carried out at 225° C or 240° C with N_2 flow rate of 40 ml/min with an FID detector.

Spectroscopy. Infrared spectra (IR) were recorded on a Beckman IR-33 spectrometer and calibrated with the bands of a polystyrene film. Proton magnetic resonance spectra (PMR) were obtained with a Bruker HX-90E (90 MHz), ultraviolet spectra (UV) with a Varian 635 spectrophotometer, and mass spectra (MS) with a DuPont GC mass spectrometer 21–491 B at 70 eV.

Extraction and Isolation of Active Principles. The single needles on long shoots (1.5 kg) were extracted with methanol (MeOH), then MeOH was evaporated *in vacuo* at 50° C (Figure 1). The aqueous residue was extracted with ethyl acetate (EtOAc) (1 liter) and the EtOAc layer was dried over anhydrous Na_2SO_4. The solvent was removed to give an EtOAc-soluble fraction (30 g). The fraction thus obtained showed the deterrent activity in bioassay at a concentration of 1 g needles equivalent (1 g N eq) per ml which corresponded to 20.2 mg of EtOAc-soluble fraction per ml of solvent.

To the fraction in EtOAc (50 ml), silica gel 1000 (30 g) was added, and EtOAc was evaporated *in vacuo* at 50° C for 2 hr to give a greenish powder into which 60 ml of *n*-hexane was added to give a suspension. The suspension was placed on top of a silica gel 100 column (150 g, 3 cm ID, 120 cm length) packed in *n*-hexane. The column was eluted with *n*-hexane containing an increasing ratio of EtOAc in a 700-ml volume per fraction. Each fraction thus obtained was concentrated to 37.5 ml (40 g N eq/ml) and bioassayed. The deterrent activity was found in the fractions eluted with 10, 20, and 30% EtOAc in *n*-hexane (F-2, F-3, and F-4) (Table 1).

These fractions were further purified by chromatography on silica gel 100, silica gel 100 impregnated with $AgNO_3$, charcoal, silica gel H, and by preparative TLC and pTLC (Figure 1) to give compound I, II, III and mixture IV (Figure 2) as glassy resins in the yields of 0.025, 0.032, 0.0040, and 0.013%, respectively, as compared to original needles and shoots.

Chemical Reactions. To a portion of the sample (carboxylic acid) in ether an excess amount of ethereal diazomethane was added (Fieser and Fieser, 1967). The reaction mixture was allowed to stand overnight at room temperature. The solvent and excess diazomethane were evaporated in a hood. Usually ca. 5 ml ethereal diazomethane was added to 20 mg of sample in 1 ml ether.

Detection of Spots on TLC. Each TLC plate developed was checked for spots by using UV light (254 nm). Analytical TLC plates were sprayed with 5% vanillin–H_2SO_4 prepared as follows: 5 g of vanillin was added to 50 ml of concentrated H_2SO_4 and the mixture was allowed to stand for 2 hr at room temperature. The reagent thus obtained was stored in a dark-colored glass

TABLE 1. ELUTION PATTERN OF FEEDING DETERRENTS OF EtOAc-SOLUBLE FRACTION FROM SILICA GEL 100 COLUMN AND ACTIVITY OF EACH FRACTION[a]

Fraction number	Solvent eluted with % EtOAc in *n*-hexane	Activity (number of experiments)				
		Volume eluted	Conc. (g N eq/ml)[b]	Percent deterrence[c]	Conc. (g N eq/ml)	Percent deterrence
F-1	0	700	40	65(8)*[e]	20	65(4)NS
F-2	10	700	40	74(8)***	20	80(4)NS
F-3	20	700	40	90(8)***	20	90(4)***
F-4	30	700	40	82(8)***	20	78(4)**
F-5	40	700	40	73(8)**	20	60(4)NS
F-6	75	700	40	48(4)NS	20	65(4)NS
F-7	100	700	40	57(4)NS	20	65(4)NS
Total[d]			10	98(4)***	5	75(4)NS

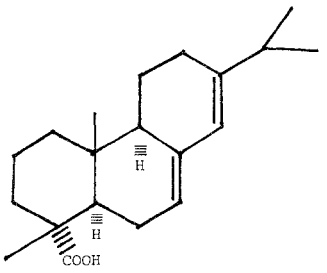
[a] See Figure 1 for overall scheme for purification and designation of fractions.
[b] Concentrations of test solutions were expressed in gram needle equivalents (g N eq) per ml of solvent.
[c] Expressed as the percent of larvae found on the solvent-treated end of the twig.
[d] The combined fraction from F-1 to F-7.
[e] Significance levels (Student's *t* test): NS, not significant; *, significant 0.10 level; **, significant 0.05 level; ***, significant 0.01 level.

bottle. After spraying with the reagent, the plates were heated and the changes in the colors of the spots were noted. The characteristic colors of the compounds are shown in Table 3 together with their R_f on TLC and R_t on GLC.

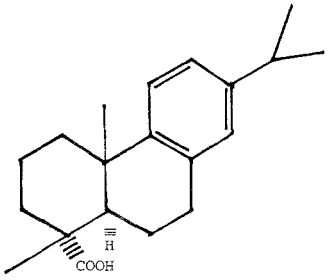
Quantitative Analysis. GLC was used for all quantitative analyses; compound III, methyl ester, however, did not give a sharp peak on GLC, and as such the quantity of compound III was measured by absorption intensity of its methyl ester on UV (at 240 nm).

To eliminate impurities, 250 mg of each EtOAc extract was chromatographed on a silica gel 100 (5 g) column eluted with 40% EtOAc in *n*-hexane in a 60-ml volume and 100% EtOAc in a 60-ml volume, successively. All compounds to be analyzed were eluted completely in the 40% EtOAc fraction (GLC and TLC). One quarter of each fraction thus obtained was methylated with excess diazomethane, and the reaction mixture was chromatographed on a precoated TLC plate by developing it with *n*-hexane–EtOAc (12:1). The plate was sectioned into five parts (F-A, F-B, F-C, F-D, and F-E, corresponding to R_f ranges 1.00–0.92, 0.92–0.63, 0.63–0.57, 0.57–0.43, and 0.43–0.00, respectively) by monitoring the pattern of UV absorption zones. F-D thus obtained consisted of almost pure compound III methyl ester (on TLC). F-C included methyl esters of all other deterrent compounds. These two fractions were submitted to UV and GLC analysis.

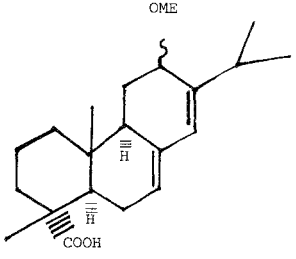
For quantitative GLC analysis, the triangulation method of McNair et al. (1969) was utilized. Before analysis of the sample, the standard straight lines of methyl esters of compounds I, II, and mixture IV were made with pure compounds (99% or more) at various concentrations. Each F-C fraction was made up to 0.2 ml with EtOAc and 0.5 μl was injected in the column. Two replicates were used for quantity determination. The quantity of each compound was expressed by the weight of its methyl ester per 10 g of needles.



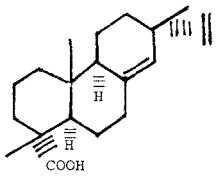
I. Abietic acid



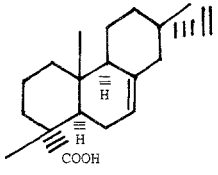
II. Dehydroabietic acid



III. 12-Methoxy abietic acid



Sandaracopimatic acid



Isopimaric acid

Mixture IV

FIG. 2. Resin acids isolated from tamarack single needles.

TABLE 2. FRACTIONATION PATTERN OF F-3 ON TLC AND ACTIVITY OF FRACTIONS

Fraction[a]	R_f range	Activity[b]
F-3-1	1.0–0.8	−
F-3-2	0.8–0.7	±
F-3-3	0.7–0.4	±
F-3-4	0.4–0.1	++
F-3-5	0.1–0.0	−

[a] The fractions obtained by preparative TLC of F-3 developed with chloroform–EtOAc (10:1).
[b] The activity was tested at a concentration of 20 g N eq/ml, and is represented as −, ±, and ++ which indicate deterrent activity: <65%, 65–70%, and 75–85%, respectively.

RESULTS

The overall scheme of purification to isolate the biologically active substances that deter larval feeding is summarized in Figure 1. The deterrent was spread among several fractions (Table 1). To identify the substances that gave highest levels of feeding deterrence, F-3 fraction was initially chosen. On purification of F-3 on silica gel-TLC, an active fraction (F-3-4) was obtained (Table 2). Upon methylation of the fraction with diazomethane, the compounds changed their chromatographic behavior. The result was interpreted as an indication that these compounds are carboxylic acids. GLC analysis of the methylated fraction showed the fraction consisted of three compounds (two major compounds, I and II, and a minor one, III). Their chromatographic behaviors, together with those of compounds discussed in this paper, are

TABLE 3. CHROMATOGRAPHIC BEHAVIOR OF CANDIDATE COMPOUNDS AND THEIR METHYL ESTERS

Compounds	R_f[a]	Solvent	UV	Detection of the Spots: 5% Vanillin–H_2SO_3	Detection of the Spots: After heating	R_t (min) at 225°C
I	0.71	n-hexane 7.5	+	Red purple	Purple	
II	0.65	EtOAc 2.5[b]	−	None	Dark purple	
III	0.46		+	Blue	Blue purple	
IV	0.57		−	Red purple	Red purple	
I-Me	0.64	*n*-hexane 10	+	Red purple	Purple	9.0
II-Me	0.60	EtOAc 1	−	None	Dark purple	7.9
III-Me	0.41		+	Blue	Blue purple	
IV-Me	0.64		−	Red purple	Red purple	6.1 and 7.0

[a] R_f values were calculated from the plates developed on precoated TLC.
[b] With this solvent system a double development technique was used for TLC.

summarized in Table 3. Further purification of F-3-4 by using chromatography on charcoal followed by silica gel gave compounds I, II, and III in large quantities for spectroscopic analyses. Throughout these chromatographic processes GLC was used on methylated products to monitor the progress of purification. The UV, IR, and PMR data for each of these compounds are summarized in Table 4. It was concluded that compounds I and II are abietic and dehydroabietic acid, respectively (Yamaguchi, 1972; Narayanan, 1965; Chien, 1960; Suzuki, 1972). These are common resin acids, and it is not particularly surprising that they exist in the foliage of tamarack. Also methyl esters of these compounds, whose spectral data are also shown in Table 4, behave in identical manners as the authentic samples in the gas and thin-layer chromatographic systems tested. Although identification of compound III, present in the smallest quantity (Figure 1), is not absolute, it was tentatively assigned as 12-methoxyabietic acid from the MS, UV, and PMR spectra. The PMR spectrum was similar to that of abietic acid except for the difference in the shift of the isopropyl group (Table 4). In the abietic acid spectrum the protons of two methyl groups at C-15 were equal in shift, while those for compound III were two doublets at 1.08 and 1.04 ppm. These data support the diagnosis that there is an extra methoxy group at the C-12 position.

Another eluate fraction from the silica gel column that showed significant deterrence was F-2. Further purification of F-2 on preparative TLC gave an active fraction F-2-4 (Table 5), which contained mainly four compounds as judged by GLC. The isolation of these compounds also was attempted. Two of the compounds using column chromatography on $AgNO_3$-impregnated silica gel, charcoal, or pTLC (Figure 1), were isolated as pure. The remaining two were obtained as a mixture (designated as mixture IV). The former two were the already identified compounds I and II, respectively. Although it was not possible to separate the latter two compounds in large enough quantities by chromatography, it was concluded that the mixture consisted of sandaracopimaric acid and isopimaric acid as a result of extensive spectroscopic examination (see Table 4) (Yamaguchi, 1972; Wenkert, 1965; Chien, 1959).

Determination of the active compounds in F-4 was unsuccessful because of the loss of the activity on further purification. The presence of compound III, however, was confirmed by GLC analysis. Isolation of the compound was attempted and the methods are described in Figure 1.

The individual and combined activities of candidate antifeedant compounds are listed in Table 6. Compounds I, II, and Mix IV actively inhibited feeding at 20 mg/ml. The role of compound III is uncertain because insufficient sample was available for bioassay. A combination of the four compounds, in their approximate naturally occurring ratio, was also effective at 20 mg/ml.

In analyzing the role of these chemicals in affecting the behavior of larch

TABLE 4. SPECTROSCOPIC CHARACTERISTICS OF ACTIVE FEEDING DETERRENTS ISOLATED FROM SINGLE NEEDLES AND THEIR METHYL ESTERS

Compound	UV in MeOH nm (ε)	IR (cm^{-1})[a]	PMR (δ ppm)[b]	MS (*m/e*)
I	233 (18,300) 240 (18,600)	3300–2900 (br.), 2800–2100 (br.), 1700, 1650–1600 (br.), 1470	5.78 (1H, br.s.) 5.36 (1H, br.s.) 1.26 (3H, s.) 1.01 (3H, d., J = 6.8 Hz) 0.83 (3H, s.)	
II	237 (sh. 900), 248 (sh. 790) 265 (840), 274 (810)	KBr: 3250, 1700, 1500, 1460–1480	7.15 (1H, d., *J* = 8.3 Hz) 6.97 (1H, br. d., *J* = 8.3 Hz) 6.89 (1H, br. s.) 1.25 (3H, s.) 1.22 (6H, d., *J* = 6.8 Hz) 1.21 (3H, s.)	
III	234 (sh. 23,600) 241 (24,000)	3300–2900 (br.), 2800–2100 (br.), 1700, 1650–1600 (br.), 1470	5.85 (1H, br. s.) 5.47 (1H, br. s.) 3.79 (1H, br. s.) 3.37 (3H, s.) 1.25 (3H, s.) 1.08 (3H, d., *J* = 6.8 Hz) 1.04 (3H, d., *J* = 6.8 Hz) 0.81 (3H, s.)	
IV	None		Signals originated from isopimaric acid 0.88 (3H, s.), 0.92 (3H, s.), 129 (3H, s.) 5.36 (1H, br. s.), 4.9–5.1 (2H, m.) and 5.5–6.1 (1H, d.d., *J* = 16 and 10 Hz) due to the group λ. Signals originated from sandaracopimaric acid 0.85 (3H, s.), 1.05 3H, s.), 1.22 (3H, s.), 5.24 (1H, br. s.), 4.9 ~ 5.1 (2H, m.) and 5.5–6.1 (1H, d.d., *J* = 16 and 10 Hz) due to the group λ.	
I-Me		3200, 2950, 1730, 1680–1630 (br.)	5.77 (1H, br. s.) 5.35 (1H, br. s.)	316($C_{21}H_{32}O_2$)

			3.63 (3H, s.) 1.26 (3H, s.) 1.01 (6H, d., J = 6.6 Hz) 0.83 (3H, s.)	
II-Me		2980, 2960, 2940, 1730, 1610, 1500, 1460	7.18 (1H, d., J = 8.3 Hz) 6.98 (1H, br. d., J = 8.3 Hz) 6.89 (1H, br. s.) 3.66 (3H, s.) 1.28 (3H, s.) 1.23 (6H, d., J = 6.8 Hz) 1.21 (3H, s.)	314 ($C_{21}H_{30}O_2$)
III-Me	231 (12,800) 240 (13,000)	2940, 1730, 1460	5.85 (1H, br. s.) 5.46 (1H, br. s.) 3.77 (1H, br. s.) 3.62 (3H, s.) 3.36 (3H, s.) 1.26 (3H, s.) 1.08 (3H, d., J = 6.8 Hz) 1.03 (3H, d., J = 6.8 Hz) 0.81 (3H, s.)	346 ($C_{22}H_{34}O_3$)
IV-Me			Signals originated from methyl isopimarate 0.86 (3H, s.), 0.90 (3H, s.), 1.26 (3H, s.), 3.65 (3H, s.), 5.35 (1H, br. s.), 4.9–5.1 (2H, m.) and 5.5–6.1 (1H, d.d., J = 16 and 10 Hz) due to the group λ **Signals originated from methyl** sandaracopimarate; 0.83 (3H, s.), 1.03 (3H, s.), 1.20 (3H, s.), 3.67 (3H, s.), 5.21 (1H, br. s.) 4.9–5.1 (2H, m.) and 5.5–6.1 (1H, d.d., J = 16 and 10 Hz) due to the group λ.	316 ($C_{21}H_{32}O_2$)

[a] Unless stated otherwise, all spectra were taken as films.
[b] All of the PMR were taken in a $CDCl_3$ solution with TMS as an internal standard. Singlet, doublet, double doublet, broad singlet, and broad doublet are abbreviated as s., d., d.d., br. s., and br. d., respectively.

TABLE 5. FRACTIONATION PATTERN OF F-2 ON TLC AND ACTIVITY OF FRACTIONS

Fraction[a]	R_f range collected	Activity[b]
F-2-1	1.0–0.8	−
F-2-2	0.8–0.7	±
F-2-3	0.7–0.4	±
F-2-4	0.4–0.1	++
F-2-5	0.1–0.0	−

[a] The fractions obtained by preparative TLC of F-2 developed with *n*-hexane–EtOAc (7:1).
[b] The activity was tested at a concentration of 20 g N eq/ml, and is represented as −, ±, and ++ which show less than 65%, 65–70% and 75–85% deterrent activity, respectively.

sawfly larvae, it is important to establish that there is a significant difference in the levels of the chemicals between single and tufted foliage. As a result of quantitative analysis the levels of the compounds were found to be quite different between the two types of foliage (Figure 3). The amount of these chemicals in single needles is roughly tenfold more than in tufted foliage. Quantities of all these resin acids tend to decline at the end of the growing season.

The overall level of the total resin acids in single needles was approximately 10 mg/10 g in July and reached the maximum at 20 mg/10 g in late August (Figure 3). This level generally agrees with the one estimated from the effective concentration of F-2 and F-3 present in single needles on long shoots collected in July (see Table 1). Thus it may be concluded that the levels of these resin acids in single needles in July and August are such that they can significantly contribute to the deterrent property of the foliage against the sawfly larvae.

DISCUSSION

The phenomenon that many *Neodiprion* sawfly larvae feed only on old foliage of pines and leave current foliage alone is well known. It has been generally interpreted to mean that for these specialized pests, which depend on the availability of the host plants, coevolutional development of such an insect–host plant relationship is a likely consequence. In other words, the species that spare the new foliage and thereby assure the availability of later oviposition and feeding material for the next generation have selective advantages for their species survival over totally destructive species that consume both current year and mature foliage.

Since the major defense mechanism of coniferous trees against generalist herbivores is the production of large quantities of resin material [i.e.,

TABLE 6. ANTIFEEDANT ACTIVITY OF COMPOUNDS I, II, III, and MIX IV, COMBINED MIXTURES AND COMMERCIAL ABIETIC ACID

	Inhibitory activity (mean % larvae on untreated end)										
Compound	30 mg/ml	20 mg/ml	15 mg/ml	10 mg/ml	7.5 mg/ml	5.0 mg/ml	3.75 mg/ml	2.5 mg/ml	1.875 mg/ml	1.25 mg/ml	0.938 mg/ml
I, abietic acid	NT[a]	74**[b]	72.5*[c]	30	NT	40	NT	47.5	NT	50	NT
II, dehydroabietic acid	70**	68.8***	59.4**	55	57.5	47.5	NT	NT	NT	NT	NT
III, methoxyabietic acid	NT	NT	NT	NT	52.5	NT	55	NT	55	NT	72.5
Mix IV, sandracopimaric acid isopimaric acid	NT	74.3***	70	NT	75	NT	70	NT	55	NT	NT
Combination I + II + III + Mix IV, 35:44:8:13	NT	85***	NT	55	NT	57.5	NT	65	NT	52.5	NT
Combination I + II, 2:3	NT	62.5	NT	NT	NT	NT	NT	NT	NT	NT	NT
Comercial abietic acid	NT	80*	81.7***	NT	73.8*	NT	78.8*	NT	65.1	NT	62.5

[a] NT = not tested.
[b] Significance level: *, **, ***, 0.1, 0.05, 0.01, respectively (Student's t test).
[c] Concentration of 17.5 mg/ml.

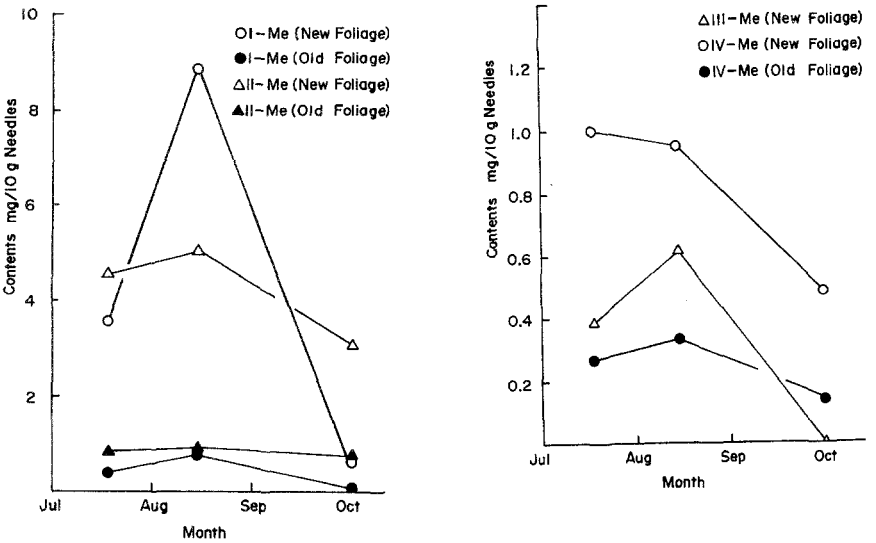


FIG. 3. Seasonal variation of methylated compunds I, II, III, and IV (see Figure 2 for their identities) present in single and tufted foliage of *Larix laricina*. (Compound III not analysed in tufted needles.)

"quantitative" plant defense mechanism of Feeny (1976) and Rhoades and Cates (1976)], they are vulnerable to specialized pests which have developed mechanisms to "crash" through such a chemical defense barrier. Indeed, some of the insects are known to use these substances as the chemosensory marker to locate their host. The diprionid sawflies are a very good example of such an adaptation in that they exclusively feed on coniferous trees, and their host selection behavior is attuned to the chemicals present in these host plants.

The question, then, is how the plants develop the second line of defense against these specialists. In the case of *Neodiprion rugifrons* and *N. swainei*, we reported (Ikeda et al., 1977) that the host tree, jack pine, produces a large quantity of a novel resin acid in the new foliage which specifically inhibits larval feeding. This compound (designated as compound A) is present in a very low concentration in old foliage, suggesting a mission to protect the growing portion of the trees.

What we have found with tamarack is rather different. That is, the single needles contain higher levels of not one but several resin acids which are also present in the tufted foliage. Clearly then there is more than one method to develop the second line of defense against the specialists.

Although at this stage there is no concrete evidence to explain the difference between these two cases, some discussion on the possible reasons for the difference may be in order. The most compelling factor in developing

the second line of defense must be the degree of specialization of the insect pests and the probability of attack by other not so specialized herbivores. That is, the activity spectrum of the secondary chemical produced to protect the vital part of the plant is narrow for a specific chemical (e.g., compound A for jack pine) working only for a few species, whereas those multiple chemicals (e.g., four resin acids for tamarack) may be effective against many other specialized insects. If tamarack is attacked by greater numbers of specialized insect pests, this phenomonon can be explained. It must be pointed out, however, this defense method is more costly to the energy budget of the host tree than the type of defense developed by the jack pine against the two sawfly species. Another aspect is that tamarack is a deciduous coniferous tree unlike the pines and some other coniferous species. The long-shoot foliage as it is termed in this paper, has a shorter time span in which to grow compared to the short shoots. A major difference between needle types is the location and morphology; long-shoot foliage is on the growing shoot with evenly spread single needles along the twig and short shoots are on the older twigs and form tufts. By contrast, the old foliage in pines may be more than one year old and has different physiological characteristics from the new foliage. It follows then that the foliage of tamarack, whether on long shoots or short shoots, is likely to have a qualitatively similar biochemical makeup. Thus, it might be more difficult for tamarack to develop a completely different biochemical machinery to produce qualitatively separate deterrent substances for the single and tufted needles than it is for jack pine.

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A FOUR-COMPONENT PHEROMONE BLEND FOR OPTIMUM ATTRACTION OF REDBACKED CUTWORM MALES, *Euxoa ochrogaster* (GUENÉE)

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Abstract—Four acetates, *Z*-5-decenyl acetate, *Z*-5-, *Z*-7-, and *Z*-9-dodecenyl acetates, in microgram ratios of 1:200:2:1 or 1:200:6:2 were excellent, specific sex pheromone blends for capturing male redbacked cutworm moths in cone traps. Blends in ratios of 1:200:2:1 and 2:200:2:1 at 1000 μg/rubber septum dispenser remained highly effective for 6 weeks under field conditions. The essential minor components, *Z*-5-decenyl, *Z*-7-, and *Z*-9-dodecenyl acetates, became inhibitory at concentrations of about 10% in the blends, and this may be an important general phenomenon in lepidopteran pheromones. Blends involving a parapheromone, *Z*-5-undecenyl acetate, with *Z*-5-, *Z*-7-, and *Z*-9-dodecenyl acetate, in microgram ratios of 8:200:2:1 or 20:200:6:2 were also excellent specific attractants for this species. The *Z*-8-dodecenyl acetate had no obvious effect on the attraction of the redbacked cutworm males.

Key Words—Redbacked cutworm, *Euxoa ochrogaster*, Lepidoptera, Noctuidae, sex pheromone blend, sex attractant, parapheromone, *Z*-5-decenyl acetate, *Z*-5-undecenyl acetate, *Z*-5-dodecenyl acetate, *Z*-7-dodecenyl acetate, *Z*-9-dodecenyl acetate.

INTRODUCTION

The redbacked cutworm (RBC), *Euxoa ochrogaster* (Guenée) (Lepidoptera: Noctuidae), is a major pest of cereals and several other crops throughout Canada and the northern parts of the United States. Struble and Swailes (1978) reported a species-specific sex attractant for RBC males. This was developed by systematically screening the relative attractiveness of about 2000 binary and tertiary combinations of highly purified (ca. 98–99%) saturated and monoolefinic acetates and alcohols of the C_{10} to C_{16} series that are

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common to lepidopteran attractants and pheromones (Tamaki, 1977). The reported attractant that gave the most consistent catches of males consisted of *Z*-5-undecenyl acetate (*Z*5–11:Ac), *Z*-5-dodecenyl acetate (*Z*5–12:Ac), and *Z*-8-dodecenyl acetate (*Z*8–12:Ac) (99% pure) in a ratio of 1:25:50 at 1 mg/dispenser. The addition of 14% *Z*7–12:Ac as a fourth component in this blend inhibited the attraction of males. The use of 0.1–1.3% *Z*5–10:Ac rather than *Z*5–11:Ac in these blends was an effective attractant, but the addition of 6% *Z*5–10:Ac also inhibited the attraction of males (Struble et al., 1980).

The following compounds were identified in the ovipositor washes of the RBC females (approximate ratios in parentheses): 10:Ac (11), 12:Ac (11), *E*5–12:Ac (4), *Z*5–12:Ac (100), *Z*7–12:Ac (4), and *Z*9–12:Ac (trace, ca. 0.6) (Struble et al., 1980). The ovipositor washes contained a trace (ca. $< 0.5\%$) of a decenyl acetate, and electroantennographic detector (EAD) and single receptor recordings with synthetic chemicals indicated that this was probably *Z*5–10:Ac. The *Z*5–11:Ac and *Z*8–12:Ac were not detected in the female ovipositor washes, although strong EAD antennal responses were recorded for *Z*5–11:Ac.

Analyses by high-resolution capillary gas chromatography (GC), GC-mass spectrometry (GC-MS), and ozonolysis showed that the 99% pure *Z*8–12:Ac used in the field screening tests (Struble and Swailes, 1978) contained 0.4% *Z*7–12:Ac and 0.2% *Z*9–12:Ac (Struble et al., 1980). It was not established whether the *Z*7-, *Z*8-, or *Z*9–12:Acs were essential for the attraction of the RBC males.

This report describes extensive field tests to determine the relative attractiveness of many combinations of synthetic chemicals based upon those present in the methylene chloride washes of the RBC female abdomen tips and tests of the synergistic or inhibitory effects of the corresponding alcohols. The relative effectiveness of the attractive four-component blend at various quantities per dispenser was determined over a 6-week period. The function of *Z*5–11:Ac and *Z*8–12:Ac in the attraction of RBC males was further investigated, and *Z*3- and *Z*7–10:Ac were tested as possible synergists in place of *Z*5–10:Ac.

METHODS AND MATERIALS

The chemicals were synthesized here (Struble and Swailes, 1975), except *Z*8–12:Ac and *Z*9–12:Ac, which were purchased from ChemSampCo., Columbus, Ohio. They were purified by argentation liquid chromatography using Lewatit® SP1080 resin impregnated with silver nitrate (Houx et al., 1974), and samples of all chemicals (ca. 100 mg) were further purified by preparative GC using columns 2 m × 5.3 mm ID with 5% FFAP or 5% OV-17 on Gas Chrom Q 60/80 mesh. Chemical purities ranged from 99.0 to 99.8% as

determined by GC and GC-MS using glass columns 20 m × 0.3 mm ID coated with Silar 10C, Carbowax 20M TPA, or OV-17 by the method described by Grob et al. (1977).

Chemical blends were prepared in *n*-hexane (spectral grade, ca. 5 mg/ml), and red rubber septa (Arther H. Thomas Co., catalog no. 8753-D22) were used as dispensers. The "cone-type" traps (Struble and Swailes, 1975) were made of FRE® duct (10.2-cm size, Canadian General Electric) with No. 8 mesh metal screen end-cones with an apical hole ca. 1.0 cm in diameter. Replicated treatments were in randomized block designs within single or double rows in a north–south direction, with the traps at a height of 1 m, 15–20 m apart, and with one end facing the prevailing west wind. In all tests, at least one trap baited with the best attractant was used at each end of the row of traps, beyond the replicated treatments. Catches in these "guard traps" were not considered in the experiments. The tests were conducted in three fields within 15 km of the Lethbridge Research Station, Alberta, in 1979. The traps were emptied and the catches recorded daily. Analyses of variance were done on the data or the transformed data [$\log (x + 1)$], and treatment means were compared using Tukey's test or the least-significant-difference (LSD) test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Initially, two experiments were done with a total of 50 duplicate treatments involving combinations of two to seven of the acetates at ratios similar to those found in the females, and some of the results are summarized in Table 1. Four-component blends of *Z*5–10:Ac, *Z*5-, *Z*7-, and *Z*9–12:Ac in microgram ratios of 2:200:2:1 and 2:200:6:1 attracted large numbers of males. When the quantities of *Z*7- or *Z*9–12:Ac were increased to 20 μg in these blends, the catches of males were reduced indicating the inhibitory effects of these components at higher concentrations. When any one of *Z*5–10:Ac, *Z*7-, or *Z*9–12:Ac was omitted from these blends, the trap-catches were dramatically reduced and a two-component blend of *Z*5- and *Z*7–12:Ac was essentially nonattractive. Omission of *Z*5–10:Ac or *Z*9–12:Ac from the three- to five- and four- to seven-component blends also resulted in the capture of fewer males. Blends including *E*5–12:Ac, 10:Ac, and 12:Ac attracted similar ($P > 0.05$) numbers of males as the four-component blend alone. Two other chemicals *Z*3- and *Z*7–10:Ac were included in some blends in place of *Z*5–10:Ac, but only small numbers (<45) of males were captured. As these two chemicals had no obvious synergistic effects, they were not included in any other tests.

These initial results clearly illustrated the importance of low concentrations of *Z*5–10:Ac, *Z*7-, and *Z*9–12:Ac with *Z*5–12:Ac for the

TABLE 1. REDBACKED CUTWORM MALES CAPTURED WITH VARIOUS COMBINATIONS OF ACETATES DETECTED IN FEMALE PHEROMONE WASHES, DURING TWO TEST PERIODS (TWO REPLICATIONS/TEST)

Acetates (μg) added to 200 μg of Z5–12:Ac						Mean males captured/test[a]	
C_{10}		C_{12}				I	II
10:Ac	Z5-	12:Ac	E5-	Z7-	Z9-	(July 31–Aug. 16)	(Aug. 1–16)
Four components							
	2			2	1	102 a	119 a
	2			6	1	—	115 a
	2			20	1	—	58
	2			2	4	—	96
	2			2	20	—	48
	2			6	4	—	80
Two and three components							
	0			2	1	5	—
	2			0	1	8	—
	2			2	0	23	—
	0			2	0	1	—
Three to five components							
	2		6	2	1	110 a	128 a
	2		12	2	1	—	91
	2		6	2	4	—	100
	2		12	2	4	—	78
	2		6	2	0	34	—
	0		6	2	0	1	—
Four to seven components							
16	2	16	6	2	1	98 a	152 a
16	0	16	0	2	0	1	—
16	2	16	0	2	0	30	—
16	2	16	6	2	0	40	—

[a] Within columns, mean males captured per treatment followed by a letter do not differ ($P > 0.05$) from the first treatment listed by LSD test.

attraction of the RBC males in the field. That these essential minor components were also inhibitory at concentrations of 10% relative to Z5–12:Ac was consistent with the previously reported inhibitory effects of Z5–10:Ac and Z7–12:Ac (Struble and Swailes, 1978) and Z9–12:Ac (Steck et al., 1980), although these identical blends were not involved in these earlier reports.

The previously reported attractant for RBC males consisted of blends of Z5–10:Ac or Z5–11:Ac with Z5–12:Ac and Z8–12:Ac in ratios of 2 or 8:200:400 (Struble and Swailes, 1978). The Z8–12:Ac was at least 99% pure,

but it contained 0.4% Z7-12:Ac and 0.2% Z9-12:Ac as impurities (Struble et al., 1980). Blends involving Z5-10:Ac or Z5-11:Ac with Z5-12:Ac and the same batch of 99% pure Z8-12:Ac and with 99.9% pure Z8-12:Ac were repeated to determine whether Z8-12:Ac had any effect on the attraction of RBC males. Treatments involving 99.9% pure Z8-12:Ac, which contained ca. 0.05% Z7-12:Ac and undetectable quantities of Z9-12:Ac, captured 56 and 80 males (Table 2). When Z7- and Z9-12:Ac were added to the blends containing 99.9% pure Z8-12:Ac, 230 and 217 males, respectively, were captured. It was concluded from these results that the small amounts of the positional isomers, Z7- and Z9-12:Ac, which were fortuitously present as impurities in the 99% pure Z8-12:Ac used in the earlier tests (Struble and Swailes, 1978), were essential for the attraction of RBC males. Furthermore, Z8-12:Ac had no effect on the trap catches in these present tests and, since Z8-12:Ac was not detected in the female pheromone (Struble et al., 1980), it was not included in any other attractant blends for this species.

Based upon the initial results summarized in Table 1, several other ratios of the seven acetates were tested in three experiments at different locations in an attempt to optimize the captures of RBC males (Table 3). The blend of Z5-10:Ac, Z5-, Z7-, and Z9-12:Ac in a ratio of 2:200:2:1 was used in each of the experiments for comparison purposes. The greatest numbers of males were attracted to these four compounds in ratios of 1:200:6:1, 1:200:6:2,

TABLE 2. REDBACKED CUTWORM MALES CAPTURED WITH BLENDS INVOLVING 99% AND 99.9%[a] PURE Z8-12:Ac (TWO REPLICATIONS, AUGUST 1-16)

Quantities (μg) of acetates added to 200 μg of Z5-12:Ac						
C_{10}	C_{11}	C_{12}				Mean males captured[b]
Z5-	Z5-	Z7-	Z8- (99%)	Z8- (99.9%)	Z9-	
2		2			1	119 a
2		1.6[c]	400		0.8[c]	111 a
2		0.2[c]		400	0	28
2		2		400	1	115 a
	8	1.6[c]	400		0.8[c]	97 a
	8	0.2[c]		400	0	40
	8	2		400	1	109 a

[a]The 99% pure Z8-12:Ac contained 0.4% Z7-12:Ac and 0.2% Z9-12:Ac as impurities, while 99.9% pure Z8-12:Ac contained 0.05% Z7-12:Ac as an impurity and Z9-12:Ac was undetectable.

[b]Mean males captured per treatment followed by a letter do not differ ($P > 0.05$) from the first treatment listed by LSD test.

[c]These quantities of Z7-12:Ac and Z9-12:Ac were present as impurities in the Z8-12:Ac.

TABLE 3. REDBACKED CUTWORM MALES CAPTURED WITH COMBINATIONS OF *Z*5–12: Ac (200 μg) WITH VARIOUS QUANTITIES (μg) OF OTHER ACETATES DURING THREE TEST PERIODS (FOUR REPLICATIONS/TEST PERIOD) IN DIFFERENT FIELDS

Acetates (μg) added to *Z*5–12: Ac (200 μg)						Mean males captured/test period[a]		
C_{10}		C_{12}				I	II	III
10: Ac	*Z*5-	12: Ac	*E*5-	*Z*7-	*Z*9-	(Aug. 16–27)	(Aug. 15–27)	(Aug. 24–Sept. 5)
	2			2	1	88	108 a	217
	1			2	1			245
	1			6	1		101 a	
	1			6	2			251
	4			2	1	95		
	6			6	1		59	
	12			6	1		49	
	2			6	1	105	84	
	2			6	2		111 a	
	2			2	6	92		
	2			6	6		80	
	2		3	2	1	114		
	1		3	2	1			217
	1		3	6	2			270
	2		6	2	1	97		
	2		6	6	1		95 a	
16	2	16	3	2	1	100		
16	2	16	6	2	1	83		
16	2	16	6	6	1		94 a	

[a] Test period II mean catches followed by a letter and mean catches within columns for the other test periods do not differ ($P > 0.05$) from the first treatment by LSD test.

and 2:200:6:2 (although $P > 0.05$). In general, the trap catches by the four-component blends were reduced when quantities of *Z*5–10: Ac and *Z*9–12: Ac were increased to 12 μg and 6 μg (6% and 3% relative to 200 μg of *Z*5–12: Ac).

Trap catches with the treatments involving 10: Ac, 12: Ac, or *E*5–12: Ac indicated that 3 μg of *E*5–12: Ac in the blends may be marginally beneficial (although $P > 0.05$); however, with *E*5–12: Ac at 6 μg and 10: Ac and 12: Ac at 16 μg, the trap catches were slightly reduced. These results indicated that low concentrations (3%) of *E*5–12: Ac would certainly not interfere with the attraction of RBC, which is important for future large-scale tests. Low concentrations of *E*5–12: Ac would not need to be removed from *Z*5–12: Ac, the main component of the blend. Since 10: Ac, 12: Ac, and *E*5–12: Ac are produced by the female moths, they may be involved with other aspects of the mating behavior (Baker et al., 1976; Roelofs, 1978), but their ethological roles are not known.

The results shown in Table 1 established that very small quantities of Z5-10:Ac, Z7-, and Z9-12:Ac in blends with Z5-12:Ac had a tremendous influence on the trap catches of RBC males. The importance of each component in the four-component blend was dramatically demonstrated by the experiment summarized in Table 4. Omission of any one of the acetates from the four-component blend resulted in large reductions ($P < 0.01$) in the numbers of captured males. These results definitely established that the optimum captures of RBC males were with the four-component blend.

The two-component blends of Z5- and Z7-12:Ac also captured very few ($P < 0.01$) moths (Table 4). This was in agreement with the data in Table 1 and with the results reported earlier (Struble et al., 1980). However, these results are contrary to the optimized attractant for this species developed by field testing of chemical combinations reported by Steck et al. (1980). They reported optimum attraction of males to a two-component blend of Z5- and Z7-12:Ac in a ratio of about 100:1. There is no obvious explanation for this discrepancy, unless the chemicals used in their tests contained trace quantities of at least Z9-12:Ac that may have been formed by their method of synthesis (Chisholm et al., 1978). They did not include Z5-10:Ac in their blends.

The synergistic and inhibitory effects of the alcohols of the corresponding acetates in the four-component blend were determined (Table 5). Each alcohol was added to a blend at quantities from 0.5 to 10% of the corresponding acetate and, in general, the Z5-10:OH, Z7-, or Z9-12:OH had no obvious effects on the trap catches of RBC males and other lepidopteran species were not captured. The Z5-12:OH at up to 5% may be somewhat beneficial (although $P > 0.05$). This may be important for future large-scale tests as trace quantities (1-2%) of the corresponding alcohols are often present in the synthetic acetates. The main component of the pheromone

TABLE 4. REDBACKED CUTWORM MALES CAPTURED WITH VARIOUS COMBINATIONS OF FOUR COMPONENTS, Z5-10:Ac; Z5-, Z7, AND Z9-12:Acs FROM SEPTEMBER 4 TO 14 (FOUR REPLICATIONS)

Quanty (μg)/septum				Mean males captured[a]
Z5-10:Ac	Z5-12:Ac	Z7-12:Ac	Z9-12:Ac	
1	200	6	2	238 a
	200	6	2	8 c
1		6	2	1 c
1	200		2	2 c
1	200	6		82 b
	200	2		5 c
	200	6		1 c

[a] Mean catches followed by different letters differ ($P < 0.01$) by Tukey's test.

TABLE 5. REDBACKED CUTWORM MALES CAPTURED WITH BLENDS A, B, AND C, WITH ADDITION OF VARIOUS QUANTITIES OF CORRESPONDING ALCOHOLS (FOUR REPLICATIONS)[a]

Alcohols added (μg)	Mean males captured with blends[b] A (Aug. 28–Sept. 9)	B (Sept. 4–14)	C (Aug. 28–Sept. 9)
None	138	176	92
Z5-10:OH (0.1)	134	160	
Z5-11:OH (0.4)			103
Z5-12:OH (1)		182	
(2)		193	
(6)		223	
(10)	145		93
Z7-12:OH (0.2)	148		86
(0.3)		169	
(0.6)		173	
Z9-12:OH (0.1)	155	184	85

[a] Blends A and B were Z5-10:Ac, Z5-, Z7-, and Z9-12:Ac in microgram ratios of 1:200:2:1 and 1:200:6:2, and blend C was Z5-11:Ac, Z5-, Z7-, and Z9-12:Ac in a microgram ratio of 4:200:2:1.

[b] Within columns, the mean number of males captured do not differ ($P > 0.05$) from the first treatment by LSD test.

blend, Z5-12:Ac, could contain up to 5% of its corresponding alcohol, Z5-12:OH, without interfering with the attraction of RBC males. Blends containing the four acetates and four alcohols were not tested, so that any combined effect of the alcohols was not determined.

The four-component blend is an excellent trap bait for monitoring the population densities of the RBC males, and it was important to establish the quantity of the blend per septum dispenser that functioned the best throughout an average flight period of 6–8 weeks. The four-component blend was tested at ratios (A) 1:200:2:1 and (B) 2:200:2:1 with 50, 200, and 1000 μg/septum from August 22 to October 1 (Table 6). The blends at 1000 μg/septum attracted the greatest numbers ($P < 0.05$) of males throughout the test period. There were only small differences between the captures to the two ratios A and B at each of the three quantities of 50, 200, and 1000 μg/dispenser.

The treatment of 50 μg of ratio A was replaced by dispensers freshly treated with the same blend at 200 μg after the third week of the test. During the fourth and fifth weeks of the test, this freshly prepared treatment of 200 μg captured more males ($P < 0.05$) than the remaining original treatment of 50 μg (ratio B), but it captured similar numbers ($P > 0.05$) as the 200- and

1000-μg treatments. This indicated that the gradual decrease in catches to the original 50- and 200-μg treatments over the first 5 weeks of the test were probably due to insufficient attractant on the dispensers rather than a gradual decrease in the moth flight. The catches to the 1000 μg/septum remained nearly constant during the test period.

The large decrease in trap catches during the sixth week of the test (5 days only) indicates the reduction in the moth flight due to lower night temperatures. Trap catches were also low during the first week of the test; this was attributed to a reduced moth flight due to rain. It was concluded that either of these four-component blends at 1000 μg/rubber septum dispenser was effective for at least 6 weeks in the field. The ratio of 1:200:6:2 for the four components was not included in this test because the initial tests involving this ratio (Table 2) were done concurrently.

The *Z*5–11:Ac was involved in the previously reported attractant in combinations with *Z*5–12:Ac and *Z*8–12:Ac (99% pure) (Struble and Swailes, 1978) (Table 2). Although strong electroantennographic antennal responses were recorded with *Z*5–11:Ac as a stimulus, it was not detected in the RBC female pheromone washes (Struble et al., 1980). The parapheromone activity was confirmed when it was included in the present four-component blends in place of *Z*5–10:Ac (Table 7). This resulted in the species-specific attraction of RBC males to all of the blends and *Z*5–11:Ac, *Z*5-, *Z*7-, and *Z*9–12:Ac in a ratio of 8:200:2:1 or 20:200:6:2 attracted the most males.

TABLE 6. MEAN NUMBERS OF REDBACKED CUTWORM MALES PER WEEK FROM AUGUST 22 TO OCTOBER 1 CAPTURED WITH BLENDS OF *Z*5–10:Ac; *Z*5-, *Z*7-, *Z*9–12:Ac AT RATIOS OF (A) 1:200:2:1 AND (B) 2:200:2:1 AT THREE QUANTITIES (μg) PER SEPTUM (FOUR REPLICATIONS)

Quantity (μg) of *Z*5–12:Ac/septum	Mean males/weak[a]						Total males captured
	1	2	3	4	5	6[b]	
Ratio A							
50[c]	87 a	133 ab	80 e	113 ab	178 a	28 ab	2552
200	76 ab	144 ab	113 cd	114 ab	106 bc	20 bc	2288 b
1000	59 ab	171 a	166 ab	169 a	164 ab	36 ab	3056 ab
Ratio B							
50	54 b	104 b	91 de	75 b	76 c	10 c	1640 c
200	58 ab	132 ab	132 bc	114 ab	113 bc	22 ab	2276 b
1000	63 ab	183 a	224 a	184 a	196 a	42 a	3580 a

[a] Within columns, means followed by the same letter do not differ ($P > 0.05$) by Tukey's test.
[b] This was only a 5-day period, as the test was terminated due to exceedingly low moth flight.
[c] This was replaced with a freshly prepared treatment of ratio A at 200 μg of *Z*5–12:Ac/septum after the third week, and the total males captured by this treatment were not included in the statistical analysis of the total males captured.

TABLE 7. REDBACKED CUTWORMS MALES CAPTURED WITH *Z*5–12:Ac (200 μg) IN COMBINATIONS WITH VARIOUS QUANTITIES OF OTHER ACETATES INCLUDING *Z*5–11:Ac, A PARAPHEROMONE COMPONENT, DURING TWO TEST PERIODS (FOUR REPLICATIONS/TEST PERIOD)

Acetates (μg) added to *Z*5–12:Ac (200 μg)					Mean males captured/test[a]	
C_{10}	C_{11}	C_{12}			I	II
*Z*5-	*Z*5-	*E*5-	*Z*7-	*Z*9-	(Aug. 22–Sept. 4)	(Aug. 24–Sept.5)
1			2	1	227 a	245 a
1			6	2		251 a
	1		2	1	80 c	
	4		2	1	148 b	185 abc
	4	3	2	1		180 abc
	4		6	2		123 b
	4	3	6	2		102 c
	8		2	1	187 ab	
	20		2	1	180 ab	
	20		6	2		188 abc
	20	3	6	2		227 ab

[a]Within columns, mean catches followed by the same letters did not differ ($P > 0.05$) by Tukey's test.

These blends captured fewer males (75–82%) than blends of *Z*5–10:Ac, *Z*5-, *Z*7-, and *Z*9–12:Ac at ratios of 1:200:2:1 and 1:200:6:2. The addition of 3 μg of *E*5–12:Ac to the blends involving *Z*5–11:Ac had little effect on the capture of males. The synergistic and inhibitory effects of the alcohols of the corresponding acetates in the four-component blends involving *Z*5–11:Ac were determined but the quantities of alcohols tested had no obvious effects (Table 5).

The use of *Z*5–11:Ac as a parapheromone component in blends with *Z*5-, *Z*7-, and *Z*9–12:Ac provides an excellent alternate attractant for RBC males, which may be very useful for maintaining species-specificity if the situation ever arises where other species are attracted to the pheromone blend.

All of the four-component blends involving *Z*5–10:Ac or *Z*5–11:Ac were essentially species-specific for RBC males as only a few, ca. 20, of the 91,500 males captured in these tests in 1979 were of other species. Several species were known to be in the test area. Furthermore, RBC males with all possible wing color patterns (Hardwick, 1965) were attracted to all of the four-component blends. Males of any one pattern did not show a preference for any of the treatments. The overall mean catch of RBC males per trap per day was 11.9 for all experiments in 1979.

This is one of the few instances where several pheromone-like com-

ponents have been detected in the female pheromone gland washes and where four of these were essential components for optimal attraction of males in the field. Similar instances have recently been reported for three other species by Cardé et al. (1979) and Klun et al. (1979).

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INSECT SEX PHEROMONES: Evaporation Rates of Alcohols and Acetates from Natural Rubber Septa[1]

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Abstract—The half-lives ($t_{\frac{1}{2}}$) of alcohol sex pheromones, 1-alkanols, acetate sex pheromones, and an epoxide (disparlure) were determined on natural rubber septa. The $t_{\frac{1}{2}}$ values for the homologous alcohols from decanol to heptadecanol increased regularly from 2.2 to 1117 days, but the $t_{\frac{1}{2}}$ of octadecanol was 609 days. The $t_{\frac{1}{2}}$ values of (*Z*)7-, (*E*)7-, and (*Z*)9-tetradecen-1-ol acetates were 154, 168, and 199 days, respectively, whereas those of five other tested 14-carbon acetates ranged from 310 to 350 days. The dependence of $t_{\frac{1}{2}}$ values on chain length and double-bond position is consistent with the hypothesis that molecular size is an important variable affecting $t_{\frac{1}{2}}$ values. Also, in accordance with the hypothesis, when a *Z*-alkenyl compound has a much shorter $t_{\frac{1}{2}}$ than the corresponding saturated compound, the $t_{\frac{1}{2}}$ values of the *Z* compound and its *E* isomer may be quite different. Thus, (*E*)-9-tetradecen-1-ol acetate had a $t_{\frac{1}{2}}$ of 331 days. The $t_{\frac{1}{2}}$ of disparlure was 180 days. The effect of the *cis*-7,8 epoxide group is apparently similar to that of the olefin group in lowering the $t_{\frac{1}{2}}$ below the value that would be expected solely on the basis of chain length.

Key Words—Insect sex pheromones, insect sex attractants, Lepidoptera, pheromones, pheromone formulations, insect population monitoring.

INTRODUCTION

Natural rubber septa are an excellent substrate for the controlled release of many insect sex pheromones used in monitoring traps. Septa release by a

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

first-order process (release rate is proportional to the amount of pheromone present) with long half-lives for most pheromones of Lepidoptera. Septa dosed with pheromone are inexpensive and can be easily prepared by the user without specialized equipment. Previously, we reported the half-lives of the acetate pheromones of many important insect pests on rubber septa (Butler and McDonough, 1979). Such data can be used to estimate evaporation rates, the length of time that the evaporation rates will be within a given range, and, for multicomponent pheromones of differing volatility, the ratio of components in the vapor. The study was conducted so that the relationship between half-life and molecular structure could be determined. We expected that a plot of the logarithm of the retention times versus the number of carbon atoms of the members of a homologous series would be linear. Although the plot for the C_{10} to C_{15} 1-alkanol acetates was linear, there were two important anomalies: 1-hexadecanol acetate had a smaller $t_{\frac{1}{2}}$ than 1-pentadecanol acetate, and the pink bollworm, *Pectinophora gossypiella* (Saunders), pheromone (*Z*7,*Z*11–16:Ac + *Z*7,*E*11–16:Ac, 1:1) (code for compounds is explained in Table 1 footnote) had a considerably smaller $t_{\frac{1}{2}}$ than either 16:Ac or *Z*11–16:Ac.

We hypothesized that cross-linking of the rubber accounted for these anomalous half-lives. According to the hypothesis, half-lives should decrease significantly for either tetradecenyl or hexadecenyl acetates as the double bond is moved closer to the center of the molecule but should decrease negligibly or relatively much less for dodecenyl acetates. Also this hypothesis required that 1-octadecanol possess a shorter half-life than 1-heptadecanol.

We therefore undertook this study to test our hypothesis and to provide half-life data which would be useful for preparing efficacious formulations of pheromones containing alcohol components.

METHODS AND MATERIALS

These were identical to those reported earlier (Butler and McDonough, 1979) except for the additional use of a gas chromatographic column to determine *cis–trans* isomer content: a 2.3-mm × 6.1-m stainless-steel column containing 15% polycyanopropylphenylsiloxane (OV-275) on 100/120 mesh Chromosorb® P, AW-DMCS (Supelco Inc., Bellefonte, Pennsylvania).

RESULTS AND DISCUSSION

Effect of Chain Length of Alcohols on $t_{\frac{1}{2}}$. The half-lives ($t_{\frac{1}{2}}$), 95% confidence limits of $t_{\frac{1}{2}}$, and correlation coefficients for a first-order plot (logarithm of amount of pheromone remaining versus time) are given in Table 1 for the compounds determined in this study. Also, a plot of ln $t_{\frac{1}{2}}$ versus the

TABLE 1. HALF-LIVES FOR EVAPORATION OF PHEROMONES AND RELATED COMPOUNDS IMPREGNATED IN RUBBER SEPTA

Compound[a]	$t_{1/2}$ (days) Average	95% confidence limit	Correlation Coefficient	No. of data points	No. of days evaporation monitored
8: OH	0.90	0.87–0.94	0.986	26	4
10: OH	2.2	2.1–2.3	0.992	25	8
9–10: OH	3.0	2.9–3.0	0.992	39	15
*E*5–10: OH	2.2	2.2–2.3	0.988	36	8.3
11: OH	6.8	6.6–6.9	0.994	56	22
12: OH	14.8	14.3–15.3	0.994	33	55
*Z*7–12: OH	13.5	13.0–13.9	0.993	41	51
*E*8,*E*10–12: OH[b]	27.0	26.4–27.6	0.994	45	126
14: OH	130	126–134	0.988	70	318
*Z*11–14: OH	117	113–121	0.980	60	325
15: OH	269	252–288	0.962	60	302
16: OH	399	327–511	0.725	81	523
*Z*11–16: OH	432	410–456	0.950	78	519
17: OH	1117	825–1731	0.645	42	272
18: OH	609	513–749	0.870	42	277
*Z*9–12: Ac	44.8	43.9–45.7	0.994	52	127
*E*9–12: Ac	38.4	37.5–39.3	0.995	33	136
Z/*E*7–14: Ac(1: 1)	161	151–172	0.978	39	185
*Z*9–14: Ac	202	189–216	0.962	60	265
Disparlure[c]	180	168–194	0.963	47	258

[a]The letters after the colon indicate functional group: OH, alcohol; Ac, acetate. The number between the dash and colon indicates the number of carbon atoms in the longest continuous chain. The letters and numbers before the dash indicate the configuration and position of the double bonds.
[b]Data from Maitlen et al. (1976).
[c]Racemic *cis*-7,8-epoxy-2-methyloctadecane.

number of carbon atoms in the 1-alkanols is given in Figure 1. The plot is similar to that obtained for the acetates (Butler and McDonough, 1979) and is linear, with a slight scatter of points, for the C_{10} to C_{17} alcohols. 1-Octadecanol had a shorter $t_{\frac{1}{2}}$ than 1-heptadecanol. In the acetate series, 1-hexadecanol acetate had a shorter $t_{\frac{1}{2}}$ than 1-pentadecanol acetate. Since 1-heptadecanol and 1-pentadecanol acetate have the same chain length, and 1-octadecanol and 1-hexadecanol acetate have the same chain length, the results we obtained with these compounds support our hypothesis that molecular size is one of the principal molecular features determining evaporation rates in rubber septa. (Molecular weight and molecular polarity are the other two factors.) When a molecule is large enough, it cannot diffuse within much of the cross-linked lattice and, because of this lesser entangle-

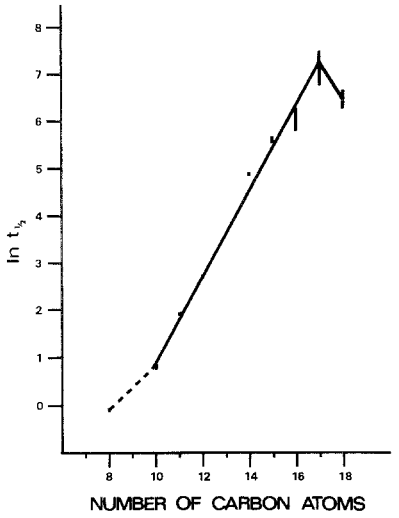

FIG. 1. Values for $\ln t_{\frac{1}{2}}$ versus number of carbon atoms for 1-alkanols. The bars define the 95% confidence limits.

ment, evaporates faster than would be expected solely on the basis of its molecular weight.

In the plot of $\ln t_{\frac{1}{2}}$ versus chain length of the acetates, the slope of the curve for the C_6 through C_{10} acetates was less steep than that for the C_{10} through C_{15} acetates. We proposed that for acetates of shorter chain length than 1-decanol acetate, diffusion would be unhindered by cross-linking, but that for acetates of longer chains up to 1-pentadecanol acetate diffusion would be increasingly hindered by cross-linking. If this proposal were correct, then the slope for a corresponding plot for 1-alkanols (Figure 1) should show an inflection at 1-dodecanol. Instead we found that, if an inflection did appear, it appeared at 1-decanol. Thus, changes in slope for compounds with relatively short chain lengths appear to be associated with some other, as yet unidentified, factor.

Effect of Double-Bond Position on $t_{\frac{1}{2}}$ of Acetates. Our expectation that the half-lives of 1-dodecenol acetates would be similar, but that the half-lives of the 1-tetradecenol acetates might decrease as the double bond became more centrally located is substantiated by the data of Table 1. For the 1-dodecenol acetates, the following $t_{\frac{1}{2}}$ ratios were found: *Z*7-12:Ac/12:Ac, 0.95 (Butler and McDonough, 1979); *E*9-12:Ac/12:Ac, 1.03; *Z*9-12:Ac/12:Ac, 1.20. In contrast the following ratios were obtained for the 1-tetradecenol acetates: *Z*/*E*7-14:Ac(1:1)/14:Ac, 0.46; *Z*9-14:Ac/14:Ac, 0.58; *Z*11-14:Ac/14:Ac, 0.89. Thus, the two main predictions (concerning chain length and double-bond positions) based on our hypothesis that molecular size influences evaporation rate in natural rubber were borne out.

Effect of Double Bond on $t_{\frac{1}{2}}$ of Alcohols. The previously tested monounsaturated acetates (Butler and McDonough, 1979) had half-lives quite similar to the saturated. This is also true for the tested alcohols. Thus, the following $t_{\frac{1}{2}}$ ratios were obtained: 9-10:OH/10:OH, 1.36; *E*5-10:OH/10:OH, 1.00; *Z*7-12:OH/12:OH, 0.91; *Z*11-14:OH/14:OH, 0.90; *Z*11-16:OH/16:OH, 1.08. Inasmuch as the position of the double bond in the 1-tetradecenol acetates has now been shown to have a substantial effect on $t_{\frac{1}{2}}$, similar effects must be assumed for the alcohols, particularly the 1-hexadecenols and 1-octadecenols.

Effect of Z,E Isomers on $t_{\frac{1}{2}}$. The *Z*/*E*7-14:Ac (1:1) applied to the septa was analyzed for *Z*/*E* ratio at the time of application and after aging in septa for 325 days. After this time the ratio was 0.532 *E*:0.468 *Z*. From these data and the 161-day half-life of the combined isomers (Table 1), half-lives of 168 days for *E* and 154 days for Z were calculated. Similarly from the 202-day half-life of *Z*9-14:Ac and levels of 3.3% *E* initially and 5.1% *E* after 325 days, half-lives of 199 days for *Z* and 331 days for *E* were calculated. The $t_{\frac{1}{2}}$ of *Z*11-14:Ac was reported previously to be 310 days (Butler and McDonough, 1979). Initially the *E* content was 2.80% and after 367 days it was 2.84% of the *Z*. So the $t_{\frac{1}{2}}$ of *E*11-14:Ac was, within experimental error, the same as that of *Z*11-14:Ac. These results are consistent with previous expectations that if a *Z* isomer has a $t_{\frac{1}{2}}$ similar to the corresponding saturated compound, then the *E* isomer will also have a similar $t_{\frac{1}{2}}$, whereas if the *Z* isomer has a substantially smaller $t_{\frac{1}{2}}$ than the saturated, then the *E* isomer might be expected to have a significantly different $t_{\frac{1}{2}}$ from that of the *Z* isomer. Since both the *Z*/*E*7 and *Z*/*E*9 isomeric pairs were candidates for *Z*/*E* isomer differences, it is not obvious why the *Z*/*E*7 isomer had similar $t_{\frac{1}{2}}$ values whereas the *Z*/*E*9 isomers were quite different. It is interesting that the $t_{\frac{1}{2}}$ of *E*9-14:Ac was similar to the values for 14:Ac (350 days), *Z*9,*E*12-14:Ac (319 days), and *Z* and *E*11-14:Ac (310 days) (Butler and McDonough, 1979). If more precise predictions of $t_{\frac{1}{2}}$ are to be made, the relative proportions of all the molecular conformations of each of the isomers would probably have to be determined.

Disparlure. The $t_{\frac{1}{2}}$ of disparlure (*cis*-7,8-epoxy-2-methyloctadecane) (Bierl et al., 1970) was 180 days. Its chain length of 18 carbon atoms is the same as that of pentadecanol acetate or 1-heptadecanol. We attribute the much shorter $t_{\frac{1}{2}}$ of disparlure to the effect of the *cis*-7,8-epoxy group, which would be expected to limit the conformational possibilities to those having a smaller average volume than that of *n*-octadecane alone, 1-pentadecanol acetate, or 1-heptadecanol.

Septa Capacity. Because septa release most pheromones at moderate to very slow rates and fairly high release rates are optimum for some pest species, high septa loadings are occassionally needed. For example, Sharma et al. (1971) reported optimum release rates of 18-60 μg/hr for the cabbage looper *Trichoplusia ni* (Hübner) pheromone (*Z*7-12:Ac), depending on type of trap

used. On the basis that $t_{\frac{1}{2}} = 35$ days, these release rates correspond to loadings of 22–73 mg/septum. In a study on the feeding attractant of the checkered flower beetle, *Trichodes ornatus* (Say), Davis et al. (1979, and unpublished) found that large septa (11 × 17-mm Thomas rather than the West No. 1) were not overloaded when impregnated with 400 or 500 mg of the attractant cyclohexyl phenylacetate. Since large septa weigh 2.36 g and small septa (West Co.) weigh 0.79 g, quantities of pheromone as large as 125 mg may not overload small septa. When the small septa were dosed with 75 mg of *Z* 7–12: Ac, the zero-day analysis indicated 61.5 mg (82%) had been retained. This retention is within the normal range of 90 ± 10% retention obtained for a variety of compounds at lower loadings. After 1 and 3 days, the amounts left were 61.0 and 58.0 mg. If the capacity of the septa was exceeded and some of the pheromone had not become dissolved, we would expect an initially very fast evaporation. Instead, the data indicate a normal release pattern and that the capacity of the septa was not exceeded.

Cross-linked versus Non-cross-linked Polymer. Daterman (1974) reported an equation describing the loss of *E* 9–12: Ac from polyvinyl chloride (PVC) (a non-cross-linked polymer): $\ln y = 4.1 - 0.047X$, where y is the evaporation rate in ng/min and X is time in days. The equation for a first-order loss is $\ln kM = \ln kM_o - kt$ (McDonough, 1978) where kM is the rate of evaporation at time t, and kM_o is the initial rate of evaporation ($k = t_{\frac{1}{2}}^{-1} \ln 2$). Therefore, Daterman's equation demonstrates a first-order loss with $k = 0.047$, and $t_{\frac{1}{2}} = 14.7$ days. In contrast, in rubber septa (Table 1) we found that $t_{\frac{1}{2}}$ for *E* 9–12: Ac is 38.4 days. This difference in $t_{\frac{1}{2}}$ may be a result of cross-linking in rubber, although perhaps part of the difference may be a result of a difference in size between the PVC pellet and the rubber septum (Lloyd and Mathysse, 1966; McDonough, 1978). Because the cross-linking effect on retarding evaporation of such pheromones as 11–14: Ac and 11–16: Ac is more substantial than for *E* 9–12: Ac, the difference in $t_{\frac{1}{2}}$ in rubber and PVC should be more pronounced for such compounds. For insects which require relatively high release rates of pheromones, such compounds might be better formulated in PVC or other non-cross-linked polymer, because a lower dosage would be required to achieve the desired release rate.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS: X. Laboratory Studies on Manipulation of Host-Finding Behavior of *Trichogramma pretiosum* Riley[1] with a Kairomone Extracted from *Heliothis zea* (Boddie) Moth Scales[2,3]

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Abstract—The behavioral response of *Trichogramma pretiosum* Riley females to the kairomone found in *Heliothis zea* (Boddie) moth scales is examined.

Key Words—Kairomone, parasitoids, biological control, *Trichogramma pretiosum*, Hymenoptera, Trichogramma tidae, *Heliothis zea*, Lepidoptera, Noeturidae.

INTRODUCTION

Interest in *Trichogramma* spp. as biological control agents has a long history. As with most parasitoids, however, the host selection behavior is largely unknown. Salt (1935, 1937) studied the interactions of *Trichogramma evanescens* Westwood and its hosts, but all of his work dealt with the interaction after contact with the host. Laing (1937), however, investigated the

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process of host finding, the stage of host selection occurring prior to host contact, by *T. evanescens*. She found that visual cues are important at distances of less than 2–3 cm and also demonstrated that an odor (kairomone) left by the adult moths was a critical factor in host finding and that the host egg itself was a stimulus for an intensified search pattern. Lewis et al. (1971) demonstrated that moth scales were the source of kairomone(s) used by *T. evanescens* to detect the presence of hosts. Lewis et al. (1972, 1975a,b, 1979) and Gross et al. (1975) demonstrated that kairomones could be used in a variety of ways to increase rates of parasitization by *Trichogramma* spp. in the field and greenhouse. That the kairomone(s) elicited a more intensified search pattern, but did not act as an attractant for *Trichogramma pretiosum* Riley has also been demonstrated (Lewis et al., 1975b). Lewis et al. (1979) demonstrated that it is not only the presence or absence of the kairomone(s) that is critical to increased parasitization, but also its distribution between oviposition sites. Still, the actual behavioral changes producing these increases in parasitization had not been investigated.

In order to fully understand the role of these kairomones in the host-finding behavior of the parasitoids and to develop techniques for their use, the behaviors which they influence must be determined. The purpose of this study was to examine the behavioral changes that kairomones elicited in *Trichogramma* and define their role in the overall host-finding sequence.

METHODS AND MATERIALS

The *Trichogramma* used in this study were *T. pretiosum* and of the same genotype as used in the previous papers in this series. The parasitoids were less than 24 hr old when taken from the rearing chamber. The sex was determined and females were placed in 2-dr shell vials and stored at ca. 18° C. They were used within the next 24 hr. All experiments were performed in the laboratory under overhead fluorescent lights at ca. 22° C.

The *Heliothis zea* (Boddie) eggs used in these studies were obtained from laboratory cultures, processed with a sodium hypochlorite wash (Burton, 1969), irradiated with 25 krad (^{60}Co source) when 8–36 hr old, and stored at ca. 10° C. They were used in experiments within the next 24 hr. The host eggs were applied to the substrate for the various experiments with a camel's hair brush moistened with water, saliva, or Plantgard® (Polymetrics Intl., New York, New York) (Nordlund et al., 1974). The method of Lewis and Redlinger (1969) was used to determine percentage of parasitization.

In some of the experiments, Anakrom C-22 (Analabs, Inc., North Haven, Connecticut) diatomaceous earth particles (40/50 mesh) impregnated with moth scale extract (Jones et al., 1973) were used to simulate moth scales. These particles were prepared according to the techniques described by Lewis

et al. (1979). Untreated particles were used as controls. Both types of particles were attached to the substrate with an artist's brush moistened with water. For treatments in other experiments, a standard 1/1000 dilution of the moth-scale extract (MSE) was used. A syringe or a chromatographic sprayer was used to apply the MSE to the surfaces.

The paired or unpaired *t*-test was used for statistical analyses, unless otherwise noted. Arcsin transformations were made on all percentages prior to analysis.

RESULTS

Petri-Dish Studies. Experiments demonstrating increased rates of parasitization caused by kairomones have been performed by Lewis et al. (1970, 1972, 1975b) in 150 × 15-mm Petri dishes. This size Petri dish was chosen as the experimental environment in which to begin these behavioral studies.

The bottoms of the dishes were lined with Whatman No. 1 filter paper. Treatments consisted of spraying the filter paper with 0.25–0.50 ml of MSE [using a chromatographic sprayer (Lewis et al., 1972)], unless otherwise noted. Various activities of the parasitoid were timed. Tracings of the parasitoid's movements were made onto a sheet of acetate overlaying the Petri dish. Measurements of the tracings of the parasitoid's path were made with a map mileage measurer. Speed was calculated by dividing the distance traveled by time.

To identify the types of behavior influenced by the kairomone, one *T. pretiosum* female was released into a Petri dish containing 5 eggs, equally

TABLE 1. TIME SPENT IN VARIOUS HOST-FINDING ACTIVITIES BY FEMALE *T. pretiosum* IN KAIROMONE-TREATED VS. CONTROL PETRI DISHES

Activity	Mean time (sec) ± ($s_{\bar{x}}$)[a]	
	Treated	Control
Time before first egg contact	300.6 (±113.1)a $N = 10$	1008.3 (±182.2)b $N = 10$
Total time moving	782.2 (±11.6)a $N = 14$	1079.8 (±155.4)a $N = 13$
Average time between eggs	279.2 (±39.2)a $N = 19$	312.5 (±59.9)a $N = 10$
Average time spent on eggs	348.0 (±35.1)a $N = 19$	523.4 (±157.0)a $N = 10$

[a]Treated and control figures followed by different letters are significantly different ($P < 0.05$) as determined by the unpaired *t* test.

spaced on a treated or control filter-paper liner, and observed for 30 min. The proportion of the 30-min period spent in each of the various activities was recorded. The results of this test are shown in Table 1. Although the efficiency of *Trichogramma* in the presence of kairomone(s) appeared to improve for all activities measured, the only period of activity in which a significant response ($P < 0.05$) occurred was that period before contact with the first egg.

To further investigate this, the accumulated time spent by *T. pretiosum* on treated vs. control filter-paper liners was measured for a 15-min test period in the absence of eggs. The results (Table 2) show that the amount of time before reaching the edge of the liner and the total time spent on the liner was significantly greater ($P < 0.05$) in the treated dishes. It is apparent that the presence of the kairomone(s) on the substrate caused the parasitoid to spend more time on that substrate. Tracings of the movements of parasitoids on acetate sheets overlaying Petri dishes during 15-min periods were also made. The distance the insects traveled on the sides of the dish was discounted because the difference between the treated and controls was considered to be insignificant. The distances traveled on the top and bottom of the dish were recorded separately. Although there was no difference in the total distance traveled on top and bottom of the dishes, the parasitoids covered a greater distance on the bottom of the treated dishes than in the control dishes ($N = 10$, $P < 0.05$).

One possible cause for the retention of the parasitoid on the treated surface is an overriding or positive phototaxis and/or negative geotaxis. To test this, two experiments were performed in Petri dishes in which parasitization was measured. In both cases, five eggs were attached to both the top and bottom of unlined Petri dishes with Plantgard. In the first experiment, only the bottoms of the treated dishes were sprayed with MSE (1 ml/dish) using a chromatographic sprayer. Control dishes received no treatment. Two *T. pretiosum* females were placed in each dish for 1 hr. In the second test, both tops and bottoms of the treated dishes were sprayed with MSE (1 ml/surface).

TABLE 2. PROPORTION OF A 15-MIN PERIOD SPENT BY FEMALE *T. pretiosum* ON FILTER-PAPER LINER OF A PETRI DISH[a]

Filter paper surface	Time (sec) spent on filter paper ($\pm s_{\bar{x}}$)	Time (sec) before reaching edge of filter paper ($\pm s_{\bar{x}}$)
Treated	478.1 (±50.6)a	118.2 (±17.8)a
Control	228.8 (±28.8)b	71.9 (±24.0)b

[a] Means of 10 replications. Control vs. treated entries followed by different letters are significantly different ($P < 0.05$) as determined by the unpaired t test.

TABLE 3. PERCENTAGE PARASITIZATION OF *H. zea* EGGS BY FEMALE *T. pretiosum* ON TOP VS. BOTTOM OF PETRI DISHES USING 2 PATTERNS OF KAIROMONE TREATMENT

Treatment location	Parasitization (%) and ratio (top/bottom)[a]			
	Treated	Ratio	Control	Ratio
Top and bottom[b]	64.75/60.70	1.07a	55.69/34.75	1.60b
Bottom only[c]	54.33/73.33	0.74a	71.42/49.67	1.43b

[a] Treated and control entries followed by different letters are significantly different ($P < 0.05$) as determined by the paired t test.
[b] Means of 80 replications.
[c] Means of 60 replications.

The eggs from the top and bottom of each dish were collected separately and dissected to determine percentage of parasitization. In both experiments, the top–bottom (T/B) ratio of parasitization is higher in untreated dishes (Table 3), indicating a preference for the top in these cases. In the dishes in which both the top and bottom were treated, the T/B ratio is approximately 1, indicating no preference. When the treatment was placed on the bottom of the dishes, a preference for the bottom of the dish was noted. These results indicate that kairomones cause *T. pretiosum* females to parasitize more eggs on treated surfaces within the Petri dish. This effect counters the tendency seen in females in control dishes to parasitize eggs on the upper surfaces (highest or nearest light) at a greater frequency.

These results indicate that the increased rates of parasitization in earlier Petri-dish studies (Lewis et al., 1975) were probably due to increased retention of the parasitoids in the bottom of the dish, which reduced the time prior to contact with the first egg and prevented the functional loss of the parasitoid into a wandering pattern on the top of the dish.

Additional tracings were made of the parasitoid's movements after being released onto a 3.8-cm treated spot on a filter-paper liner within a Petri dish. These tracings indicated a pattern of movement in which the parasitoid concentrated its search around the treated spot and returned to it over and over again. This behavior was not evident when the entire bottom surface was treated.

To further examine this, a basic study of the reaction of *T. pretiosum* females to the kairomone was undertaken. In an open Petri dish lined with filter paper, the response of the parasitoid to a 2.5-cm-diameter spot of MSE was studied. Based on over 50 uncontrolled observations, it was noted that females contacting a kairomone-treated surface usually halted their forward movement within 2–5 mm of the point of contact. The insect appeared to remain immobile for 5–20 sec, after which it began to move with further

intermittent halts of similar duration. This type of behavior gradually changed to wider ranging movements with fewer halts.

In an attempt to study this behavior more closely, the responses of 20 individual parasitoids to 2.5-cm spots of moth scale extract placed on filter paper in an open Petri dish were observed through a dissecting microscope. In 17 of the 20 observations, the immediate response to the treated area was a close investigation of the substrate with the antennae. The body was sloped forward, and the insect remained stationary or moved very slowly. This initial investigation lasted 5–20 sec, during which the antennae were extended straight forward and held closer together than normal (Figure 1). The movement of the insect was broken by halts which were separated by longer and longer periods of movement. Eventually, the parasitoid left the treated surface. In 13 of the 20 observations, the insect returned. However, in other experiments when larger (7.6 cm) spots of MSE were used, return to the treated area was much more common. This was observed in over 50 cases. In at least 30 other uncontrolled observations of parasitoids in contact with small, capillary tube-applied dots of kairomone, return was quite rare. It appeared that the larger the area, the greater the likelihood of return.

Typing-Paper Environment. In view of indications that the Petri dish was too small for the parasitoid to fully express its behavioral response to the

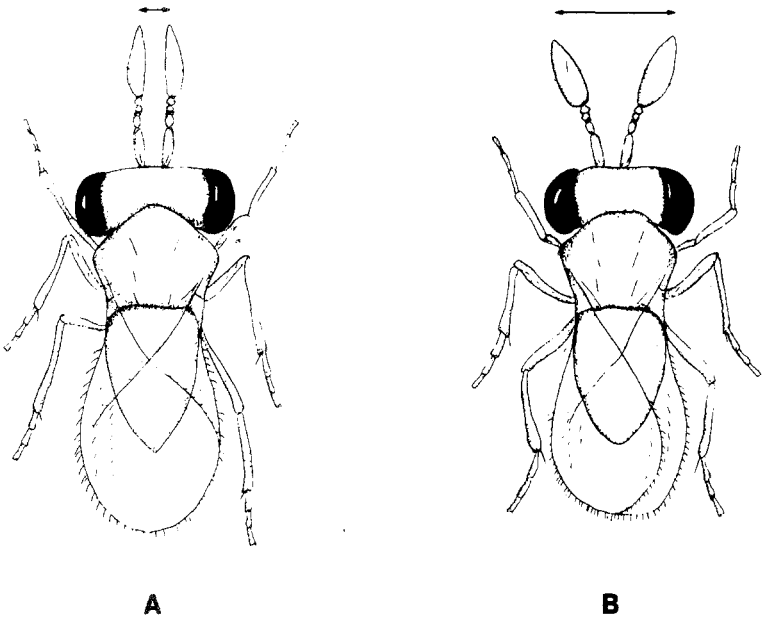


FIG. 1. *Trichogramma pretiosum* female responding to kairomone-treated substrate (A) in a normal position (B).

TABLE 4. ACTIVITIES OF FEMALE *T. pretiosum* ON AND AROUND 2 CONCENTRIC CIRCLES (7.62 AND 15.26 CM); INNER CIRCLE TREATED OR CONTROL AS INDICATED[a]

Measurement	Time (sec) or number of visits ($\pm s_{\bar{x}}$)[b]	
	Treated	Control
Time to leave inner circle	67.2 (±14.3)a	33.2 (±9.8)b
Time to leave outer circle	230.0 (±34.0)a	44.7 (±10.0)b
Number of returns to inner circle	9.7 (±2.0)a	0.2 (±0.2)b
Total time spent in inner circle	157.6 (±23.8)a	33.2 (±9.8)b

[a] Means from 12 replications.
[b] Treated and control entries followed by different letters are significantly different ($P < 0.05$) as determined by the unpaired t test.

kairomone, a larger experimental environment was needed where movement was unrestricted. This was provided by using 8 × 10.5-in. bond typing paper on a laboratory countertop from which escape was possible. The test pattern consisted of two concentric circles of 7.6 cm and 15.3 cm in diameter. In the treated universe, the inner circle was treated with 0.5 ml MSE. A parasitoid was released into the center of the inner circle after which the times required for the parasitoid to leave the inner circle and then the outer circle were recorded. The number of returns to the inner circle and the total time spent in the inner circle were also recorded. The results are given in Table 4.

In addition to the data presented in Table 4, five of the 12 control insects took flight prior to reaching the outer circle, while none of the parasitoids released onto treated surfaces flew prior to reaching the outer circle. This inhibition of flight is considered to be a major factor in the mode of kairomone action. The importance of this behavioral modification via the use of kairomones has also been demonstrated by Gross et al. (1975).

Expanded Laboratory Universe. To study the effects of the kairomone in an environment more like the field, a large sheet of white butcher paper, 117.5 × 30.4 cm in size, was used as a substrate. Three host eggs were placed on the sheet and 10 parasitoids released as described by Lewis et al. (1978). Three paired comparisons of two treatment patterns applied to the arenas were conducted at one time. The total number of parasitoids remaining on three butcher paper sheets constituted one replication for each treatment. Counts were made at 0.5–1.5, 5, 10, 15, and 30 min.

Five different treatment patterns were tested: (1) control, only eggs applied to the sheet; (2) solid spray, each sheet was sprayed with ca. 25 ml of MSE and eggs applied; (3) spot, a 7.6-cm spot, centered on each egg site, treated with 0.25 ml of MSE (with a pipette) and eggs applied; (4) spot + treated particles, spot treatment with the addition of 144 treated

particles evenly distributed (ca. 5.5-cm spacing) over the sheet and eggs applied; and (5) spot + control particles, same treatment as (4) but with untreated particles.

The spot treatment and the spot + control particles were considered to be equivalent.

The following comparisons were made: (I) solid vs. control; (II) solid vs. spot; (III) spot + treated particles vs. spot + control particles; and (IV) solid vs. spot + treated particles.

The results of these tests are given in Figure 2. The readings at most time intervals were significantly different ($P < 0.05$) in each comparison. There

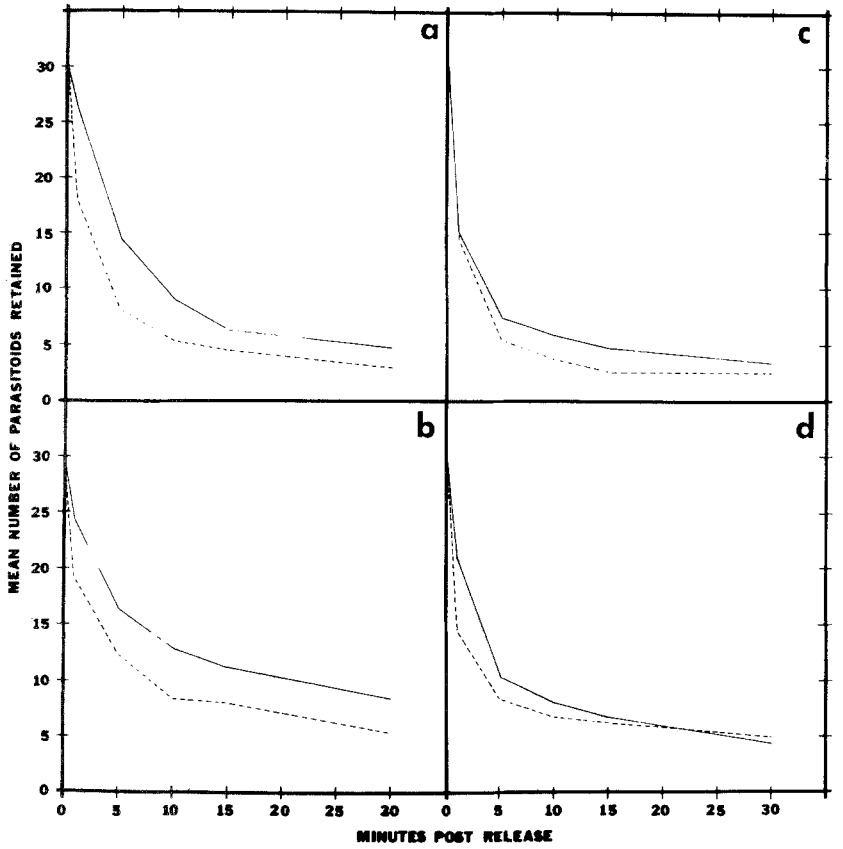


FIG. 2. Mean number of parasitoids remaining on substrate. (a) Solid treated (——) vs. control (– – –) substrates. (b) Solid treated (——) vs. spot treated (– – –) substrates. (c) Spot + impregnated particle treated (——) vs. spot + untreated particle treated (– – –) substrates. (d) Solid treated (——) vs. spot + impregnated particle treated (– – –) substrates.

were two important exceptions to this, however. The 30-min reading for parasitoids on solid vs. spot + impregnated particle in comparison IV (Figure 2d) was not significantly different. This indicates the possibility that retention after the initial departure of some parasites may be no stronger on solid treatment than it is on spot + treated particles. The second exception involves comparison III (Figure 2c) in which control and treated particle treatments are shown to have the same number of insects present at the initial reading. This can be explained by the fact that at this time the insects in the treated particle treatment had not yet contacted a kairomone source and thus were responding as if none was present.

During the comparison II (Figure 2b) experiment, the number of parasitoids within the oviposition sites (7.6-cm-diameter circles around each egg) was recorded at each time interval. There were 24 replications of this experiment with each individual universe constituting one replication. The mean of 0.8 females in the oviposition sites under the 30-min test period on the spot-treated universes was significantly higher ($P < 0.01$, 2-way ANOV) than the mean of 0.2 on the solid-treated universes. Using the same laboratory universe, Lewis et al. (1979) demonstrated higher rates of parasitization on spot than solid treatment. The present experiment reveals that factors other than retention are important in the increased rates of parasitization observed in the spot-treated universe as opposed to solid-treated universe. These factors are collectively referred to as efficiency of parasitoid movement.

Further Examinations of Behavior. Microscopic examinations of the response of *T. pretiosum* females to treated particles, small spots of MSE (applied with a capillary tube) on the filter paper, or actual moth scales revealed no differences in the basic behavioral response. The response to moth scales appeared to be slightly more intense (visual and tactile influences possibly operating), and parasitoids would occasionally pass over extremely small spots of MSE (applied with a capillary tube) with no response.

For macroscopic observations, a different experimental environment was used. This consisted of a 61.0-cm-square sheet of butcher paper with a plate of glass supported 1.9 cm above. Simulated oviposition sites consisted of 7.6-cm-diameter circular areas treated with MSE with an *H. zea* egg placed in the center. Four such sites were placed symmetrically 22.4 cm from a central release point. Treatment consisted of control particles or treated particles distributed between the simulated oviposition sites. The particles were distributed in a grid pattern 3.6 cm apart. Movements of individual parasitoids were traced and time from time of contact with the first particle. Along the recorded path of movement, 30-sec time intervals were marked. The total distance traveled within an arbitrary 7.6-cm-diameter circle around the first particle contacted was measured. The results of these studies are given in Table 5.

TABLE 5. DISTANCE TRAVELED/TIME, VECTOR DISTANCE/TIME, LENGTH OF TRAVEL, DISTANCE TRAVELED/AREA AFTER CONTACT AND DEPARTURE FROM A PARTICLE BY *T. pretiosum*

Particle type	$\bar{x}$ distance in cm ($\pm s_{\bar{x}}$)[a]		
	Traveled distance (30 sec)	Vector distance (30 sec)	Traveled within 3.81-cm. radius of particle
Treated	12.22 (±1.44)a $N = 14$	3.38 (±0.32)a $N = 32$	11.09 (±1.11)a $N = 38$
Control	11.29 (±1.22)a $N = 32$	8.34 (±1.17)b $N = 16$	3.46 (±0.36)b $N = 27$

[a] Treated and control entries followed by different letters are significantly different ($P < 0.05$) as determined by the t test.

A similar experiment compared control surfaces to solid-treated surfaces. Measurements were made from the time the parasitoid was released onto the test surface. The results are given in Table 6.

DISCUSSION

Although the Petri-dish environment was too small to allow the complete expression of kairomone-induced behavior, it did serve to demonstrate several features of the parasitoid's response. Retention of the parasitoid in the target area is important to the mode of action of the kairomone, as it apparently overrides positive phototaxis and/or negative geotaxis. In tests with host eggs present, the kairomone reduced the amount of time before contact with the first egg. The time between eggs, however, was not reduced by the kairomone. This is probably attributable to the intensified search pattern created by the egg itself (Laing, 1937), which apparently at least equals the stimulation provided by the kairomone within the Petri-dish environment.

Observations of the parasitoid's behavior reveal the following characteristic responses to a treated surface: (1) arrested forward movement, and antennal examination of the substrate (5–20 sec); (2) locomotion (5–20 sec); (3) antennal and leg cleaning and/or further close antennal examination of the substrate (3–30 sec); (4) further movement punctuated with halts with further antennal examination of the substrate; (5) departure and subsequent return to the treated substrate; and (6) a series of departures and returns (time spent in stationary examination, after the first and second departures, diminishes with each return).

TABLE 6. DISTANCE TRAVELED AND VECTOR DISTANCE TRAVELED BY *T. pretiosum* FEMALES FROM POINT OF PLACEMENT ON SURFACE IN SPECIFIC TIME PERIODS

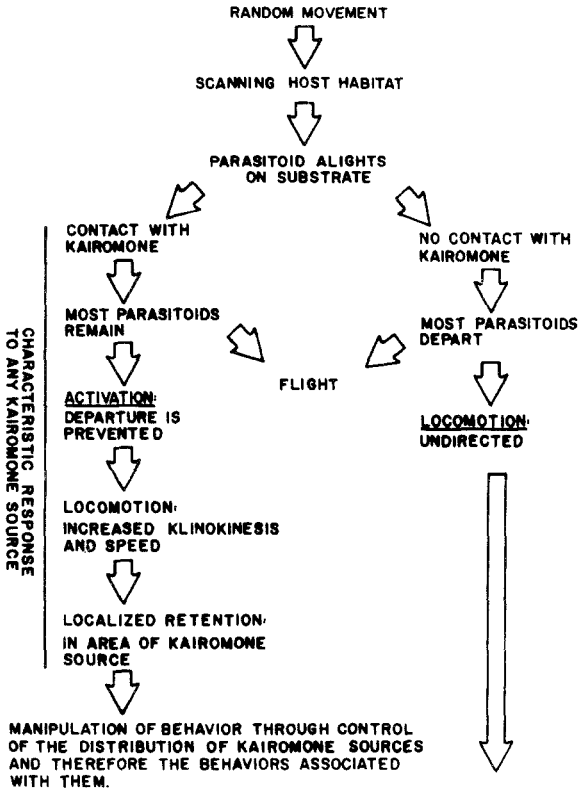
Treatment type	Distance in cm ($\pm s_{\bar{x}}$)[a]				
	Distance traveled			Vector distance traveled	
	0–30 sec	30–60 sec	60–90 sec	30 sec	60 sec
Solid treated	5.95 (±0.60)a ($N = 12$)	15.33 (±1.37)a ($N = 12$)	16.65 (±0.96)a ($N = 12$)	3.23 (±0.55)a ($N = 12$)	5.37 (±1.10)a ($N = 12$)
Untreated	11.79 (±1.10)b ($N = 9$)	10.67 (±1.65)b ($N = 9$)	9.09 (±2.81)b ($N = 5$)	9.56 (±1.55)b ($N = 9$)	14.47 (±2.83)b ($N = 9$)

[a]Treated and untreated entries followed by different letters are significantly different ($P < 0.05$) as determined by the t test.

This type of behavior, which is similar to that of *Venturia* (=*Nemeritis*) *canescens* (Gravenhorst) as reported by Waage (1978), clearly contributes to the retention of the parasitoid, and it is this retention that is primarily responsible for the increased parasitism seen in previous Petri-dish experiments (Lewis 1971, 1972, and 1975a). Lewis et al. (1979), however, demonstrated that spot treatment produced higher rates of parasitization than solid treatment in the expanded laboratory universe, as well as in the greenhouse and the field. Using the same laboratory universe, data from Table 5, and Figures 2b and 2d indicate that, although larger numbers of parasitoids are retained on the solid treatment, their efficiency of movement to oviposition sites is reduced in comparision to the spot treatment and spot + particle treatment. This indicates that there is more than retention alone involved in increasing parasitization in large areas.

The characteristic behavioral responses, inhibition of flight and induced klinokinesis, appear to be the main features of kairomone-induced behavior that are subject to manipulation. It is not the intensity of these behavioral mechanisms that is important, but rather where and how frequently their expression occurs. The behavior of the parasitoid upon contact with molecules of kairomone is the same regardless of dosage (Lewis, unpublished data), but the alteration of the distribution of kairomone sources can alter the host-seeking behavior. Retention is slightly stronger on solid-treated surfaces than when the surface is treated with simulated moth scales (Figure 2d), but treatment with simulated moth scales apparently results in a more efficient search pattern (Lewis et al., 1979). This leads to the conclusion that it is not only the presence or absence of the kairomone that is critical to increased parasitism, but also its distribution. When the frequency of the kairomonal contacts is controlled, as with simulated moth scales, it is possible to change the overall performance of *Trichogramma* in a large area. However, the localized response to each kairomone source is not changed.

A generalized scheme comparing the responses of a parasitoid to selected distributions of a kairomone vs. no kairomone is given in Figure 3. Most of the parasitoids that do not contact the kairomone depart immediately. A great majority of those that do contact the kairomone are activated by it and perform the characteristic investigation of the area. Locomotion with increased klinokinesis (and speed in the case of solid treatment) results in localized retention in the area of the kairomone source. At this point, the characteristic behavioral response to kairomones is complete. It is only through manipulation of the distribution of kairomone sources that retention and efficiency can be improved. Solid treatment causes retention but highly inefficient movement between oviposition sites. Spot + treated particles cause less retention with more efficient movement between oviposition sites. Spot treatment alone causes still less retention with more efficient movement. Control produces the least retention, but the few parasitoids that remain have a rapid straight-line



	SOLID TREATMENT	SPOT + PARTICLES	SPOT	CONTROL
OVERALL RETENTION	HIGH	ADEQUATE	LOW	VERY LOW
EFFICIENCY OF PARASITOID MOVEMENT	VERY LOW	ADEQUATE	HIGH	HIGH BUT UNDIRECTED

FIG. 3. Generalized scheme showing how the behavioral response of *Trichogramma pretiosum* females is altered by several types of kairomone distribution.

movement pattern which is highly efficient. This movement, however, is undirected and, on an untreated surface, the chances of a parasitoid contacting an egg are relatively small.

For optimal parasitization, the desired effect is a balance of retention and efficient movement. To attain this, the frequency of contacts with kairomones and the resultant display of klinokinesis must be reduced below the levels seen when the parasitoid is in continuous contact with the kairomone, as in the case of the solid treatment. At the same time, the insect must receive more stimulus than spot treatment. Particle treatment provides this effect by reducing the number of times the characteristic behavioral response is exhibited. The result

over a large area is that the parasitoid contacts the stimuli at a sufficient frequency to retain it in the habitat while permitting it to search in a pattern so that it can concentrate its search in high-probability locations.

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Journal of Chemical Ecology, Vol. 7, No. 3, 1981

Letter to the Editor

HOMEOCHEMICS? PLEASE RECONSIDER!

Irwin Martin (1980) has proposed a new term, "homeochemic," as a way of rescuing a usefully limited definition of the word "pheromone." I grant the current disarray, but two considerations give me pause. First, it seems likely that "pheromone" is already so broadly applied that an additional, overlapping term comes too late to be very helpful. Second, and more emphatically, I am afraid that a word as labored as "homeochemic" will not soon find many enthusiastic adherents.

Professor Martin has certainly pointed out a problem in current terminology. However, I urge him to retract "homeochemic" and coin us a word that will roll more easily off our tongues and ring more euphoniously in our ears!

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REFERENCE

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Book Review

Chemical Signals: Vertebrates and Aquatic Invertebrates. D. Müller-Schwarze and R. M. Silverstein (eds.). New York: Plenum Press, 1980, 405 pp., illus., $42.00.

This book presents the papers of an international symposium on chemical signals held at the State University of New York at Syracuse in the summer of 1979 and follows an earlier publication also by Plenum entitled *Chemical Signals in Vertebrates* which appeared in 1977 as a consequence of a similar gathering of the great and the good the previous year.

In spite of its title, this volume principally concerns terrestrial vertebrates. There is just one full paper (plus a few abstracts) on aquatic invertebrates. This is from Morse et al. and concerns research on contact-dependent chemical signals of algal origin which induce planktonic abalone larvae to settle and undergo metamorphosis. Interesting as this is, it does seem to be something of an aquatic invertebrate out of water in the present text.

On the vertebrate front there are some valuable contributions. There is, for example, a group of papers on priming pheromones. Here Vandenbergh provides a nice overview of how chemical signals influence the onset of puberty in both females and males, and Bronson and Coquelin discuss a conceptual framework of a single cueing system to take account of the various mouse priming pheromone effects. Chemical studies reported by Novotny et al. on the identity of male urine components which accelerate puberty in female mice have yet to reach a breakthrough point. Activity is associated with peptide (?) fractions of mass less than 1000, yet in spite of the rigorous purification processes employed, active fractions still retain a mouse odor detectable by the human nose. Headspace analysis of urine volatiles is also leading to further identifications and to the plotting of differences in profile associated with sex and endocrine status. The 2-alkyl-4,5-dihydro-1,3-thiazoles previously identified in, and unique to, male mouse urine are without puberty-accelerating activity.

There is also a fascinating account from Yamazaki et al. of studies with genetically uniform inbred strains of mice in which they find that mice can detect genetic differences by olfaction and that this affects mating preferences. The odor-determining genes studied in this work are those of the major

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histocompatibility complex (H-2) which also plays an important role in regulating immune responses.

It is good to see the volume contains a section on field studies, for laboratory studies can never be wholly adequate. Stoddart starts off with some perplexing thoughts on predator odors and describes how it seems that the woodmouse is rashly prone to ignore the odor of weasel, but not so the short-tailed vole—clearly a good source of children's stories if research funds give out—and is followed by one of a number of contributions on red fox odor. Here Henry reports how he pursued foxes through the snows of Saskatchewan noting in detective fashion evidence of urine marking. Of the various parameters he studied, only one stands out as showing a significant change over the breeding season. This is urine odor. The intensity of the urine mark of either sex increased sharply with the start of the breeding season and remained elevated for three weeks before diminishing. Wilson et al., reporting on the responses of wild fox to synthetic mixtures of compounds previously identified in fox urine headspace volatiles, show that the organosulfur volatiles, isopent-3-enyl methyl sulfide and 2-phenylethyl methyl sulfide, are particularly important in eliciting fox overmarking, no such response being associated with synthetic mixtures lacking these substances. However, Bailey et al., in the most significant chemical study reported here, have monitored seasonal changes in the profiles of fox urine headspace volatiles and note in male urine a massive increase during the breeding season, not of these compounds, but of a component which they identified as the closely related 3-methylbutyl methyl sulfide. Clearly these studies indicate one area at least where chemists and biologists are pointing in the same direction.

There is, of course, much else of interest in this tome. There is a substantial section on the vomeronasal organ, with contributions on reptiles (Burghardt), hamster (Meredith), and guinea pig (Beauchamp et al.). In the latter paper the communicatory possibilities of involatile urine components are discussed, and it is shown that involatile, fluorescent rhodamine hydrochloride added to urine and presented to these animals quite clearly reaches their vomeronasal organs. Finally Johns summarizes experiments implicating the vomeronasal organ in the reception of rodent priming pheromones.

Elsewhere in the volume, Müller-Schwarze reviews his studies on deer alarm substances (no chemistry unfortunately) and we learn about chemical signaling in the vicuna (Franklin) and the camel (Wemmer and Murtaugh), and of course Epple talks interestingly about tamarins. Leon discusses the development of olfactory mother attraction in young rats, while Nyby and Whitney demonstrate just how much of the male mouse behavioral response to female odors is learned, and Rudy and Cheatle consider odor aversion learning in neonatal rats. There are also three papers on food responses, on the chemosensory searching of rattlesnakes for rodents (Chiszar and Scudder), on

"what the nose learns from the mouth" (Garcia and Rusiniak) and on the effects of preweaning taste and odor experience on a young rat's food consumption (Bronstein and Crockett).

Apart from the papers already listed there is just one further chemical study. This is by Singer et al. and concerns the pheromonal components of hamster vaginal secretion. Apart from the potent male attractancy associated in large measure with the volatile dimethyl disulfide component (5 ng/female), they report a separate "mounting pheromone" activity which stimulates copulation. The active substances here lack volatility and are associated in some way with high-molecular-weight protein fractions, although lower-molecular-weight substances may also be involved. In this and other papers in this volume one notes a new and developing awareness of the potential communicatory significance of high-molecular-weight or poorly volatile substances.

These 24 full papers are capped with a number of tantalizingly brief abstracts. The value of the condensed paper is very considerable, but perhaps abstracts are not the answer. The elegance of lucid brevity is greatly to preferred to the tedium of unbalanced, excessive detail and to the weight of papers burdened with sections of text which are little more than repeat presentations of other publications, unfortunately all too common a feature of much modern scientific literature for reasons we all understand. And let us note in passing that the present volume could have benefited had the editors been rather tougher in these regards with some of the contributors. However, it would have been good to read just a little more, for example, of Anna Marchlewska-Koj's thoughts on mouse pregnancy block pheromone peptides, or of the ideas of Bubenik et al. on chemical communication in the moose than these abstracts actually provide.

In all, an interesting collection. We see vertebrate semiochemistry as a new synthesis of old disciplines. This is its opportunity and its challenge. The opportunity is clear, for only together can we advance into new and unexplored fields. Have not biologists been undisturbed long enough in their awareness of the central importance of chemical signals without having the first notion of what these chemicals are? And have not chemists been locked away for too long in their smelly laboratories pursuing their structural minutiae happy to forget the complexities of the natural world from which the materials they manipulate have arisen? Of course, we thought these thoughts long ago, and it would be expected that we would do things differently now. Did not the need for interdisciplinarity emerge loud from the first Syracuse symposium? Was it not echoed in the findings of the 1978 NATO Advanced Research Institute on Chemical Ecology? But what is the situation today? What does the present symposium show?

The challenge we face is that the necessary synthesis is not easy. Resistance to integration in any depth is both personal and institutional.

Experience suggests that too often the profound divide occurs between those whose world is organisms and those whose world is molecules, at the biologist–chemist interface. In a paragraph headed "lack of serious interest in mammalian semiochemicals among chemists," Mykytowycz only begins to touch the tip of the problem when he writes in connection with the NATO Institute,

> I suspect that many efforts to cooperate broke down . . . because of the difficulty in communication. Often the specialists expect too much of one another. The chemist on the one hand hopes that on the laboratory bench mammals will behave like automata . . . and the biologist believes that modern techniques and instruments make the routine analysis of odors a comparatively easy task.

The gulf clearly remains. I am frankly dismayed to see how little chemistry, with its deep insights and its immense technical power, is represented in this symposium, and in vertebrate semiochemical studies generally. Changes must occur if the subject is to take off.

Clearly symposia of the type arranged at Syracuse in 1976 and again in 1979 can have a most important role in integrating our efforts, in taking stock of progress made, in confronting together major issues, in enabling us to listen and to learn as discipline talks to discipline. I am sure the recent symposium was all this for its participants, and I very much hope that the book which has appeared will permit others to share in this process.

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SEX PHEROMONE OF THE SALTMARSH CATERPILLAR MOTH, *Estigmene acrea*[1,2]

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Abstract—Three compounds have been identified as components of the sex pheromone emitted by females of the saltmarsh caterpillar moth, *Estigmene acrea* (Drury). These are (*Z*,*Z*)-9,12-octadecadienal (I), (*Z*,*Z*,*Z*)-9,12,15-octadecatrienal (II), and (*Z*,*Z*)-3,6-*cis*-9,10-epoxyheneicosadiene (III). In female tip extract they were found in a ratio of 1:6:25, respectively, and in trapped female effluvia the ratio was 1:6:27, respectively. Combinations of III with either I or II elicited sustained upwind flight in a wind tunnel, but none of these compounds by themselves did so. There is evidence that the antennal acceptor site for III is chiral.

Key Words—Saltmarsh caterpillar moth, *Estigmene acrea*, (*Z*,*Z*)-9,12-octadecadienal, (*Z*,*Z*,*Z*)-9,12,15-octadecatrienal, (*Z*,*Z*)-3,6-*cis*-9,10-epoxyheneicosadiene, fall webworm moth, *Hyphantria cunea*, insect sex pheromone, *cis*-9,10-epoxyheneicosane, *trans*-9,10-epoxyheneicosane, linolealdehyde, linolenaldehyde, Lepidoptera, Arctiidae.

INTRODUCTION

The saltmarsh caterpillar moth, *Estigmene acrea* (Drury), occurs throughout the North American continent and appears to be unknown elsewhere. The larvae feed on the foliage of a wide variety of garden and field crops and are usually a pest of minor economic importance (Metcalf et al., 1962).

The sex pheromones of only a few species in the Arctiidae family have been reported to date. These include members of the *Holomelina aurantiaca* complex, *Holomelina laeta* (Guerin), and *Isia isabella* (J. E. Smith) (Guerin)

[1]Lepidoptera: Arctiidae.

[2]Supported in part by the Rockefeller Foundation and by National Science Foundation Grants GB-38020 and PCM 78-13241.

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(formerly *Pyrrhactia isabella*), for all of which 2-methylheptadecane has been reported as a pheromone component (Roelofs and Carde, 1971). We initiated an investigation of the sex pheromone system of *E. acrea* because it represented another arctiid species. Initial tests involving electroantennogram (EAG) studies of materials obtained from female tip extracts indicated that 2-methylheptadecane probably was not involved, so a full-scale investigation of the *E. acrea* sex pheromone system was undertaken.

METHODS AND MATERIALS

Solvents were distilled through a 10-plate Oldershaw column. Temperatures are given in °C. Gas chromatography (GC) columns were glass, either 2 mm × 1.8 m, 2 mm × 3.6 m, or 4 mm × 1.8 m, and were packed with one of the following: OV-1 or OV-101 (methyl silicone, 3% on 100–120 mesh Gas-Chrom Q); Hi-Eff (Hi-Eff 8BP, cyclohexanedimethanol succinate, 3% on 100–120 mesh Gas-Chrom Q); XF-1150 (GE XF-1150, 50% cyanoethyl, methyl silicone, 10% on 100–120 mesh Chromosorb W-AW-DMCS), Porapak Q (100–120 mesh) or Tenax (Tenax GC, 60–80 mesh). The carrier gas for GC was nitrogen and hydrogen flame ionization was used. Mass spectra (MS) were determined with either a Perkin-Elmer Hitachi model RMU6 interfaced with an OV-1 GC column, or with a Finnigan 3300 dual quadrupole mass spectometer interfaced with an OV-101 GC column (Cornell University Mass Spectrometry Center). The infrared spectra were recorded using either a Perkin-Elmer model 257 grating infrared spectrophotometer, a Nicolet 7199 Fourier Transform Interferometer® located at the USDA Laboratories in Gainesville, Florida, or a Nicolet Fourier Transform Interferometer® located at the USDA Laboratories in Beltsville, Maryland. Proton magnetic resonance (PMR) spectra were either from a Varian HA 100 or a Varian XL 100 equipped with FT located at the School of Forestry, Syracuse, New York. Ultraviolet (UV) spectra were determined with a Carey model 15 recording spectrophotometer.

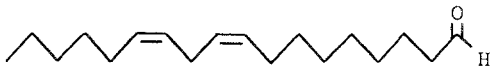
Electroantennograms (EAGs) were determined as described by Roelofs (1977, and references therein). The sustained flight tunnel, or wind tunnel, has been described previously (Miller and Roelofs, 1978) and was modeled after those described by Kennedy (1977) and Farkas et al. (1974).

Insects were reared on a pinto bean diet (Shorey and Hale, 1965). These insects originated from two different sources. One was from a culture previously maintained in Texas (Texas culture), which was used during the first phase of this investigation, and the other was from a single female collected from a blacklight trap in Geneva, New York (Geneva culture); this culture was supplemented occasionally with additional field-collected specimens, and was the one used during the later phase of this investigation, including the wind tunnel bioassays.

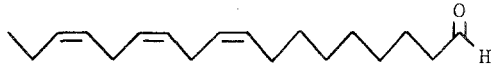
Pupae were segregated according to sex and were allowed to emerge at ca. 20–25° C under a 16:8 light–dark cycle. Females were collected during the first part of their scotophase at 1–3 days posteclosion; each abominal tip was extruded manually, snipped off, and extracted with methylene chloride. Alternately, the females were collected at any time during their light phase, and the glands were pulled out from the insects' abdomens with dissecting forceps. The glands occur as a pair of tubes about 3 mm long by about 0.2 mm OD, with separate openings at either side of the mid-dorsal line on the last abdominal segment. These also were extracted with methylene chloride. Extracts were stored in a freezer. Males were held 1–10 days at 20–25° C under a 16:8 light–dark cycle. They were removed any time during the last half of their scotophase, brought into light (>1 lux), and then bioassayed in the flight tunnel starting about 15 min after removal from scotophase conditions. The bioassay period lasted 30–60 min.

The linoleyl and linolenyl alcohols used for synthesis of the corresponding aldehydes were purchased from NuCheck Corp. (Elysian, Minnesota). Acetaldehyde, propanal, nonanal, and dodecanal were purchased from Aldrich Chemical Co. Pentadecanal and octadecanal were prepared from the corresponding alcohols, which were purchased from Aldrich Chemical Co. Aldehydes were prepared from the corresponding alcohols by treatment with pyridinium chlorochromate (PCC) by the method of Corey and Suggs (1975). The (*Z*)-9-tetradecenyl acetate was purchased from Farchan Chemical Co.

Compound III [Figure 1, (*Z*,*Z*)-3,6-*cis*-9,10-epoxyheneicosadiene] was prepared by B. Kovalev via a route described elsewhere (Hill et al., 1981).



I. (Z,Z)-9,12-octadecadienal



II. (Z,Z,Z)-9,12,15-octadecatrienal

III. (Z,Z)-3,6-cis-9,10-epoxyheneicosadiene

FIG. 1. Structures of I, II, and III.

The two enantiomers of *cis*-7,8-epoxy-2-methyloctadecane (disparlure) were prepared by Dr. K. Mori (Mori et al., 1979).

Microchemical reactions were usually carried out in 1-dram vials having Teflon-lined screw-caps and routinely involved the use of a substantial excess of the reagents involved. Some of these reactions have been described in detail elsewhere, such as ozonolysis (Beroza and Bierl, 1967) and acetylation with acetyl chloride (Hill et al., 1977). Catalytic hydrogenation was with Pd/$CaCO_3$ in methanol or ethanol at 1 atmosphere of H_2 and room temperature. Epoxides were converted to diols by treatment with 0.5% H_2SO_4 in 50% aqueous tetrahydrofuran [THF: freshly distilled form lithium aluminum hydride (LAH)] for 4 hr at room temperature; the product was recovered by dilution with H_2O and extraction with Skellysolve-B (petroleum ether 60–68°) (Skelly B). Reductions with LAH were carried out either in dry Et_2O or in dry, purified THF for 10 min to 1 hr at room temperature; products were recovered by addition of dilute, aqueous NaOH (1–5%) at ice-bath temperature under N_2 and extraction with Skelly B. Hydroxy compounds were converted to the corresponding bromides by reaction for 1 hr or longer at room temperature with triphenylphosphine dibromide (TPPDB), freshly prepared from triphenyl phosphine and bromine in CH_2Cl_2 (Sonnet and Oliver, 1976); products were recovered by evaporation of the solvent in a stream of N_2 and extraction of the product with Skelly B. Hydroxyl groups were trimethylsilated with hexamethyldisilazane (HMDS) and trichlorosilane (TCS) in pyridine (Sweeley et al., 1963); the products were either analyzed directly by GC or were recovered by evaporation in a stream of N_2, addition of water, and extraction with Skelly B.

Synthesis of cis- *and* trans-*9,10-Epoxyheneicosanes*. 1-Decyne (1.7 g, 12.3 mM; Farchan Research Laboratories) in dry dioxane (50 ml) was treated with lithium amide (1.1 g, 48 mM; Alpha Inorganics) for 3.5 hr at reflux, after which it was cooled to room temperature and 1-bromoundecane (6.4 g, 27 mM; Chemical Samples Co.) was added dropwise over a period of ca. 30 min. The mixture was kept at reflux overnight. After cooling, the mixture was diluted with water and extracted twice with Skelly B; the Skelly B layers were combined and washed successively with dilute nitric acid (ca. 1%), water, saturated sodium bicarbonate, and water. Filtration through a magnesium sulfate bed was followed by evaporation of the solvent. The recovered crude product, 9-heneicosyne (6.1 g), was filtered through Florisil (petroleum ether), and the recovered materials were converted to the alkenes without further purification. Reduction with sodium–liquid ammonia (Warthen and Jacobson, 1973) yielded (*E*)-9-heneicosene, which still contained large amounts of the alkyne. The *E* isomers required were purified by GC collection from Hi-Eff. Catalytic reduction with Pd/$CaCO_3$ and quinoline (Green et al., 1967) yielded (*Z*)-9-heneicosene. The epoxides were formed from alkenes using *m*-chloroperbenzoic acid.

RESULTS

Initially, an aliquot (ca. 10 FE) of a crude female tip extract from *E. acrea* was collected in timed fractions from a GC column and the fractions were assayed for EAG activity. Using an OV-101 column, one distinct area of EAG activity was always evident, with a second, earlier, active area sometimes present. This early area was more distinct and reproducibly detected when gland extracts were used instead of whole tip extracts. Female effluvium recovered from Porapak Q also reproducibly showed two distinct areas of activity when collected from OV-101. When each of the two EAG-active materials was recovered and re-collected from a Hi-Eff column, the early OV-101 area of activity resolved into two EAG-active materials, whereas the late OV-101 fraction showed only one EAG-active component that had a longer retention time on Hi-Eff than either of the other two components. These components were designated I, II, and III, in their order of elution from Hi-Eff. Prominent GC peaks coincident with each of these areas of EAG activity were evident in GC tracings of these extracts. Their equivalent carbon numbers were calculated to be the following (using a series of *n*-hydrocarbons as reference standards): on OV-101, 200°, 19.73 for I and II, and 22.35 for III; on Hi-Eff, 200°, 23.6 for I, 24.1 for II, and 25.3 for III. The approximate ratios between the three components were calculated to be as follows: for a whole tip extract 1:6:25, for a gland extract 1:6:10, and for recovered female effluvium 1:6:27.

Identification of I and II. Compound I was identified as (*Z*,*Z*)-9,12-octadecadienal (linolealdehyde, CAS registry No. 2541-61-9), and II was identified as (*Z*,*Z*,*Z*)-9,12,15-octadecatrienal (linolenaldehyde, CAS registry No. 2423-13-4), as described below. These structures are presented in Figure 1. Samples of I and II were purified by collection of the crude pheromone extract from an OV-1 or an OV-101 column followed by re-collection of the EAG-active materials from a Hi-Eff column, which completely resolved the two components.

Catalytic hydrogenation of I and of II yielded indistinguishable products. In both cases the product had the same GC retention time as *n*-octadecanal on OV-101 and on Hi-Eff. The CI-MS (methane) of hydrogenated I, hydrogenated II, and *n*-octadecanal were identical (*m*/*e* 269, MH^+, as the one prominent ion).

CI-MS (isobutane) of I and II showed MH^+ ions at 265 and 263, respectively, indicating that I is diunsaturated and II is triunsaturated. These spectra were very similar to those obtained for linolealdehyde and linolenaldehyde, respectively. The GC retention times observed for I and II on OV-101, Hi-Eff, and XF-1150 were within 0.1 min or less of those for linolealdehyde and linolenaldehyde, respectively.

Samples of I and II were purified and each was subjected to the following sequence of reactions: (1) treatment with LAH in dry Et_2O to produce the alcohols, (2) acetylation with acetyl chloride to produce the acetates, and (3) microozonolysis to produce a compound identified as 9-acetoxynonanal. After each reaction, the products were examined by GC on a nonpolar column and a polar column (6.7 min at 170° and 4.0 min at 170°, respectively) and matched those of the expected products within 0.1 min or less. Prior to the ozonolyses, the acetates from I and II were collected from OV-101. Authentic 9-acetoxynonanal was prepared by ozonolysis of (*Z*)-9-tetradecen-1-yl acetate. The CI-MS (isobutane) of all three ozonolysis products were, for all practical purposes, the same (*m/e* 201 MH^+, with other ions all less than 20% of this). These data support a 9-position double bond in I and II.

Ozonolysis of purified I produced *n*-hexanal, as determined by comparison of the GC retention times of the product with synthetic hexanal on XF-1150, 100° (5.2 and 5.3 min, respectively). These data indicate a 9,12 double-bond system in compound I.

Purified II was also ozonized to produce *n*-propanal, which was identified by its GC retention time on XF-1150 (4.4 min at 50°) compared to that for synthetic *n*-propanal (4.35 min). These data indicate that the end double bonds of the three in compound II are in positions 9 and 15.

A UV scan of II (cyclohexane) showed no evidence of conjugation. The GC retention times of II on the various columns also confirmed the absence of conjugation in II. This leaves only positions 9,12, and 15 for the double-bond system in compound II.

An FT-IR spectrum of II revealed that the double bonds in II are all *Z* double bonds (Figure 2). There was strong carbonyl absorption at 1731 cm^{-1}.

Identification of III as (Z,Z)-3,6-cis-9,10-Epoxyheneicosadiene. Samples of III were purified for the various analyses by collection of the crude female tip extract from OV-1 or OV-101 followed by re-collection from Hi-Eff. In some instances, the collections were preceded by chromatography on Florisil, from which III eluted with ca. 5–10% Et_2O in Skelly B (the effective mixture was dependent on the activity of the Florisil). This was consistent with an epoxide structure.

A mass spectrum (EI, 70 eV) of III showed a molecular ion at *m/e* 306, with an ion at *m/e* 288 (M-18), consistent with an epoxide structure. Hydrogenation of III over $Pd/CaCO_3$ produced a compound with a shorter retention time than III on OV-101 and Hi-Eff (equivalent carbon number of 21.2). The mass spectrum (EI, 70 eV) of hydrogenated III had a molecular ion at 310, and an ion at *m/e* 292 (M-18). This was consistent with the presence of two double bonds in III.

To establish that III is unbranched, hydrogenated III was treated successively with LAH in Et_2O, triphenylphosphine dibromide (TPPDB) in CH_2Cl_2, and then again with LAH. This sequence reductively removed the

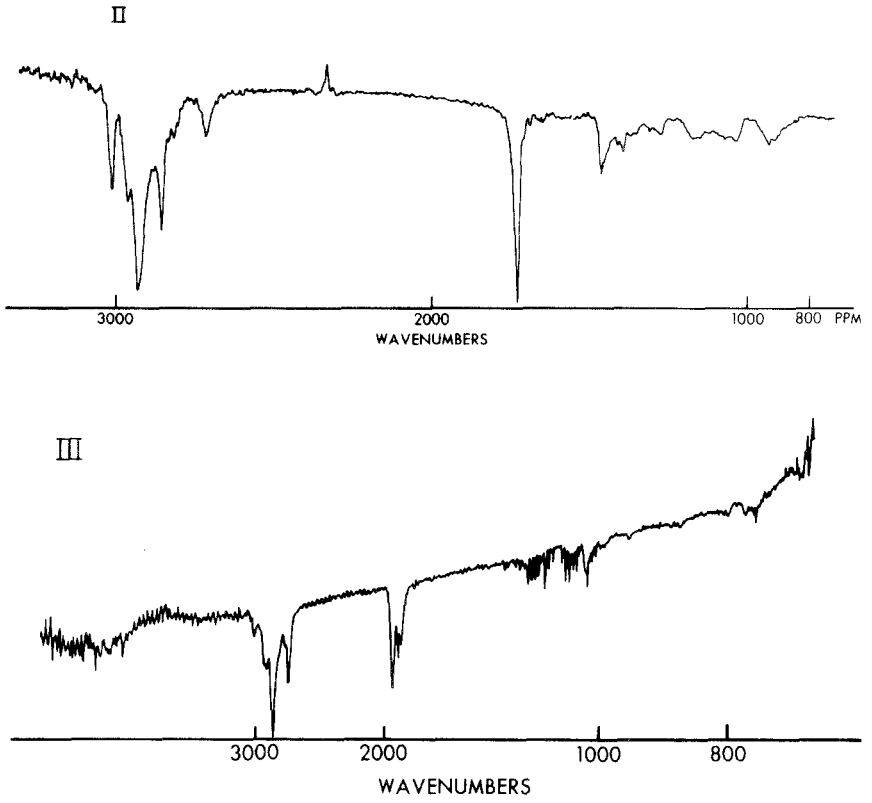


FIG. 2. FT-IR of II and III (as films).

epoxide group and produced a compound with the same GLC retention times on OV-101, 181° (20.7 min), and Hi-Eff, 181° (9.1 min) as *n*-heneicosane. In addition, the mass spectra (E1, 70 eV) of *n*-heneicosane and of treated III were the same (*m/e* 296, M^+, and sequence ions characteristic of an unbranched saturated hydrocarbon chain).

The presence of the epoxide group in III was confirmed, and its location on the 21-carbon chain was established, by the following route: treatment of catalytically hydrogenated III with 0.5% sulfuric acid in 1:1 aqueous THF followed by preparation of the trimethylsilyl derivative using HMDS–TMCS–pyridine. The mass spectrum (E1, 70 eV) of this trimethylsilated diol showed two prominent peaks at *m/e* 215 and 257, corresponding to the fragments produced by cleavage between carbons 9 and 10. This type of fragmentation is well-documented (Capella and Zorzut, 1968; Eglinton et al., 1968).

A sample of catalytically hydrogenated III had the same retention time on Hi-Eff, 170° (25.2 min) as a synthetic sample of *cis*-9,10-epoxyheneicosane.

Synthetic *trans*-9,10-epoxyheneicosane has a different retention time (24.0 min) on this column than the corresponding *cis* isomer (25.2 min).

The location of the double bond closest to the epoxide in III was established by reductive removal of the epoxide group and subsequent ozonolysis. The epoxide group was removed, without alteration of the double bonds, by the following sequence of reactions: (1) treatment of III with LAH in dry Et_2O to produce a secondary alcohol, (2) reaction of this alcohol with freshly prepared TPPDB in benzene to produce the corresponding bromide, and (3) treatment with LAH in dry Et_2O to remove the bromide group. The product was collected from Hi-Eff, 190°, at 3.2–4.25 min (with *n*-heneicosane at 2.9 min). This material had an equivalent carbon number of 20.9 on OV-101, 210°. This purified hydrocarbon was ozonized in CS_2 to produce as the major product a material with retention times on OV-101, 170° (6.5 min), and on XF-1150, 170° (4.25 min), similar to those of synthetic *n*-pentadecanal (6.4 min and 4.35 min on the two columns, respectively).

The position of the other double bond was determined by ozonolysis of untreated III in CS_2, which yielded a product with a similar retention time on XF-1150 (4.4 min at 50°) as propionaldehyde.

An FT-IR of III showed that the two double bonds are of the *Z* configuration, since there was no peak in the 960–980 cm^{-1} region (Figure 2).

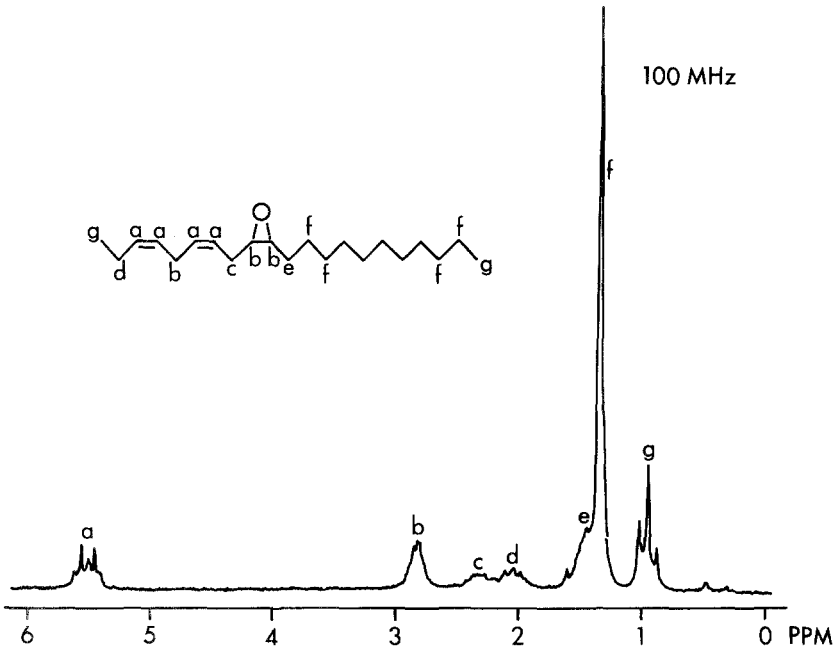


FIG. 3. FT-PMR of III (C_6D_6), with chemical shift assignments.

A [^{1}H]NMR spectrum (FT) of III is shown in Figure 3 with the chemical shift assignments, which are all consistent with the nonconjugated diene-epoxide structure assigned to III.

All the above data lead to the unequivocal structure assignment of (*Z*,*Z*)-3,6-*cis*-9,10-epoxyheneicosadiene for III, which can exist in two enantiomeric forms, 9*S*,10*R* and 9*R*,10*S*. Tests have, as yet, not established the enantiomeric composition of III produced by female *E. acrea*. It also is possible that minor quantities of the various geometric isomers of I, II, and III are present in the gland extract.

EAG Responses of Male Antennae to (+)- and (−)-Disparlures. Typical EAG responses given by a male *E. acrea* antennae to (+)-disparlure, (−)-disparlure and III are presented in Figure 4. Structures of these compounds are given in Figure 5. The two disparlure samples were evaporated into the stream of air going over the test antenna from filter paper cartridges having 100 g of each material; the sample of III was from a capillary tube (GC collected). The highest response was to III (1.8–2.9 mV), followed by that to (−)-disparlure (1.0–1.4 mV), and then by a distinctively lower response to the (+)-disparlure (0.2–0.6 mV). In addition, the response to (−)-disparlure showed the same slow recovery as the response of III, indicative of a binding interaction with the acceptor site.

Observations of Male Flights in a Wind Tunnel. Table 1 presents the results obtained when male *E. acrea* moths were exposed in a flight tunnel to I, II, III, and various combinations of these materials; this includes the samples isolated from female *E. acrea* as well as synthetic samples of the three compounds (III was racemic).

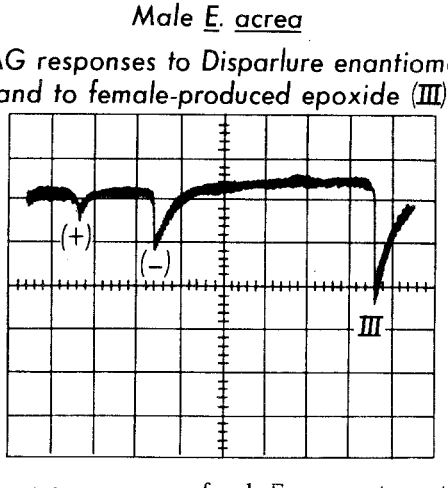


FIG. 4. Electroantennogram responses of male *E. acrea* antenna to III, (+)-disparlure, and (−)-disparlure.

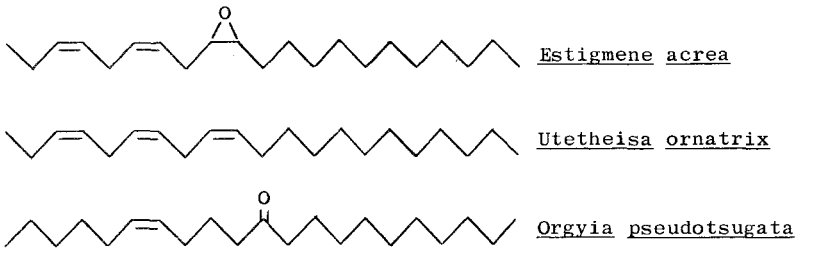


FIG. 5. Comparison of III with other female-produced sex pheromone components known for Lepidoptera.

It is clear that males will not fly to III alone, but that III in combination with either I or II will cause the males to fly up to the source. They will also hover near it, extend their claspers while walking on the source with vigorous wing fanning, and even attempt to mate with other individuals either at the source or at the site of flight initiation. After flying to the source, individuals could be placed downwind repeatedly and again would fly to the source. They could also be kept in flight for several minutes (up to 10–15 min) by moving the striped floor underneath them backwards (Miller and Roelofs, 1978). The period of this flight activity started about 15 min after initiation of the photophase and lasted about 1 hr.

TABLE 1. RESPONSES OF MALE *E. acrea* MOTHS IN A FLIGHT TUNNEL TO I, II, III, AND COMBINATIONS THEREOF

Sample[a]	No. of observations	No. of male responding[b] (% of total)		Average flight time to source[c]
		Fanning	Flying at source	
E. acrea, effluvium	3	3 (100)a	3 (100)a	
E. acrea, tip extract	2	2 (100)a	2 (100)a	
I	4	2 (50) b	0 (0) b	
II	2	0 (0) b	0 (0) b	
III ( )	10	8 (80) b	0 (0) b	
III ( ) + I	14	13 (93)ab	8 (57)a	226 sec
III ( ) + II	20	20 (100)a	14 (70)a	659 sec
III ( ) + I + II	13	13 (100)a	10 (77)a	204 sec
III ( ) + Z9-18:ALD	5	1 (20) b	0 (0) b	
III + I + II	6	6 (100)a	5 (83)a	300 sec

[a]Samples were synthetic unless indicated to be from females. Samples from moths were purified by GC collection. Z9-18:ALD is (Z)-9-octadecenal. Synthetic III was racemic.
[b]Figures followed by the same letter in each column are not significantly different, according to the method of Ryan (1960), at the 5% experimental error rate.
[c]Total numbers of flights timed were 7, 9, 10, and 1, respectively (for samples 6, 7, 8, and 10).

Observation of Calling E. acrea *Females and Location of Female Sex Pheromone Gland.* Females were kept routinely on a 16:8 light–dark cycle. Under these conditions, females were seen calling just before scotophase and also just after scotophase. Calling could be initiated in the females by removal of the females from scotophase at any time during the last 5 hr of scotophase; when this was done, the females would call starting about 15 min after initiation of the photophase.

The calling stance is one in which the female elevates her wings slightly, sometimes flutters them while walking around, and her abdominal tip is pushed out slightly and retracted in a pulsing motion. The rate of pulsing at room temperature was timed at about 80 pulses per min (three individuals were observed).

Various crude dissections of female abdominal tips were carried out and each portion was extracted and analyzed for the pheromone components. Only a pair of tubes, existing dorsally at the base of the penultimate abdominal segment, were found (using GC tracings) to contain any appreciable amounts of the sex pheromone components, I, II, and III. These tubes are visible under the cuticular abdominal covering under slight magnification (25×). Each is about 3 mm in length and about 0.2 mm OD, translucent, and almost colorless (light cream color). They appear to be similar to the tubular glands of the geometrid moth, *Rheumaptera hastata* (L.) (Werner, 1977), except the pair of tubes in *E. acrea* exist separately, whereas the tubes of *R. hastata* join and have a common funnel-shaped opening.

DISCUSSION

The three compounds (I, II, and III; Figure 1) identified as sex pheromone components for *E. acrea* and *Hyphantria cunea* (Drury) (Hill et al., 1981) have not been reported previously as components of other sex pheromone systems. Compounds I and II are the aldehydes corresponding to the widely distributed polyunsaturated fatty acids linoleic and linolenic acids, which are known to be essential fatty acids for insects (Downer, 1978). The epoxide component, III, although hitherto unknown, is similar to the compounds shown in Figure 5, which have been reported as sex pheromone components for other lepidoptera: one is from another arctiid, *Utetheisa ornatrix bella* (L.) (Conner et al., 1980); the other two are from lymantriids, the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough) (Smith et al., 1975) and the gypsy moth, *Lymantria dispar* (L.) (Bierl et al., 1970). The top two pheromones in Figure 5 and compound III are all unbranched 21-carbon chain structures with a *Z* double bond at the 6 position. The *U. ornatrix* component also has a (*Z*)-3 double bond in common with III, and its other double bond, at position 9, occurs at the same locus on the carbon chain

and has the same *Z* configuration as the epoxy group of III. The points of similarity between III and the *O. pseudotsugata* pheromone component are fewer. Although the oxygen functionalities of these two compounds are not in exactly the same place along the carbon chain, they do occur at vicinal positions. The only other epoxide so far reported as an insect sex pheromone, *cis*-7,8-epoxy-2-methyloctadecane (disparlure), is that from *L. dispar*. Field data (Miller et al., 1977; Cardé et al., 1977) strongly support assignment of the 7*R*,8*S* structure [(+)-disparlure] as that of the natural pheromone. No other sex pheromone components for lymantriids have been reported, although males of one other species in this family, the nun moth, *Lymantria monacha* (L.), has been captured in traps baited with disparlure.

The only other known arctiid sex pheromone structure is 2-methylheptadecane, which has been reported for the banded woolly bear moth, *Isia Isabella* (J. E. Smith; formerly *Pyrrarctia isabella*), and for a number of tiger moths in the *Holomelina* genus (Roelofs and Cardé, 1971). This hydrocarbon has the 2-methyl branch feature in common with the disparlure structure.

Male *E. acrea* antennae can discriminate between the levo- and dextrorotatory isomers of *cis*-disparlure, with the (−)-isomer consistently producing EAG responses that are 2–5 times greater in amplitude than those produced with the (+)-isomer. The acceptor site is, presumably, that for III, so this acceptor site appears to be chiral. If the structures of the two isomers of III and the two enantiomers of *cis*-disparlure can be correlated as shown in Figure 6, then a prediction of the absolute configuration of III seems possible. The correspondence of the two longer-chain portions of each compound, shown in Figure 6, to the right of each epoxide group, is made on the assumption that the 10-carbon chain portion of the *cis*-disparlure molecule is too bulky to be accomodated at the locus on the acceptor site that ordinarily

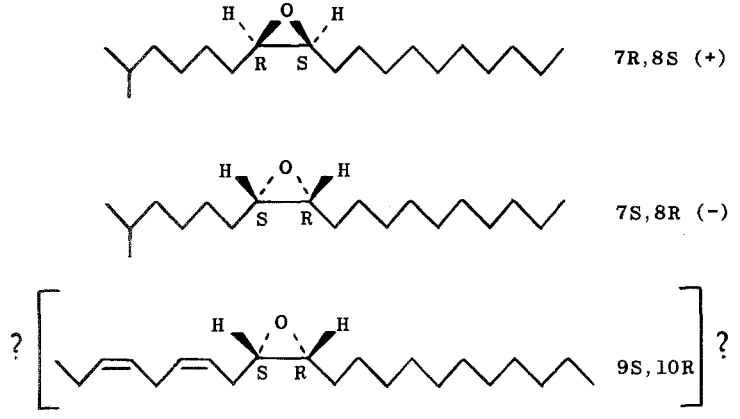


FIG. 6. Stereochemical relationships between III and the disparlure enantiomers.

fits the 8-carbon unsaturated portion of III. Based on these assumptions, a 9*S*,10*R* configuration at the epoxide group of III is predicted. Additionally, because of the marked difference in the EAG responses to (+) and (−) disparlures, it seems reasonable to expect that III produced by female *E. acrea* will be found to be exclusively or predominantly the 9*S*,10*R* enantiomer.

Wind tunnel observations of male *E. acrea* flights have demonstrated that upwind flight by males is initiated and sustained in the presence of III in combination with either I or II and that racemic, synthetic III is effective. Since all three components are emitted by the females, but only two of the three appear to be required for sustained upwind anemotaxis, the functional reason for emission of both aldehydes by the female is not clear at present.

At this stage it is evident that all three compounds, I, II, and III, are emitted by female *E. acrea* and that all three can be perceived by the males and mediate the specific behavior of upwind flight (amenotaxis) by them. Further testing of the biological significance of these components, including field trapping tests and investigation of the biosynthesis of these compounds, will be carried out.

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IDENTIFICATION OF THE CUTICULAR HYDROCARBONS OF THE HORN FLY[1] AND ASSAYS FOR ATTRACTION[6]

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Abstract—Horn fly cuticular paraffin and monoolefin hydrocarbons were chemically identified and assayed for biological activity as attractants. The majority of the paraffins were odd-numbered, straight-chain molecules 21–29 carbons in length; much smaller amounts of even-numbered, straight-chain molecules 22–28 carbons in length and methyl-branched compounds were also present. At least 80% of the monoolefin consisted of straight-chain molecules 23, 25, and 27 carbons in length, two of which have been identified as sex pheromones in other muscoid species. The hydrocarbon profiles among sexes and strains (laboratory and wild) were very similar except for wild females, which showed quantitative differences from the other sources. However, only females showed significant (albeit low) responses to some test materials, both synthetic and natural, and activity appeared to be centered in the monoolefins.

Key Words—Horn fly, olefins, mating pheromone, paraffins, *Musca autumnalis*, sex attractant.

[1]Diptera: Muscidae.
[4]A portion of a dissertation by the first author to the Graduate School of the University of Florida in partial fulfillment of the requirements for the PhD degree.
[6]Mention of a proprietary or commercial product does not constitute an endorsement by either the University of Florida or the U.S. Department of Agriculture.

INTRODUCTION

The horn fly, *Haematobia irritans* (L.), is a serious pest of cattle in the U.S. and elsewhere. The blood-feeding habits of the adults cause irritation, worry, and blood loss in host animals, and this commonly results in reductions in weight gain and milk production (Bruce, 1942, 1964). In recent years, control of this pest has become increasingly difficult because of legal restrictions placed on insecticide use.

The utilization of sex attractants in insect control programs offers promise for reducing dependence on insecticides. To date, certain of the cuticular hydrocarbons from a number of related muscoid flies have been isolated and identified as sex attractants and/or mating stimulants (Carlson et al., 1971, 1978; Muhammed et al., 1975; Uebel et al., 1975a–c, 1978a,b). Furthermore, Mayer et al. (1972) found attraction of male house flies, Musca domestica L., in an olfactometer to horn fly cuticular hydrocarbons.

In view of these findings, we determined the chemical nature of horn fly cuticular hydrocarbons, and investigated their potential as horn fly attractants.

METHODS AND MATERIALS

Chemical Analyses. Crude lipid extracts were obtained separately from 3- to 4-day-old sexually mature, virgin, male and female flies from the colony at the University of Florida, hereafter called Florida Laboratory Strain (L), that were immobilized by freezing at −20° C and rinsed with *n*-hexane (Phillips, 20 flies/ml). The hexane washes were reduced to dryness on a rotary evaporator, weighed, and then reconstituted with *n*-hexane. The hydrocarbons were obtained by open-column liquid chromatography (LC) by eluting 1–2 g of extract from a 2 × 45-cm column of silica gel (60–200 mesh, J.T. Baker Chemical Co.) with 200 ml *n*-hexane. The hydrocarbons were then eluted on a 1.3 × 36-cm column of 20% $AgNO_3$-impregnated silica gel (60–200 mesh, Hi-Flosil-Ag, Applied Science Laboratories) with 60 ml *n*-hexane to obtain the paraffins, followed by 100 ml of 2% ether in hexane to obtain the olefins. Separation and purity of all eluted fractions were confirmed by thin-layer chromatography (TLC) on silica gel plates (250 μm Anasil, Analabs, or 150 μm Uniplates, Analtech) or $AgNO_3$-impregnated silica gel plates (250 μm AG Anasil, Analtech). No polyolefins were detected.

The hydrocarbons were analyzed by gas chromatography (GC) using a Varian model 2100 with a 1.8-m × 2-mm ID glass column containing 3 or 5% SE-30 on 120–140 mesh Gas Chrom Q, and flame ionization detection. The major *n*-paraffins and chain lengths of branched paraffins were determined by coinjection of known paraffin standards. Chain lengths of the major monoolefins were similarly determined and retention indices (KI) assigned

(Kovats, 1966). Quantitations were obtained by comparing peak height or peak areas with standards of known concentration; peak areas were measured on a Hewlett-Packard model 3380A integrator.

Olefins from males and females consisted of four major peaks eluting at KI 2272 and KI 2292 (C_{23}), KI 2477 (C_{25}), and KI 2677 (C_{27}) and hereafter designated compounds I, II, III, and IV, respectively. Each was collected from samples of olefin from L females by preparative GC using a Varian model 90-P aerograph with a thermal conductivity detector and stainless-steel (SS) columns. The C_{23} compounds were collected from a 6-m × 4-mm ID column containing 5% SE-30 on 100–120 mesh Gas Chrom Q, and the C_{25} and C_{27} compounds collected from a 3-m × 2-mm ID column containing 3% SE-30. The individually trapped compounds were ozonized to determine sites of unsaturation and the resulting aldehyde fragments identified by GC after the method of Beroza and Bierl (1967). Aldehydes resulting from ozonolysis of compounds I and II were identified on three gas chromatographs (F and M model 810 with a 3-m × 2.2-mm ID SS column containing 5% Carbowax 20 M on 100–120 mesh Gas Chrom Q; Varian model 1200 with a 2-m × 2.2-mm ID SS column containing 5% Hi-EFF-1BP on 80–100 mesh Chromosorb W AW; and the previously described Varian model 2100 with the 5% SE-30 glass column). The short-chain aldehyde obtained from ozonolysis of compound II could not be detected on the above columns or on SS or glass columns of Porapak Q. The ozonolysis products of III and IV were determined with the 5% SE-30 column.

Electron impact mass spectra (EI-MS) of paraffins from L males and females and from wild females were obtained using a Varian MAT CH5 mass spectrometer interfaced via a membrane separator to a Varian 1400 GC equipped with 3.2-m × 2-mm ID glass columns of 3% OV-1 on 100–120 mesh Gas Chrom Q.

Chemical ionization mass spectra (CI-MS) of II and the ozonolysis products of the four olefins were obtained with a Finnigan 1015 S/L mass spectrometer, interfaced to a Systems Industries 150 data system. A Varian model 1400 GC, equipped with either a 1.8-m × 2-mm ID glass column containing 3% OV-1 on 100–120 mesh Gas Chrom Q or a 1.8-m × 2-mm ID SS column containing 5% SE-30 on Gas Chrom Q (100–120 mesh), was used as the inlet. Methane, used as the carrier and ionizing gas, was passed directly into the ion source where pressure was maintained at 1.0 torr, and the GC oven was temperature programed. The computer data system provided mass spectra, reconstructed gas chromatograms (RGC), and limited mass range searches (LMS), representing ions of specific masses plotted versus spectrum number.

Extracts from all sources were taken through the same scheme. These were (1) 3- to 4-day-old, virgin male or female flies reared from eggs obtained

from Florida wild flies (hereafter, W) and (2) hexane washes from cages heavily contaminated with fly feces.

Compound I had been synthesized previously (Carlson et al., 1971), while III and IV were obtained from other sources, for GC and TLC analysis, ozonolysis, and bioassay. Compound II [(*Z*)-5-tricosene] was prepared by the Wittig reaction as follows. 1-Bromooctadecane (Aldrich, 52 g, 0.156 mol) and 52 g (0.2 mol) of triphenylphosphine (Aldrich) were dissolved in 200 ml of acetonitrile and refluxed overnight in N_2 atmosphere. The acetonitrile was removed on a rotary evaporator, and the residue was poured into anhydrous ether. The white solid that precipitated upon stirring was collected and dried thoroughly under vacuum to give octadecyltriphenylphosphonium bromide. Octadecyltriphenylphosphonium bromide (34 g, 0.057 mol) was dissolved in 100 ml of anhydrous tetrahydrofuran (THF) in a dry flask under N_2. The solution was stirred, cooled in an ice bath, and held between 10 and 20° C, while 30 ml of butyllithium (15.16% solution in hexane, Foote Mineral Co.) was added slowly. The dark red solution was held at 15–20° C for 1 hr and then cooled to 10° C. Freshly distilled pentanal [5.6 g, 0.065 mol, bp 102–103° C (760 mm)] was added dropwise with stirring; the solution was allowed to warm to room temperature overnight. Two days later, the reaction mixture was shaken with water and hexane and separated. The hexane layer was washed with brine and dried. Rotary evaporation gave 25 g of crude oil and a few crystals of triphenylphosphine oxide. The crude oil was diluted with hexane and portions were passed 3 times through 2 × 50-cm columns of silica gel (60–200 mesh, Baker) with 200 ml hexane each time. Removal of solvents gave 13.5 g (54%) of olefin. The *cis* and *trans* isomers from a 2-g sample of the olefin were separated by $AgNO_3$-LC; the *trans* isomer was eluted with 120 ml of hexane and the *cis* isomer with 150 ml of 1% ether in hexane. Separation and purity of the eluents were confirmed by $AgNO_3$-TLC. The olefin as synthesized was estimated to contain 85% *cis* and 15% *trans* isomer of 5-tricosene by weighing the $AgNO_3$-LC eluents after removal of solvent.

Bioassays. The movement and choice response of flies over a distance of ca. 50 cm to test materials was assayed in an olfactometer. The olfactometer was a modification of the vertical types used to assay stable fly, *Stomoxys calcitrans* (L.) (Muhammed et al., 1975) and screwworm fly, *Cochliomyia hominivorax* (Coquerel) (Adams et al., 1979) attractants. It consisted of four independent units, each comprised of an insect-holding chamber (30 cm long, 7 cm ID) and two choice chambers (each 30 cm long, 3.5 cm ID) (Figure 1). Insects placed in the holding chambers moved vertically against the air flow, through the choice ports, and into treatment or control choice chambers. Partially conditioned air was directed through an activated charcoal filter to the small mixing chamber (21 × 21 × 9.5 cm); situated here was a heating element connected with a proportional temperature controller (RFL model

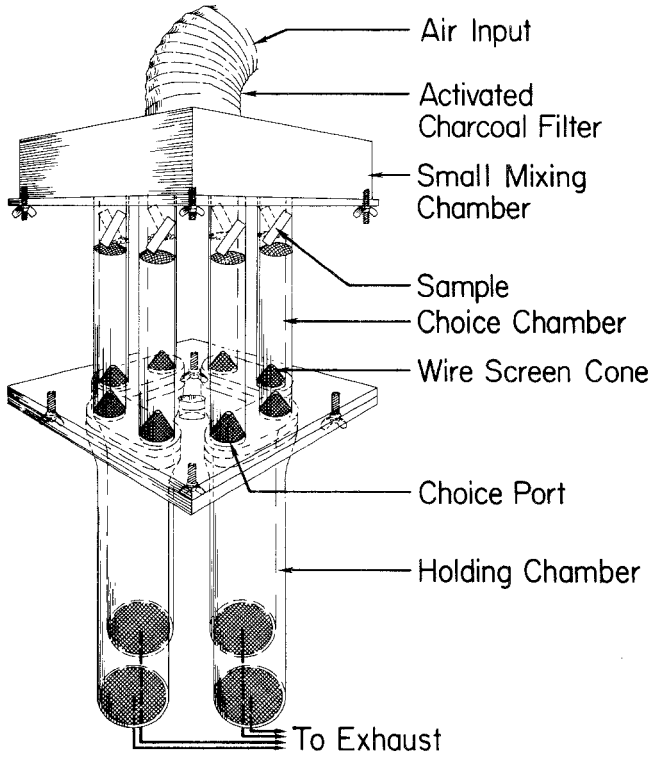

FIG. 1. The horn fly olfactometer.

879, RFL Industries), and wet and dry bulb thermistor probes to monitor temperature and humidity. Temperature and humidity were maintained at 32.5 ± 1.5° C and 60 ± 10% relative humidity, respectively. The fully conditioned air then passed into the eight choice chambers, over glass microscope slides treated with given test materials (Figure 1, Sample), through the choice ports and holding chambers, and finally was exhausted to the outside. Air velocity at the choice ports was maintained at 15 m/min. A fluorescent lamp, either a General Electric Plant Light or a Sears Cool White, was ensheathed in red plastic and illuminated the olfactometer from its central position along the axis of the choice and holding chambers.

For a given bioassay, test materials in 6- to 50-μl solutions of hexane were applied by syringe to one side of glass microscope slides, which were then placed in the treatment choice chambers. Slides treated with 25 μl hexane only were placed in control choice chambers. Lots of 20–40 virgin, 3- to 4-day-old male or female test insects, which had been offered a blood meal up until 1 hr before, were anesthetized briefly with N_2 and introduced to each of the

holding chambers. Flies entering treatment and control choice chambers were counted at the end of 30 min. Flies for a given test were used only once and then discarded. Between each test the holding and choice chambers were thoroughly washed with hot water and detergent. The materials tested included male and female crude lipids; mixed-sex hydrocarbons, nonhydrocarbons, olefins, and paraffins; and synthesized olefins. Since the hydrocarbon profiles of males and females as identified were very similar, the large quantities of extracted natural materials which were needed were recovered from mixed-sex rather than virgin flies.

Tests run concurrently in the independent units of the olfactometer were considered to be replicates. The assay of a given test material was replicated 8–43 times. Data transformations were performed ($\sqrt{X} \pm 0.5$) owing to large variation in test insect response, and the transformed values were analyzed with the paired t test.

RESULTS AND DISCUSSION

Chemical Analyses. The bulk of the paraffins from L flies were odd-numbered, straight-chain molecules 21–29 carbons in length; much smaller amounts of even-numbered, straight-chain molecules 22–28 carbons in length and methyl-branched compounds were present. Major branched isomers are listed first, and others in descending order, as estimated from heights of fragments in the EI-MS data. The lack of parent ions does not affect identification for these materials, as the KI data and characteristic mass spectra are unequivocal (Tables 1 and 2). The methods of Nelson et al. (1972) and Pomonis et al. (1978) were followed to determine the lengths of paraffins and the number of methyl branches by GC-MS. Identification was possible for all major and most branched components. The identified branched paraffins were either mono- or dimethyl, with the most common sites of methyl substitution being the 9, 11, 13, and 15 positions, with three methylene units present between branches in dimethyl components. A unique dimethyl-branched paraffin was found at KI 2750 only in the mass spectra of W females, and it eluted with the last traces of 5-methylheptacosane. The fragments at 168, 169, 196, 197, 239, and 393 (M-15) are consistent with 11,15-dimethyl-heptacosane. GC analyses showed that the latter was the most prevalent branched paraffin in W females, and other samples had less of it. Otherwise, male and female paraffins from L and W flies were qualitatively similar, since they eluted together by GC, but quantitative differences were seen between the strains and the sexes (Table 3). Paraffins in Table 3 were present at 2.8, 5.8, 3.9, and 5.4 μg/fly for L males, L females, W males, and W females, respectively, and branched paraffins comprised only 5.8, 9.6, 9.3, and 10.0% of these totals, respectively. GC profiles of paraffins recovered from cage washes (feces) were very similar to those of L flies.

TABLE 1. FEMALE L HORN FLY PARAFFINS

Kovats' index	Structure	Observed fragments		
		m/e	M-15	M
2330	9-$C_{23}H_{47}(CH_3)$	140, 141	323	none
		224, 225		
	11-$C_{23}H_{47}(CH_3)$	168, 169		
		196, 197		
2530	11-$C_{25}H_{51}(CH_3)$	168, 169	351	none
		224, 225		
	13-$C_{25}H_{51}(CH_3)$	196, 197		
2550	11,15-$C_{25}H_{50}(CH_3)_2$	168, 169	none	none
		239		
2730	11-$C_{27}H_{55}(CH_3)$	168, 169	379	none
		252, 253		
	13-$C_{27}H_{55}(CH_3)$	196, 197		
		224, 225		
	5-$C_{27}H_{55}(CH_3)$	337	379	none
	7-$C_{27}H_{55}(CH_3)$	309		
2930	11-$C_{29}H_{59}(CH_3)$	168, 169	407	422
		281, 282		
	13-$C_{33}H_{67}(CH_3)$	196, 197		
		252, 253		
	15-$C_{29}H_{59}(CH_3)$	224, 225		
2950	11,15-$C_{29}H_{58}(CH_3)_2$	168, 169	421	none
		224, 225		
		238, 239		
		295		
	13,17-$C_{29}H_{58}(CH_3)_2$	196, 197		
		266, 267		
3130	11-$C_{31}H_{63}(CH_3)$	168, 169	435	450
		308, 309		
	13-$C_{31}H_{63}(CH_3)$	196, 197		
		280, 281		
	15-$C_{31}H_{63}(CH_3)$	224, 225		
		252, 253		
3150	9,13-$C_{31}H_{62}(CH_3)_2$	140, 141	449	464
		210, 211		
		280, 281		
		352, 353		
	11,15-$C_{31}H_{62}(CH_3)_2$	238, 239		
		252, 253		
		322, 323		
	13,17-$C_{31}H_{62}(CH_3)_2$	196, 197		
		266, 267		
		294, 295		
3330	11-$C_{33}H_{67}(CH_3)$	168, 169	463	478
		336, 337		

TABLE 1. (*cont.*)

Kovats' index	Structure	Observed fragments		
		m/e	M-15	M
	13-$C_{33}H_{67}(CH_3)$	196, 197		
		308, 309		
	15-$C_{33}H_{67}(CH_3)$	224, 225		
		280, 281		
3350	11,15-$C_{33}H_{66}(CH_3)_2$	169, 169	none	none
		238, 239		
		280, 281		
		350, 351		
	13,17-$C_{33}H_{66}(CH_3)_2$	196, 197		
		252, 253		
		266, 267		
		322, 323		
3530	11-$C_{35}H_{71}(CH_3)$	168, 169	491	none

TABLE 2. MALE L HORN FLY PARAFFINS

Kovats' index	Structure	Observed fragments		
		m/e	M-15	M
2130	9-$C_{21}H_{43}(CH_3)$	140, 196		
	11-$C_{21}H_{43}(CH_3)$	169		
2200	$C_{22}H_{46}$		none	310
2300	$C_{23}H_{48}$		none	324
2330	9-$C_{23}H_{47}(CH_3)$	140, 224	none	none
	11-$C_{23}H_{47}(CH_3)$	168, 196		
2400	$C_{24}H_{50}$		none	338
2500	$C_{25}H_{52}$		none	none
2530	7-$C_{25}H_{51}(CH_3)$	113, 281	none	none
	9-$C_{25}H_{51}(CH_3)$	252, 253		
		141, 142		
	11-$C_{25}H_{51}(CH_3)$	168, 169		
		224, 225		
	13-$C_{25}H_{51}(CH_3)$	196, 197		
2550	9,13-$C_{25}H_{50}(CH_3)_2$	141, 197	none	none
		211, 267		
	11,15-$C_{25}H_{50}(CH_3)_2$	169, 239		
2600	$C_{26}H_{54}$		none	none
2730	7-$C_{27}H_{55}(CH_3)$	112, 308	379	none
	11-$C_{27}H_{55}(CH_3)$	169, 253		
	13-$C_{27}H_{55}(CH_3)$	196, 197		
		225		

TABLE 2. (*cont.*)

Kovats' index	Structure	Observed fragments		
		m/e	M-15	M
2900	$C_{29}H_{60}$		none	none
2930	7-$C_{29}H_{59}(CH_3)$	112, 337	none	none
	9-$C_{29}H_{59}(CH_3)$	141, 309		
	11-$C_{29}H_{59}(CH_3)$	168, 281		
	13-$C_{29}H_{59}(CH_3)$	196, 252		
	15-$C_{29}H_{59}(CH_3)$	225		
3130	11-$C_{31}H_{63}(CH_3)$	169, 309	435	450
	13-$C_{31}H_{63}(CH_3)$	196, 281		
	15-$C_{31}H_{63}(CH_3)$	225, 253		

TABLE 3. QUANTITIES[a] OF PARAFFINS RECOVERED FROM L AND W HORN FLIES

Kovats' index[b]	L male	L female	W male	W female
2100	19.7	10.5	7.9	1.0
2130	0.2	0	1.9	0.4
2200	1.1	0.9	2.1	0.8
2300	36.3	27.5	11.6	3.1
2330	0.6	0.4	0.4	0.1
2350	0.1	0.1	0.1	0.4
2400	0.6	0.7	1.5	1.7
2500	15.7	18.9	20.0	22.8
2550	0.3	0.2	0.4	0.4
2600	0.6	0.8	2.2	2.9
2700	13.7	22.4	25.6	42.9
2730	0.5	0.7	2.0	2.5
2750	0.2	1.7	3.6	5.3
2800	0.2	0.5	1.7	2.6
2900	4.8	6.4	3.6	5.6
2930	2.0	3.0	0.9	0.9
3100	0.5			
3130	1.9	3.5		

[a]Percent of total.

[b]Unbranched (normal) paraffins have KI values of 2100, 2200, 2300, etc., by definition; KI values of 30 (eg, 2130) are internally monomethyl branched; KI values of 50 (eg, 2150) are dimethyl branched.

All olefins were of the *cis* configuration. The major longer aldehydes resulting from ozonolysis of compounds I, II, III, and IV coeluted on GC with C_{14}, C_{18}, C_{16}, and C_{18} aldehyde standards, respectively. The shorter aldehydes from ozonized I, III, and IV coeluted with the C_9 aldehyde nonanal. Pentanal from ozonized II was not detected. CI-MS confirmed the identities of the detected aldehyde fragments (Table 4). All the major aldehydes had fragments M + 1, M − 1, M + 1–18; for some aldehydes, M + 29 and M + 41 fragment ions (addition of $C_2H_5^+$ and $C_3H_5^+$, respectively) were observed as noted. The aldehyde fragments account for the following structures: (*Z*)-9-tricosene (I), (*Z*)-5-tricosene (II), (*Z*)-9-pentacosene (III), and (*Z*)-9-heptacosene (IV). Compounds I–IV coeluted by GC with their respective synthetic counterparts as did the ozonolysis products of I and II with the ozonolysis products of their respective synthetic counterparts.

Olefins were present at 5.13, 7.17, 2.12, and 2.34 μg/fly for L males, L females, W males, and W females, respectively. The four major compounds comprised 95% of the total olefins in L flies and W males, and 80% of the total in W females. Table 5 summarizes the quantities of these compounds in each group of flies. Among L flies and W males, I was the major component (38–83% of the total); however, in W females it was only 2% of the total. In W females III and IV were 22 and 55% of the total, respectively, while two other unidentified compounds of higher molecular weight made up most of the remainder. Natural materials were not collected by preparative GC for bioassay because the response to natural olefin was low. Monoolefins extracted from fly feces showed GC profiles quite similar to those of L flies.

Bioassays. Males showed a barely significant, positive response to synthesized II but did not respond to the crude lipids, the natural olefin, or other materials containing this compound (Table 6). Female response was more consistent and significant, as positive responses to the female crude lipid, hydrocarbons, olefins, and compound I were elicited. Neither sex responded to other lipids, but no obvious repellency was observed. The lack of stronger responses to the individual olefins suggests that combinations of the compounds were necessary, that synthesized compounds were impure or too pure for the assay, or that essential compounds were missing.

(*Z*)-9-Tricosene has been previously identified as a house fly attractant pheromone (Carlson et al., 1971), and (*Z*)-9-pentacosene has been identified as the copulatory sex pheromone of the little house fly, *Fannia canicularis* (L.) (Uebel et al., 1975a). In olfactometer tests performed by Mayer et al. (1972), compound I was apparently responsible for the attraction of male house flies to horn fly hydrocarbons. Because of the success of these approaches, we felt justified in assaying synthetic olefins. We did not test olefins from horn flies other than compounds I–IV because it has been our experience that no highly potent attractants in flies have been found that work at submicrogram

TABLE 4. CI-MS OF MONOOLEFIN OZONIDES FROM FEMALE L HORN FLIES

Kovats' index	Compound	Aldehydes	Observed fragments						
			M-1-18	M + 1-18	M-1	M	M + 1	M + 29	M + 41
2272	(Z)-9-Tricosene (I)	C_9[a]	123	125	141		143[b]	171	183
		C_{14}[a]	193	195	211	212	213	241	
2292	(Z)-5-Tricosene (II)	C_{16}	221		239		241[b]	269	281
		C_{17}	235		253		255	283	
		C_{18}[a]	249		267		269	297	
2477	(Z)-9-Pentacosene (III)	C_9[a]	123	125	141		143[b]	171	183
		C_{13}			197		199[b]	227	
		C_{14}			211		213[b]		
		C_{16}[a]	221	223	239	240	241[b]	269	281
		C_{18}	249		267		269[b]	297	
2677	(Z)-9-Heptacosene (IV)	C_8		111[b]			129	157	169
		C_9[a]	123	125	141		143	171	183
		C_{15}		209	225	226		255	
		C_{16}	221	223	239		241[b]	269	281
		C_{17}	235	237	253	254	255	283	295
		C_{18}[a]	249	251	267	268	269	297	309
		C_{20}	277		295	296	297	325	

[a]Major products.
[b]Base peak.

TABLE 5. QUANTITIES OF 4 MAJOR MONOOLEFINS RECOVERED FROM L AND W HORN FLIES

Kovats' index	Compound[a]	Component/fly (μg)			
		L male	L female	W male	W female
2272	Z-9-Tricosene	1.20	0.64	0.18	0.01
2292	Z-5-Tricosene	0.76	1.04	0.35	0.02
2477	Z-9-Pentacosene	0.34	0.54	0.42	0.37
2677	Z-9-Heptacosene	0.12	0.34	0.37	0.90

[a]Compounds identified via CI-MS from L females only.

quantities. No *trans* isomers were found in flies, nor were any *trans* synthetics tested, but *trans* isomers do not appear to play an inhibitory role in response of other muscids studied (Carlson et al., 1974).

At this point, the nature of attraction of horn flies to these hydrocarbons is not clear, especially as no striking sexual dimorphism was observed in the quantity or identity of cuticular components of L flies (Tables 1, 2, 3, and 5).

TABLE 6. AVERAGE RESPONSE OF VIRGIN, 3- TO 4-DAY-OLD HORN FLIES TO TEST MATERIALS AND CHECKS IN THE OLFACTOMETER

Test material	Quantity	Sex tested	*N*	T[a]	C[b]	*t* statistic[c]
Natural products						
♂ crude lipid	20 FE	♀	16	7.9 ± 4.5	7.4 ± 5.1	0.32
		♂	16	9.3 ± 4.4	5.5 ± 4.4	1.80
♀ crude lipid	20 FE	♀	16	6.3 ± 4.5	5.6 ± 3.6	0.23
		♂	16	9.0 ± 4.7	4.8 ± 2.9	2.58**
Hydrocarbons	100 μg	♀	8	12.4 ± 5.1	8.9 ± 4.6	1.12
		♂	10	6.7 ± 3.8	2.6 ± 2.6	3.08**
Nonhydrocarbons	20 FE	♀	16	8.8 ± 4.6	7.5 ± 4.0	0.46
		♂	16	6.5 ± 4.7	6.8 ± 3.7	0.40
Paraffins	1 mg	♀	15	3.3 ± 2.1	4.0 ± 2.5	0.89
		♂	15	4.8 ± 6.7	5.6 ± 4.0	0.60
Olefins	1 mg	♀	43	5.7 ± 5.2	4.5 ± 4.9	1.44
		♂	35	7.8 ± 4.7	4.6 ± 3.0	4.77***
Synthetics						
I	50 μg	♀	18	6.4 ± 4.1	4.0 ± 3.2	2.89**
	1 mg		32	5.6 ± 4.4	8.2 ± 5.4	1.96
		♂	13	9.3 ± 4.6	7.0 ± 3.1	1.40
II	1 mg	♀	40	8.2 ± 4.5	5.8 ± 3.8	2.02*
		♀	27	6.1 ± 4.9	6.3 ± 4.6	0.25

TABLE 6. (*cont.*)

Test material	Quantity	Sex tested	*N*	T[a]	C[b]	*t* statistic[c]
Synthetics (*cont.*)						
III	1 mg	♂	16	7.3 ± 3.5	8.0 ± 4.2	0.31
		♀	16	7.9 ± 4.5	5.6 ± 3.7	1.28
IV	1 mg	♂	16	7.9 ± 4.2	9.1 ± 4.4	0.53
		♀	16	6.8 ± 5.2	8.5 ± 5.2	0.61
I:II (60:40)[d]	1 mg	♂	16	9.2 ± 4.1	7.8 ± 3.9	0.79
		♀	16	7.5 ± 3.9	7.8 ± 4.1	0.14
I:II (40:60)[e]	1 mg	♂	16	9.1 ± 3.8	7.7 ± 3.3	0.85
		♀	16	8.9 ± 3.8	8.3 ± 4.2	0.36

[a]Test materials as 6-50 μl solutions in hexane on glass microscope slides, means ($\bar{x}$) followed by standard deviation (±SD).
[b]25 μl hexane only on glass microscope slides as control, $\bar{x}$ ± SD.
[c]*, **, and *** paired *t* values at the 0.05, 0.025, and 0.001 levels of confidence, respectively.
[d]Reflects the ratio of these two compounds in virgin, mature lab-reared male horn flies.
[e]Reflects the ratio of these two compounds in virgin, mature lab-reared female horn flies.

However, Bolton et al. (1980) have shown in horn fly mating stimulant tests that males respond to female cuticular hydrocarbons but not to male cuticular hydrocarbons and crude lipids. Also, activity was shown using olefin blends which did not necessarily include I. Differences in mode of action (mating stimulation of males vs. attraction of females) and minor differences between male and female hydrocarbons may account for the different conclusions drawn by us and Bolton et al. (1980) concerning biological activity. In both efforts, branched paraffins were a minor complement to the total of compounds found, and never comprised more than 10% of the paraffin fractions. Thus, it is difficult to believe that they could contribute much to attractant activity, although a role in sex stimulant activity is possible as in the house fly where synergism of olefins by branched paraffins was observed by Uebel et al. (1976). While our L females had relatively large amounts of the more volatile olefins I and II, in which activity could reasonably be expected, W females had very little of them, but mostly III and IV, for which no activity was seen. Lower paraffins were also lacking in W females, suggesting that an essentially laboratory phenomenon was tested and both data from L and W flies must be considered. Perhaps, as some researchers have concluded about the tsetse fly, *Glossina* spp. (Bursell, 1961; Dean et al., 1969), the host animal serves not only as a food source but as an aggregation site for the sexes, thus eliminating the need for a sex attractant which operates over distance. However, the observation of similar hydrocarbons in horn fly feces suggest that the deposition and build-up of these

materials on a host's body surface is quite possible, which may affect flies coming near and/or alighting on the surface.

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ACCELERATED SOIL MINERALIZATION, NITRIFICATION, AND REVEGETATION OF ABANDONED FIELDS DUE TO THE REMOVAL OF CROP-SOIL PHYTOTOXICITY

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Abstract—In an abandoned corn field, clear-cutting of crop vegetation increased the productivity, species richness, and nonannuals in the following years after abandonment, as compared to the control plots from which crop vegetation was not removed. The increase in plant growth was apparently due to the elimination of allelopathic chemicals from the soil, which normally are released from the standing crop. Removal of vegetation also increased the soil mineralization of Ca^{2+}, Mg^{2+}, K^{+}, NH_4^{+} and NO_3^{-}-N. This situation encouraged species having higher mineral requirements to rapidly invade the fields in the successive years. Clear-cutting also increased the nitrification process by removing the inhibitors of nitrification. The number of *Nitrosomonas* was always significantly higher in the harvested plots as compared to unharvested plots. Phenolic phytotoxins were isolated from the crop residue and soil. Further, these phytotoxins were significantly higher in the unharvested crops as compared to clear-cut plots, in most samples. Whatever the direct or indirect additional explanation for increased biomass, nonannuals and richness in successive years, it is clear that the removal of standing crop has a definite influence.

Key Words—Revegetation, clear-cutting, allelopathy, nitrification, nitrifiers, mineralization, old fields, *Nitrosomonas*, phytotoxins, phenolic acids.

INTRODUCTION

Drew (1942) reported that the vegetation of an abandoned crop field in Missouri is dominated by the pioneer species for approximately three years. Most of the plants (73–75%) at this early stage are annuals, but perennial species increase during the second year and reach almost 50% by the fifth year.

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The weedy stage is then rapidly eliminated and several perennial species dominate the site. In a partially unharvested corn field, however, the weedy state is often persistent, slowing succession. The slow maturation of the abandoned field could be due to the standing crop residue which is known to contain phytotoxins in quantities inhibitory to plant growth (Guenzi and McCalla, 1962; Patrick, 1971). Such phytotoxins may possibly decrease the productivity and the organic matter accumulation rate to the point where advanced species may not enter this seral stage.

Rice et al. (1960) reported that the climax species appeared in the regeneration of old fields only after the amounts of some minerals increased in the infertile soils. It seems possible that the phytotoxicity of the standing crops could further retard the productivity of early successional plants, the accumulation of soil organic matter, and thus the invasion of advanced species.

With the above knowledge in mind, a partially unharvested corn field was established and compared with a clear-cut corn field to investigate the changes, including productivity, mineralization, nitrification, content of phytotoxins in the soil and in corn residue, and some possible role of such phytotoxins in slowing the disappearance of early vegetation.

METHODS AND MATERIALS

A one-year abandoned field in Saint Louis County, Missouri, was divided into two plots in August 1971. One plot was clear-cut, biomass removed, and considered as a test plot; the other plot was kept unharvested and treated as the control plot.

To describe quantitatively the growth of vegetation, 20 randomly located quadrats 0.25 m^2 were clipped each summer from 1972 through 1976 from each of the control and test plots. Species were separated, oven dried, and weighed.

Soil moisture, pH, available nitrogen, Ca^{2+}, Mg^{2+}, and K^+ analyses were made to see if the differences in the weedy vegetation in clear-cut and partially unharvested fields were due to physical and chemical properties of the soil. Soil moisture was determined each summer from 1972 through 1976, by taking soil samples at 0–30 cm levels. Twenty samples were taken from each plot and treated separately for each sampling time. All samples were weighed, oven dried for 48 hr at 100° C, and reweighed to determine the amount of water present. Soil moisture was calculated on the basis of the oven-dry weight of the soil.

For physical and chemical soil analysis, ten soil samples minus litter were collected at the 0–30 cm level from the corn plot and ten from the clear-cut plot, each summer from 1972 through 1976. The procedures used to determine

soil pH, NO_3^-–N, NH_4^+–N, Ca^{2+}, Mg^{2+}, and K^+ were the same as described previously (Lodhi, 1977). All calculations were based on the oven-dry weight of the soil. For the bacterial count soil samples were combined into three samples from each site and then analyzed for the numbers of *Nitrosomonas* by the method used previously (Lodhi, 1979). The procedure used to isolate and identify phytotoxins from corn followed the alkaline hydrolysis method (Lodhi, 1975a). Methods used to isolate and quantify phytotoxins from soil were the same as described by Lodhi (1975b).

RESULTS

The oven-dry weight of total above-ground plant biomass was significantly greater in the clear-cut area than in the corn field area, in all years except 1972 (Figure 1). Species richness and the numbers of nonannual species were also always higher in the clear-cut plot than in the unharvested corn field (Figure 1). No statistically significant changes occurred in soil pH from 1972 to 1976; however, the soil pH was slightly higher in clear-cut plots (6.8) as compared to unharvested plots (6.5). Percent soil moisture was never significantly lower in the clear-cut plots. There were appreciable differences in mineral content of the soil in all years except the first, 1972 (Figure 2). All cations (Ca^{2+}, Mg^{2+}, and K^+) analyzed rose sharply in 1973 in the clear-cut plots and remained significantly higher through 1976 than the unharvested plots (Figure 2). Similarly the amounts of NO_3^-–N and NH_4^+–N were significantly greater in the clear-cut plots in all sampling times with the exception of 1972 (Figure 3). The amounts of NO_3^-–N increased by a factor of 5 to 7 and NH_4^+–N by a factor of 2 in clear-cut plots as compared to unharvested plots (Figure 3). *Nitrosomonas* MPN were determined only in 1972 and 1976, and in both years the counts in unharvested plots were significantly lower (1972 = 210, 1976 = 180) than in the harvested plots (1972 = 1200, 1976 = 840).

Phytotoxins identified in alkaline hydrolysate of corn residue were caffeic, ferulic, *p*-coumaric, syringic, and *p*-OH benzoic acids (Table 1). The same compounds were also identified in the soils associated with the corn plots and the clear-cut plots (Table 1). Caffeic, syringic, and *p*-OH benzoic acids were not measured after initial analysis due to their smaller amounts as compared to ferulic and *p*-coumaric acids in soils. Various additional compounds were also identified in soil samples from 1973 to 1976. The amounts of ferulic and *p*-coumaric acids were significantly higher in the unharvested plots as compared to the clear-cut plots, with the exception of ferulic acid in 1976. The amounts of ferulic and *p*-coumaric acids showed an increase from 1972 through 1976.

TABLE 1. PHYTOTOXINS IDENTIFIED BOTH IN CORN RESIDUE AND ASSOCIATED SOILS

Compound	R_fs on Whatman No. 1[a]				UV fluorescence[b]		Reagent colors[b,c]	
		6%	BAW	IBW	Long	Short	P. nit.	Sulf. acid
Caffeic acid	0.33	0.67	0.79	0.75	bl	bl	bn black	none
Suspected caffeic acid	0.34	0.65	0.78	0.75	bl	bl	bn black	none
Ferulic acid	0.36	0.70	0.87	0.80	bl	bl	f	f tan
Suspected ferulic acid	0.36	0.71	0.87	0.79	bl	bl	f bn black	f tan
p-Coumaric acid	0.46	0.69	0.92	0.88	p abs	p abs	bn black	or red
Suspected *p*-coumaric acid	0.45	0.69	0.91	0.87	p abs	p abs	bn black	or red
p-OH benzoic acid		0.64	0.90	0.86	f abs	p abs	f wine	or red
Suspected *p*-OH benzoic acid		0.64	0.91	0.86	f abs	p abs	f wine	or red
Syringic acid		0.57	0.88	0.11		lav abs	yell	f or
Suspected syringic acid		0.57	0.87	0.12		lav abs	yell	f or

[a]6% = 6% acetic acid; BAW = 63:10:27 butanol–acetic acid–acetic acid–water; IBW = 140:20:60 isopropanol–butanol–water.
[b]bl = blue, bn = brown, f = faint, abs = absorption, p = purple, lav = lavender, or = orange, yell = yellow.
[c]Sulf. acid = diazotized sulfanilic acid, P. nit. = diazotized *p*-nitraniline.

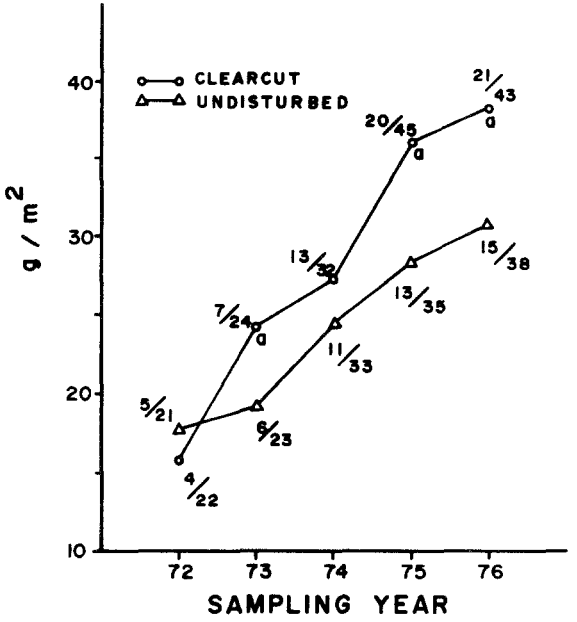

FIG. 1. Comparison of herbaceous biomass from clear-cut and undisturbed corn field during 5 years following abandonment. Denominators represent total number of species, numerators represent nonannual species. (a) Herbaceous biomass significantly different at 0.05 level in clear-cut plots from undisturbed plots in their respective sampling years after abandonment.

DISCUSSION

Experimental data of this report support the contention that clear-cutting significantly decreases the invasion time for successional stages. The nonannual species and species richness increased in the clear-cut plots as compared to unharvested plots (Figure 1). The total biomass significantly increased in the clear-cut plots in all sampling years after abandonment with the exception of 1972.

Even though the soil temperature was not determined in this study, it is quite unlikely that the soil temperature was the only determining factor for the increased biomass in the clear-cut area. Changes in soil temperature can easily influence the soil moisture, and it should be emphasized that the soil moisture was never significantly different between the control and the test plots. Further, the mean soil moisture values were rather inconsistent. As a matter of fact in 1973 the soil moisture was slightly higher in the clear-cut plots than in the unharvested plots. However, Rice and Parenti (1978) reported that the increased productivity in a tall grass prairie was due to the higher soil temperature in the mowed plots and not due to the removal of toxins. They

pointed out that the toxins in the soil and litter were not inhibitory to the dominant species, but were very inhibitory to the pioneer species. The inhibitory effects on the pioneer species are certainly comparable to the results of the current research which involves the inhibition of pioneer species (Drew, 1942).

In the current study, the amounts of Ca^{2+}, Mg^{2+} and K^{+} increased by two to fourfold, NO_3^{-}–N by five to sevenfold and NH_4^{+}–N by twofold in clear-cut plots as compared to unharvested plots (Figures 2 and 3). Lodhi (1979) found that the amounts of Ca^{2+}, Mg^{2+}, K^{+}, and NO_3^{-}–N were higher in early successional stages of coal strip mine spoil soil areas, as compared to the unmined areas. The increased amounts of NO_3^{-}–N after clear-cutting were probably due to the release from inhibition of nitrification. *Nitrosomonas*

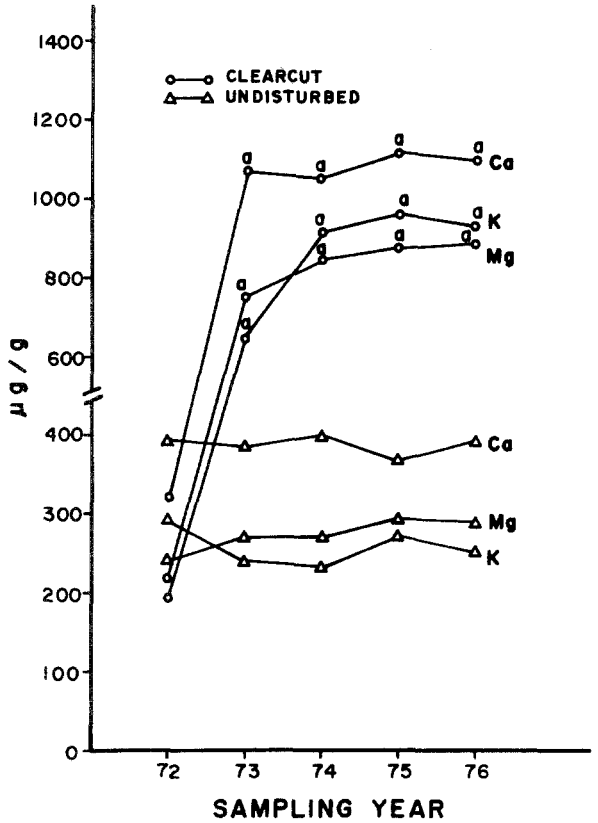

FIG. 2. Mineral analysis during study years. (a) Amounts significantly different at 0.05 in clear-cut plots from undisturbed plots in their respective sampling years after abandonment.

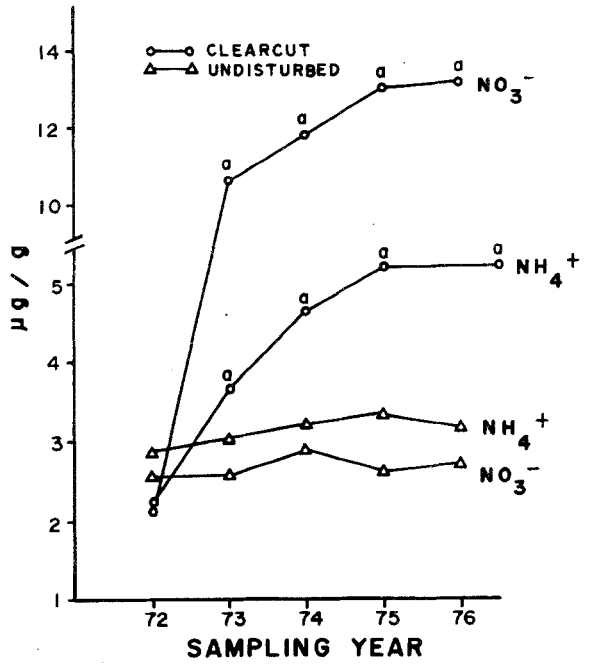

FIG. 3. Ammonium and nitrate nitrogen analysis during study years. (a) Amounts significantly different at 0.05 in clear-cut plots from undisturbed plots in their respective sampling years after abandonment.

count was always significantly higher in the harvested plots than in the unharvested plots. It is a well documented fact that the nitrification process is inhibited by the phenolic toxins released by plant parts (Boughey et al., 1964; Rice and Pancholy, 1974; Lodhi, 1977, 1978b, 1979). Many phenolic acids which are known to inhibit nitrification were released by the corn residue and accumulated in soils in large quantities. Two phenols, ferulic and *p*-coumaric acids, were significantly lower in clear-cut plots than unharvested plots in 1972 through 1976, with the exception of ferulic acid in 1976. Rice and Pancholy (1974) reported that a 10^{-8} and 10^{-4} M concentration of ferulic acid completely inhibited *Nitrosomonas* and *Nitrobacter*, respectively, for three weeks in a 10^{-1} soil suspension.

The increases through time of NH_4^+-N in the clear-cut plots were probably due to two reasons: (1) an increased amount of inhibitors of nitrifiers and (2) the mineralization was supported by slightly higher pH values in clear-cut plots, which increase bacterial growth and their activity.

The phytotoxins, caffeic, ferulic, *p*-coumaric, *p*-OH benzoic, and syringic acids, isolated from the corn residue and associated soils are also

known to reduce the growth of associated vegetation. Two phytotoxins, ferulic and *p*-coumaric acids, selected for quantitative investigations are known to inhibit the germination and seedling growth of various species (Lodhi, 1975a; Chou and Patrick, 1976; Rasmussen and Einhellig, 1977). Lodhi (1975b, 1978a) described the cycling of ferulic and *p*-coumaric acids in soils in different seasons and reported that the accumulation of such phytotoxins corresponded with the amount of decaying litter. The release of phytotoxins and their activity depended on whether they were free or bound and on their solubility. Thus, the persistent accumulation of ferulic and *p*-coumaric acids from corn residue continues to influence the soil properties unless the standing crop is removed. This crop removal would reduce inhibitors from being added to the soil (Figure 4). It should be emphasized that the toxicity level of inhibitors would be more drastic when all inhibitors operate cumulatively and simultaneously (Lodhi, 1975b; Rasmussen and Einhellig, 1977).

The mechanisms by which phytotoxins inhibit growth are not well understood. However, several reports suggest that phytotoxins interfere with enzyme activity (Gortner and Kent, 1958; Machackova and Zmrhal, 1976),

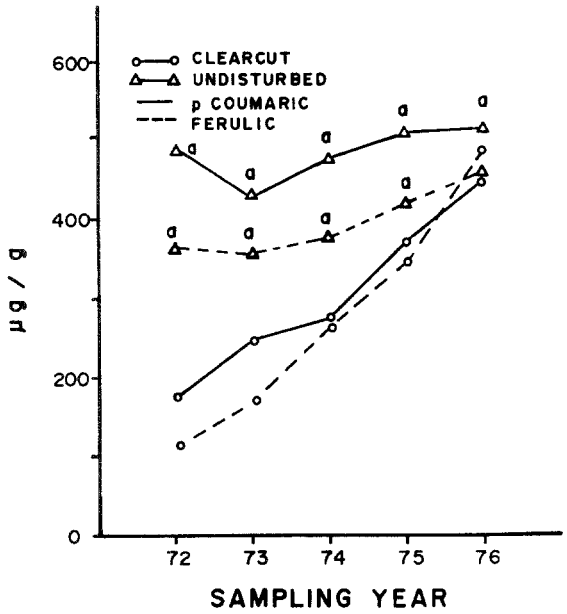

FIG. 4. Inhibitor analysis during study years. (a) Amounts of inhibitors significantly different at 0.05 level in clear-cut plots from undisturbed plots in their respective sampling years after abandonment.

photosynthesis and respiration (Einhellig et al., 1970; Lodhi and Nickell, 1973), and/or ion uptake rates (Danks et al., 1975; Demos et al., 1975).

Whatever the direct or indirect additional explanation for increased biomass, nonannuals, and species richness in successive years, it is clear that the removal of standing crop includes: (1) the removal of toxic effects of plant-produced chemicals on nitrifiers and plants, (2) an increase in the nitrification process by removing the inhibitors of nitrification, and (3) the vigorous growth of invading vegetation which may have taken advantage of accelerated mineralization of Ca^{2+}, Mg^{2+}, K^+, NH_4^+-N and NO_3^--N. The increased productivity of pioneering species and their subsequent decomposition may possibly provide an increased rate of turnover of decaying matter during the study years. Rice et al. (1960) reported that such a situation may encourage some species having higher mineral requirements to rapidly invade the fields in the successive years (Figure 1).

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SYNTHESIS AND IDENTIFICATION OF A THIRD COMPONENT OF THE SAN JOSE SCALE[1] SEX PHEROMONE

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Abstract—Two components of the San Jose scale sex pheromone had previously been identified as 7-methyl-3-methylene-7-octen-1-yl propanoate (I) and (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate (II). An isomer and various homologs have subsequently been synthesized and tested in a greenhouse bioassay. The *E* isomer of II (XI) was found to be attractive to male scales. This compound has now been isolated and identified from airborne extracts of virgin female scales. The composition of the natural pheromone was 48.5%, 46.7%, and 4.8% of I, II, and XI, respectively. In field tests in California and New York, synthetic XI was found to be attractive to male scales alone and in combination with I and II, but there was no obvious increase in trap catch when the synthetic isomers were present in the same ratio as in the natural blend.

Key Words—San Jose scale, *Quadraspidiotus perniciosus*, sex pheromone, synthesis, 7-methyl-3-methylene-7-octen-1-yl propanoate, (*E*)- and (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate, attractant, Homoptera, Diaspididae.

[1] *Quadraspidiotus perniciosus* (Comstock) (Homoptera: Diaspididae).

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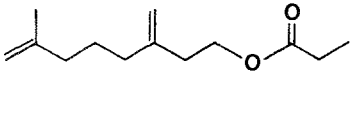
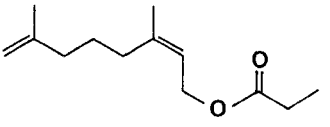
INTRODUCTION

The sex pheromone of the San Jose scale [*Quadraspidiotus perniciosus* (Comstock)], a commercially important homopterous pest, was isolated and initially identified (Gieselmann et al., 1979a) as a two-component mixture of 7-methyl-3-methylene-7-octen-1-yl propanoate (I) and (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate (II).



I



II

These structural assignments were confirmed by the synthesis of each of these ester components and comparison of the synthetic samples with the naturally occurring pheromone (Anderson et al., 1979). To establish which structural features of these pheromone components are crucial for attractancy in this species, several closely related homologs of I and II and an isomer of II were prepared. We report herein the preparation and the biological activity of these new compounds, and the resultant identification of a third component of the San Jose scale pheromone.

METHODS AND MATERIALS

Preparative thin-layer chromatography was carried out on 1-m × 20-cm glass plates coated with 1.3 mm of Merck (Darmstadt) silica gel PF-254. Silica plates impregnated with Rhodamine 6G dye were used to visualize those compounds lacking significant UV absorbance at 254 nm. Gas–liquid chromatographic analyses were performed on model 402 Hewlett-Packard instruments equipped with hydrogen flame ionization detectors. NMR spectra were determined on a Varian T-60 spectrometer. Infrared spectra were measured on a Unicam SP-200G or a Perkin-Elmer 281 spectrophotometer. Mass spectra were measured on a Hewlett-Packard model 5985 GC-MS-DS with an all glass jet separator at 70 eV ionization potential. Elemental analyses were performed by Mr. Erich Meier at the Stanford University Chemistry Department microanalytical laboratories. All temperatures are in degrees Celsius.

7-Methyl-3-methylene-7-octen-1-yl Acetate (*III*). To 6.25 ml (10 mmol) of 1.6 M *n*-butyllithium in hexane at room temperature and under a nitrogen atmosphere was added 1.49 ml (1.16 g, 10 mmol) of *N*,*N*,*N*′,*N*′-tetramethyl-

ethylenediamine (TMEDA) dropwise. After 30 min, 430 mg (5.0 mmol) of 3-methyl-3-buten-1-ol in 1 ml of hexane and another 0.75 ml of TMEDA were added, and the resulting yellow mixture was stirred 3.5 hr at room temperature. The reaction mixture was cooled to 0°, and 745 mg (5.0 mmol) of 1-bromo-3-methyl-3-butene in about 1 ml of hexane was added at once. The now colorless solution was stirred for 1 hr, and then water was added to the reaction. The mixture was poured into ether and 5% aqueous HCl. The ether fraction was separated, washed with saturated $NaHCO_3$ and brine, and dried (Na_2SO_4). Solvent was carefully removed in vacuo, and the residue was stirred for 2 hr at 60° with 0.66 ml (0.71 g, 7 mmol) of acetic anhydride and 0.8 ml (10 mmol) of dry pyridine under a nitrogen atmosphere. Ice was added to the reaction mixture, and after 30 min the mixture was poured into pentane and 5% aqueous HCl. The pentane fraction was washed with 2 M Na_2CO_3 and brine and was dried (Na_2SO_4). Removal of solvent in vacuo gave about 1 g of crude product which was applied to two 1-m × 20-cm preparative silica plates (impregnated with Rhodamine 6G) which were developed in 5% ether in hexane. Recovery of the purified ester followed by microdistillation [bp 60° (bath) 0.9 mm] gave 392 mg (2.0 mmol, 40% yield) of acetate III in a chemical purity of 97.3% as determined by GLC analysis (2 m 3% OV-17/0.4% Carbowax). IR (neat) 3075 ($C=CH_2$) and 1740 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 4.80 (br s, 2H), 4.70 (br s, 2H), 4.17 (t, 2H, $J = 7$ Hz), 2.33 (br t, 2H, $J = 7$ Hz), 2.07 (s, 3H) and 1.73 ppm (br s, 3H). MS (CI, CH_4) *m/e* (relative intensity) 137 (100). Analysis: Calc'd for $C_{12}H_{20}O_2$: C, 73.43; H, 10.27. Found: C, 73.48; H, 10.21.

(Z)-3,7-Dimethyl-2,7-octadien-1-yl Acetate (IV). A mixture of 90 mg (0.58 mmol) of (*Z*)-3,7-dimethyl-2,7-octadien-1-ol (VI) (Anderson et al., 1979), 150 μl of acetic anhydride, and 250 μl of pyridine was stirred under a nitrogen atmosphere at room temperature overnight. Ice was added to the reaction, and after 30 min the mixture was poured into ether and 5% aqueous HCl. The ether fraction was separated, washed with 2 M Na_2CO_3 and brine, and dried (Na_2SO_4). The solvent was removed in vacuo, and the residue was microdistilled [bp 55° (bath) 0.25 mm] to give 88 mg (0.45 mmol, 75% yield) of acetate IV in a chemical purity of 95.5% as determined by GLC analysis (2 m 3% OV-17/0.4% Carbowax). IR (CCl_4) 3070 ($C=CH_2$) and 1740 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 5.40 (br t, 1H, $J = 7$ Hz), 4.72 (br s, 2H), 4.57 (d, 2H, $J = 7$ Hz), 2.06 (s, 3H) and 1.75 ppm (br s, 6H). MS (CI, CH_4) *m/e* (relative intensity) 137 (100). Analysis: Calc'd for $C_{12}H_{20}O_2$: C, 73.43; H, 10.27. Found: C, 73.15; H, 10.04.

3-Methylene-7-octen-1-yl Propanoate (VII). To a suspension of 1.0 g (41.1 mmol) of magnesium turnings in 6 ml of dry ether under a nitrogen atmosphere was added dropwise 5.0 g (33.6 mmol) of 1-bromo-4-pentene in 25 ml of ether at a rate to maintain a gentle reflux of ether. Titration of the

Grignard reagent (Watson and Eastham, 1967) gave a molarity of 0.72 M (~67% yield).

To a solution of 0.68 g (3.3 mmol) of cuprous bromide–dimethyl sulfide complex (House et al., 1975) in 5 ml of dry ether and 10 ml of dimethyl sulfide cooled to −40° under a nitrogen atmosphere was added 4.2 ml (3.0 mmol) of 0.72 M 4-pentenyl magnesium bromide in ether while the temperature was maintained at −35°. After 1 hr, the mixture was recooled to −45°, and 0.41 g (3.2 mmol) of 3-butyn-1-yl propanoate in 10 ml of ether was added while the temperature was raised to −25°. After another 4 hr, 10 ml of saturated aqueous NH_4Cl was added, and the reaction was allowed to slowly warm to room temperature overnight. The mixture was filtered through a pad of Celite, which was washed several times with additional ether. The filtrate was separated into components, and the ether fraction was washed with brine and dried (Na_2SO_4). Careful removal of solvent gave 700 mg of crude product which was purified by application to two 1-m × 20-cm preparative silica plates (Rhodamine impregnated) and development in 10% ethyl acetate in hexane. Removal of a band with an R_f value of about 0.4 followed by microdistillation [bp 65° (bath) 1.0 mm] gave 276 mg (1.41 mmol, 47% yield) of propanoate VII in a chemical purity of 96.5% as determined by GLC analysis (2 m 3% OV-17/0.4% Carbowax). IR (neat) 3075 (C=CH_2) and 1730 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 6.07–5.50 (m, 1H), 5.07 (m, 1H), 4.80 (br s, 3H), 4.17 (t, 2H, J = 7 Hz), and 1.13 ppm (t, 3H, J = 7.5 Hz). MS (CI, CH_4) m/e (relative intensity) 197 ($M^+ + H$, 4), 123 (100). Analysis: Calc'd for $C_{12}H_{20}O_2$: C, 73.43; H, 10.27. Found: C, 73.66; H, 10.27.

Methyl (E)-3,7-Dimethyl-2,7-Octadienoate (IX). Lithium–1% sodium wire (224 mg, 32 mmol) was washed free of grease with hexane and was cut into small pieces upon addition to 5 ml of dry ether under an argon atmosphere. The suspension was cooled to 0° and 2.45 g (15 mmol) of 1-bromo-4-methyl-4-pentene (VIII; Anderson et al., 1979) in 10 ml of ether was added dropwise while the temperature was maintained at 0°. After 2.5 hr, titration of an aliquot (Watson and Eastham, 1967) gave a molarity of 0.75 M (~75% yield).

To a suspension of 2.28 g (12 mmol) of cuprous iodide in 10 ml of dry ether at −40° under an argon atmosphere was slowly added 13 ml (9.75 mmol) of 0.75 M 4-methyl-4-pentenyllithium followed by 1.81 g (15.6 mmol) of TMEDA. After 30 min, the reaction was cooled to −60°, and 980 mg (10 mmol) of methyl 2-butynoate in several ml of ether was added. The reaction was maintained at −60° for 1.5 hr, and then was quenched by the dropwise addition of 4 ml of methanol. The cooling bath was removed, and after the mixture had warmed to room temperature, 10 ml of saturated aqueous $(NH_4)_2SO_4$ was added. After 30 min, the mixture was filtered through a Celite pad which was washed thoroughly with additional ether. The filtrate was

separated into components, and the ether fraction was washed with water and brine and then dried (Na_2SO_4). Careful removal of solvent in vacuo followed by microdistillation [bp 70° (bath) 3.0 mm] gave 1.50 g (8.3 mmol, 85% yield) of methyl ester IX. GLC analysis (2 m 10% UCON) indicated that IX had been produced with high stereospecificity (*E*:*Z*, 99.0:1.0). IR (neat) 3090 ($C{=}CH_2$) and 1725 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 5.63 (br s, 1H), 4.67 (br s, 2H), 3.67 (s, 3H), 2.13 (d, 3H, $J = 2$ Hz), and 1.70 ppm (br s, 3H). MS (70 eV) *m/e* (relative intensity) 182 (M^+, 3), 95 (100). Analysis: Calc'd for $C_{11}H_{18}O_2$: C, 72.49; H, 9.95. Found: C, 72.37; H, 9.87.

(E)-3,7-Dimethyl-2,7-octadien-1-ol (X). To a solution of 151 mg (0.83 mmol) of methyl ester IX in 7.5 ml of benzene under a nitrogen atmosphere was added 2.5 ml (4.5 mmol) of a 27.8% solution of diisobutylaluminum hydride (DIBAH) in heptane. After 6 hr, excess hydride was quenched with several ml of methanol. The reaction mixture was poured into a mixture of ether and 5% aqueous HCl. The organic layer was separated, washed with saturated $NaHCO_3$ and brine, and dried (Na_2SO_4). Removal of solvent in vacuo gave 116 mg (0.75 mmol, 90% yield) of alcohol X. IR (CCl_4) 3620 (OH) and 3075 cm^{-1} ($C{=}CH_2$); NMR ($CDCl_3$, δ) 5.42 (t, 1H, $J = 7$ Hz), 4.68 (br s, 2H), 4.12 (d, 2H, $J = 7$ Hz), and 1.68 ppm (6H). Analysis: Calc'd for $C_{10}H_{18}O$: C, 77.87; H, 11.76. Found: C, 77.72; H, 11.70.

(E)-3,7-Dimethyl-2,7-octadien-1-yl Propanoate (XI). A mixture of 121 mg (0.78 mmol) of alcohol X, 207 mg (1.60 mmol) of propanoic anhydride, and 0.15 ml of pyridine was heated at 85° under a nitrogen atmosphere for 2 hr. Ice was then added to the reaction mixture and, after 30 min, was poured into a mixture of ether and 5% aqueous HCl. The ether fraction was separated, washed with 2 M Na_2CO_3 and brine, and dried ($MgSO_4$). Removal of solvent in vacuo and purification by TLC (1-m × 20-cm preparative silica plate impregnated with Rhodamine 6G and developed twice in 5% ether in hexane) followed by microdistillation [bp 50° (bath) 0.1 mm] gave 130 mg (0.62 mmol, 79% yield) of propanoate XI. GLC analysis (2 m 3% OV-17/0.4% Carbowax) of this ester gave a chemical purity of 97.7%. IR (CCl_4) 3070 ($C{=}CH_2$) and 1740 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 5.37 (t, 1H, $J = 7$ Hz), 4.68 (br s, 2H), 4.60 (d, 2H, $J = 7$ Hz), 1.73 (br s, 3H) and 1.13 ppm (t, 3H, $J = 7.5$ Hz). MS (70 eV) *m/e* (relative intensity) 57 (100). Analysis: Calc'd for $C_{13}H_{22}O_2$: C, 74.25; H, 10.54. Found: C, 74.14; H, 10.67.

Greenhouse Bioassay of Pheromone Components and Related Compounds. The greenhouse bioassay has been described in detail elsewhere (Gieselmann et al., 1979a). Rubber septa, impregnated with 33 μg of a synthetic chemical, were tested against each other and a solvent blank on five consecutive evenings.

Isolation and Identification of (E) 3,7-Dimethyl-2,7-octadien-1-yl Propanoate (XI). The procedures for rearing the insects, obtaining the crude

pheromone extract from Porapak Q airborne collections, liquid chromatographic fractionation on Florisil, saponification and esterification reactions, description of the gas chromatographic columns employed (OV-101 and XF-1150), and the use of Kovats' retention indices have also been described in detail in earlier work (Gieselmann et al., 1979a, and references therein). Briefly, the San Jose scale were reared on banana squash and a crude extract was obtained from a rinse of a short column of Porapak Q used in the airborne collection. This material was partially purified by fractionation on Florisil, eluting with a solvent gradient of diethyl ether in Skelly B. Aliquots were tested in a greenhouse bioassay, and those fractions attractive to male San Jose scale were combined.

This partially purified extract was concentrated and injected on an OV-101 column. A single fraction was collected from the gas chromatograph which contained I, II, and XI, whose retention times were determined from available synthetic standards. The capillary tube used to collect the material was rinsed into a 1-dram vial, and saponification was performed using dilute NaOH in 95% ethanol. The three saponified pheromone components were collected separately from the OV-101 column, and each was esterified with propanoyl chloride in carbon disulfide.

Electron impact mass spectra were obtained on a Hewlett-Packard 5882 GC-MS data system modified to accept a 30-m wall-coated open tubular capillary column of OV-101. The ionization voltage was 70 eV and source temperature was 200° C.

Field Testing in 1979. For all tests the lure consisted of a total of 100 μg of the chemical(s) soaked into a rubber septum (5 × 9-mm rubber stoppers, sleeve type, Arthur H. Thomas Co.). Five treatments were tested: I, II, XI, a 50:50 mixture of I and II, and a mixture of I, II, and XI in a ratio of 48.5:46.7:4.8, respectively. Blanks were also included in the tests.

In California, tent traps (7.6 cm × 25.4 cm) (Rice and Hoyt, 1980) were used, and testing was performed in a peach orchard. One set of caps (six replicates of each of six treatments) was placed in the orchard on April 4 in a randomized complete block design. A second, freshly prepared, set was put out in the same manner as the first on June 6. Trapping began on June 11 with the traps counted and rerandomized daily until June 18.

Testing for San Jose scale in New York was performed in apple orchards. The tent trap was similar to that used in California except that the sticky surface measured 15.2 × 15.2 cm. Four replicates of each of the five treatments were tested between July 23 and August 14 with trap counts obtained two times a week.

Raw trap counts (X) were transformed (log $X + 1$) and subjected to Duncan's multiple range test at $P = 0.05$. To test for the effects of cap aging, the California data were also subjected to a two-way analysis of variance.

RESULTS AND DISCUSSION

The scale pheromones which have been identified to date are either acetates or propanoates of terpenoid alcohols; thus, the two components of the California red scale sex pheromone (Roelofs et al., 1977) and the yellow scale sex pheromone (Gieselmann et al., 1979b) are acetates, whereas the sex pheromone of the white peach scale (Heath et al., 1979) and of the San Jose scale, I and II, are propanoates. To determine the importance of the acid component of esters I and II for biological activity, the homologous acetates III and IV were prepared from their corresponding alcohols as detailed in Figure 1. Although a synthesis of alcohol V has been previously described (Anderson et al., 1979), the one-step preparation of V shown in Figure 1 is more efficient and convenient. Thus, formation of the dianion of 3-methyl-3-buten-1-ol (isopentenol) with two equivalents of the *n*-butyllithium–*N*,*N*,*N*′,*N*′-tetramethylethylenediamine complex (Cardillo et al., 1974, 1979) in hexane followed by alkylation of the dianion with isopentenyl bromide gave alcohol V, which was then acetylated with acetic anhydride and pyridine to produce the acetate III in a 40% distilled yield from isopentenol. The isomeric acetate IV was obtained by similar acetylation of alcohol VI, prepared as previously reported (Anderson et al., 1979).

To determine the effect of structural modification in the alcohol moiety of esters I and II on attractancy, a homolog of I and the geometric isomer of II were prepared. The synthesis of a 7-desmethyl homolog of I is summarized in

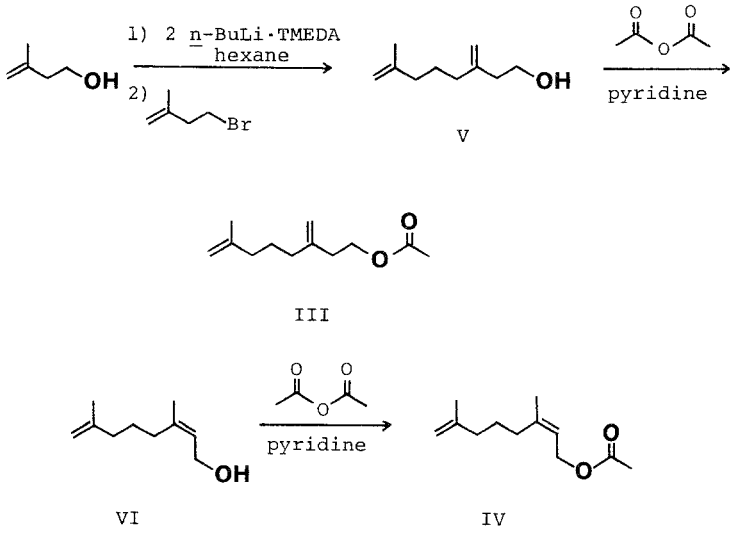
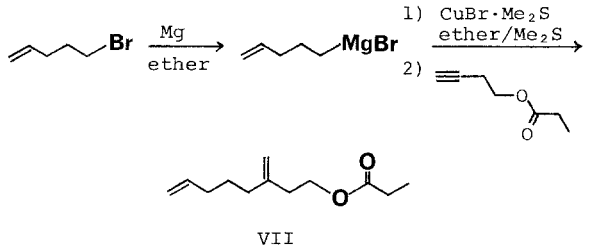


FIG. 1. Synthesis of acetate homologs of the San Jose scale pheromone components.

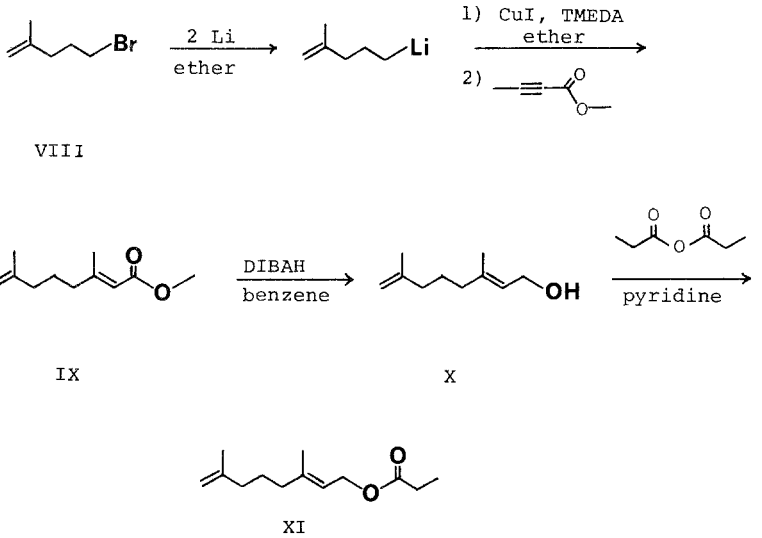


VII

FIG. 2. Synthesis of a 7-desmethyl homolog of pheromone component I.

Figure 2. In a manner similar to that of Alexakis et al. (1975), the Grignard reagent of 1-bromo-4-pentene was first reacted with the cuprous bromide-dimethyl sulfide complex, and then the organocopper intermediate was treated with 3-butyn-1-yl propanoate to give 3-methylene-7-octen-1-yl propanoate (VII).

Since olefin stereochemistry is often a key element of insect sex attractancy, it was of interest to determine if the *E* isomer of pheromone component II was at all attractive to male San Jose scale. The synthesis of this isomer is outlined in Figure 3. Thus, 1-bromo-4-methyl-4-pentene (VIII; Anderson et al., 1979) was converted to the corresponding lithium reagent which was then reacted with cuprous iodide and *N,N,N',N'*-tetramethylethylenediamine in ether. Conjugate addition of the organocopper intermediate to methyl 2-butynoate (Anderson et al., 1975) gave stereospecifically the *E* ester IX.



VIII

IX X

XI

FIG. 3. Synthesis of the *E* isomer of pheromone component II.

Reduction of IX with diisobutylaluminum hydride (DIBAH) gave allylic alcohol X which upon treatment with propanoic anhydride and pyridine yielded XI, the *E* isomer of II. GLC analysis of propanoate XI indicated that the sample contained 1.0% of the *Z* isomer, II.

Greenhouse bioassay for attractancy of the two San Jose scale pheromone components I and II, acetates III and IV, 7-desmethyl homolog VII, and *E* isomer XI was carried out, and the results are summarized in Table 1. As previously noted in the identification work (Gieselmann et al., 1979a), the two components of the San Jose scale pheromone, I and II, were independently attractive and of approximately equal potency. The acetate homologs, III and IV, as well as the 7-desmethyl homolog VII were completely unattractive to male San Jose scale. Of special interest, however, was the attractancy of the *E* isomer, XI, which was as attractive as I or II, its *Z* isomer. The results of this bioassay have led us to reexamine the pheromone blend produced by the San Jose scale.

After partial clean-up of the crude pheromone extract on a column of Florisil, a single fraction was collected from an OV-101 column which contained all isomers. Saponification of this mixture produced three new peaks, which eluted much earlier than the biologically active fraction on OV-101. The retention times of these new compounds on two GLC columns were identical to those of alcohols prepared by hydrolysis of synthetic I, II, and XI. Kovats' retention indices (KRI) for the saponified pheromone components were 1194, 1210, and 1223 on OV-101 and 1808, 1838, and 1872 on XF-1150, respectively. When the alcohols from the natural extract were collected separately from OV-101 and esterified to form the propanoates, the retention times were the same as those of the synthetic esters on both GLC columns. KRI values for I, II, and XI were 1414, 1418, and 1442 on OV-101

TABLE 1. GREENHOUSE TRAP CATCHES OF MALE SAN JOSE SCALE

Compound tested[a]	Mean number of males caught/day[b]
I	68.0 a
II	47.2 a
III	6.2 bc
IV	2.2 c
VII	10.6 b
XI	88.8 a
Solvent blank	4.6 bc

[a]See figures and text for chemical structures.
[b]Means followed by the same letter are not significantly different at $P = 0.05$ according to Duncan's multiple-range test.

TABLE 2. MEAN TRAP CATCHES IN FIELD TESTS FOR SAN JOSE SCALE PHEROMONE COMPONENTS AND MIXTURES

Treatment	Mean male catch/trap/counting period[a]	
	California	New York
Fresh lures		
I	36.8 a	346.1 a
II	38.5 a	242.8 a
XI	58.7 a	236.1 a
50:50 mix of I and II	28.7 a	
48.5:46.7:4.8 mixture of I, II, and XI	49.3[b]	262.1 a
Blank	2.2 c	4.1 b
10.5-week-old lures		
I	23.2 b	
II	24.0 b	
XI	24.0 b	
50:50 mix of I and II	22.2 b	
Blank	3.3 c	

[a]In each column, means followed by the same letter are not significantly different according to Duncan's multiple-range test at $P = 0.05$.
[b]Since this treatment was not replicated among the old lures, it was not included in the two-way analysis of variance.

and 1860, 1860, and 1888 on XF-1150, respectively. The mass spectrum of the esterified saponification product of natural XI was the same as that of synthetic XI and of II, which appears in Gieselmann et al. (1979a).

Unlike the propanoates, the three alcohols were completely resolved by GLC on an OV-101 column. Therefore, it was possible to measure the relative peak areas and determine the pheromone composition. This was found to be 48.5%, 46.7%, and 4.8% of I, II, and XI, respectively.

The results of the 1979 field tests appear in Table 2. The three components of the pheromone were individually attractive to male scale, and there did not appear to be a synergistic effect on trap catch when two mixtures of the components were used as lures. After 10.5 weeks of weathering, the attractancy of the older set of caps in the California test was significantly less than that of the fresh caps. In studies with one of the pheromone components of the California red scale, Tashiro et al. (1979) showed that loadings of 10 and 100 μg on rubber septa kept in direct sun for 16 weeks or held constantly at 43° C for six weeks showed no loss of attractancy. However, the lower molecular weight of the San Jose scale pheromone components would be expected to greatly increase their volatility.

The unexpected biological activity of synthetic (*E*)-3,7-dimethyl-2,7-octadien-1-yl propanoate as a San Jose scale attractant and subsequent identification of this ester along with its *Z* isomer and another positional isomer as components of the San Jose scale sex pheromone represent a departure from the high degree of stereoisomeric specificity of other scale pheromones. Thus, of the four stereoisomers of each of the two components of the California red scale sex pheromone, only a single stereoisomer of each component is attractive to males (Gieselmann et al., 1980).

Likewise, the *Z* isomer of the yellow scale pheromone, (*E*)-3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate, was unattractive to male yellow scale (Gieselmann et al., 1979b; Anderson and Henrick, 1979), and only one of four possible stereoisomers of the white peach scale pheromone was attractive to males (Heath et al., 1979).

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EFFECT OF DAY LENGTH AND LIGHT INTENSITY ON 2-TRIDECANONE LEVELS AND RESISTANCE IN *Lycopersicon hirsutum* f. *glabratum* TO *Manduca sexta*[1,2]

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Abstract—First instar *Manduca sexta* (L.) larvae confined on foliage from *Lycopersicon hirsutum* f. *glabratum* (accession PI 134417) plants grown under a long-day regime exhibited greater mortality than larvae on foliage from plants grown under a short-day regime. 2-Tridecanone, a toxin important in the insect resistance of PI 134417, was significantly more abundant in the foliage of plants grown under the long- than the short-day regimes. Light intensity influenced neither 2-tridecanone levels nor the expression of resistance. The density of glandular trichomes, which secrete 2-tridecanone, was influenced by an interaction between day length and light intensity.

Key Words—*Manduca sexta* (L.), Lepidoptera, Sphingidae, *Lycopersicon hirsutum* f. *glabratum*, day length, light intensity, 2-tridecanone, glandular trichome, resistance, allelochemic.

INTRODUCTION

Accession PI 134417 of the wild tomato species *Lycopersicon hirsutum* f. *glabratum* C.H. Mull is resistant to a number of arthropod pest species including the tobacco hornworm, *Manduca sexta* (L.) (Kennedy and Hender-

[1]Lepidoptera: Sphingidae.
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son, 1978, and references cited therein). The resistance to *M. sexta* has been attributed to the presence of high levels of a toxin, 2-tridecanone, in the exudate of glandular trichomes which abound on the plant's foliage (Kennedy and Yamamoto, 1979; Williams et al., 1980). However, while investigating this resistance we noticed that PI 134417 plants grown in the greenhouse under natural light from November through February were less resistant to *M. sexta* than were plants grown similarly from April through June. It was observed in identical experiments conducted in June and January that larvae fed excised foliage from a single vegetatively propagated, highly resistant plant of PI 134417 average 87% and 8% mortality, respectively, after 72 hr. A knowledge of the factors responsible for the seasonal fluctuation in the level of resistance would contribute to an understanding of the basis for resistance as well as its potential ecological and practical significance. Further, it might also permit a modification of the greenhouse environment to minimize or eliminate the observed seasonal effect.

Since *L. hirsutum* is strongly photoperiodic in its flowering response (Luckwill, 1943) and since the most dramatic seasonal changes observed in the greenhouse environment appeared to involve day length and light intensity, the present study was undertaken to determine the effect of various light regimes on the expression of resistance in PI 134417 to *M. sexta*.

METHODS AND MATERIALS

Two *L. hirsutum* f. *glabratum* lines, PI 134417 and PI 199381, and susceptible *L. esculentum* Mill. were included in this study. PI 134417 is highly resistant and PI 199381 susceptible to *M. sexta* when grown in the greenhouse during the summer months (Kennedy and Henderson, 1978). *L. esculentum* was included as a standard. All plants of each line were vegetatively propagated from a single plant and thus constituted a clone. Plants were grown in plastic pots (24.4 cm) using a substrate of 1/3 peatlite and 2/3 gravel. They were irrigated twice daily, once with distilled water and once with nutrient solution.

The studies were conducted in the controlled-environment chambers of the Southeastern Plant Environmental Laboratory at North Carolina State University, Raleigh. These chambers use a combination of cool white fluorescent and incandescent lamps to provide an illuminance of 451 hectolux at 95 cm from the lamps. In the appropriate treatments, illuminance was reduced by suspending a shade cloth over the plants.

All photoperiods were based on a high-intensity light period of 9 hr. Where appropriate, day lengths were kept effectively long by interrupting the dark period from 11:00 PM to 2:00 AM with 12 W/m^2 of photomorphogenically active radiation (41 hectolux) from incandescent lamps.

Air temperatures were maintained at ± 1/4° C of the set point. Day and night temperatures were 26 and 22° C, respectively. Relative humidity did not drop below 70%.

The experimental regimes were: (1) long day, high light (451 hectolux); (2) long day, low light (226 hectolux); (3) short day, high light; and (4) short day, low light. Six plants of each line were grown for 13 weeks under each regime.

Plants were evaluated for resistance to *M. sexta* by confining three first instar hornworm larvae on a fully expanded leaflet held in a snap-cap, plastic vial (37 mm diam × 62 mm high). A damp strip of filter paper was included to provide moisture. Leaflets were replaced with fresh foliage daily, and survivors were counted after 72 hr. Each experimental treatment was replicated 10 times in each of four evaluations made 38, 55, 69, and 76 days, respectively, following introduction of the plants into the controlled environment chambers. This bioassay procedure has been validated previously (Kennedy and Henderson, 1978).

In that the resistance of PI 134417 has been related to the presence of toxic levels of 2-tridecanone in the exudate from glandular trichomes of the foliage (Kennedy and Yamamoto, 1979; Williams et al., 1980), the effects of various light regimes on the toxicity of the trichome exudate, the abundance of 2-tridecanone, and the density of glandular trichomes were studied.

Crude foliage extracts which included the trichome exudate were collected at 35, 71, and 92 days by soaking 7 g (fresh weight) of foliage from each entry in 75 ml chloroform for 1 hr. Water was removed from the extract by passage through Whatman® phase-separating filter paper onto Na_2SO_4. The extract was then filtered (Whatman® No. 2) to remove the Na_2SO_4, evaporated to dryness, and the residue weighed and redissolved in chloroform to prepare solutions of the following concentrations for bioassay: 0.5, 1.0, 2.0, and 3.0 mg/ml. These solutions were bioassayed for toxicity by confining 20 newly hatched first instar *M. sexta* larvae on a filter paper disk (5.5 cm diam) treated with 1 ml of the solution being tested. The chloroform was evaporated prior to addition of the larvae. After confinement for 6 hr on treated filter paper, the larvae were transferred to susceptible *L. esculentum* foliage for 18 hr, after which mortality was recorded. As controls, larvae were confined in an identical manner on filter paper previously treated with 1 ml of chloroform. Two such bioassays were conducted using the extracts from each sample. Since previous results had indicated that extracts from susceptible *L. esculentum* were not toxic to *M. sexta* when bioassayed at concentrations up to 5 mg/ml, only foliage from PI 134417 and PI 199381 was extracted.

The 2-tridecanone levels present in extracts of PI 134417 foliage were analyzed using gas chromatography. Individual, single-leaflet extracts were prepared from 10 fully expanded leaflets from PI 134417 plants grown under each light regime. Extracts from *L. esculentum* and PI 199381 foliage were not

analyzed because preliminary results indicated the 2-tridecanone levels were extremely low. Individual leaflets were weighed and extracted by steeping in 2 ml chloroform for 24 hr at room temperature. Each leaflet was then rinsed with an additional 2 ml of chloroform and the rinse combined with the extract. Anhydrous Na_2SO_4 was added to each extract to remove water. To each extract, 2-pentadecanone (0.04 mg) was added as an internal standard for gas chromatographic analysis. Each sample was then passed through a Millipore® 0.2-mm type F6 fluorospore filter to remove particulates.

The amount of 2-tridecanone in each extract was determined using a Perkin-Elmer Sigma 3B gas chromatograph equipped with a Spectra Physics model SP800 liquid chromatograph data system. A dual-column system was used to maintain baseline stability. The stationary phase was 10% Carbowax 20M on 100/120 GLQ in a glass column (0.635 × 243.8 cm) (Applied Science Laboratories, Inc.). Analyses were conducted isothermally at a column temperature of 195° C using a flame ionization detector with an air–hydrogen flame. Both the detector and injector were operated at 250° C. The carrier gas was nitrogen at a flow rate of 25 ml/min. Injections consisted of 1 μl of extract. Periodic injections of a standard mixture of 2-tridecanone and 2-pentadecanone were made to monitor any change in the relative response to the two compounds.

The density of glandular trichomes on the foliage of PI 134417 plants grown under each light regime was determined by counting the number of glandular trichomes in three 7.1-mm^2 circles randomly marked on the abaxial surface of each sample leaf with a cork borer. Counts were made on each of 10 fully expanded leaflets at 79 and 86 days. Data from these two samples were pooled prior to statistical analysis. Trichome density was not measured for either *L. esculentum* or PI 199381 since neither was resistant to *M. sexta*.

All data were subjected to factorial analysis of variance. Percentage data were transformed to arcsin $\sqrt{\%}$ prior to analysis.

RESULTS

Excised Leaflet Bioassays. Across all light regimes the greatest larval mortality was observed on PI 134417; however, there was a highly significant day length–plant line interaction (Figure 1) attributable to the greater larval mortality on PI 134417 foliage grown under the long- than under the short-day regime. Day length did not significantly affect the level of resistance of either PI 199381 or *L. esculentum*. Neither the light intensity–plant line, day length–light intensity, nor the plant line–day length–light intensity interactions were statistically significant.

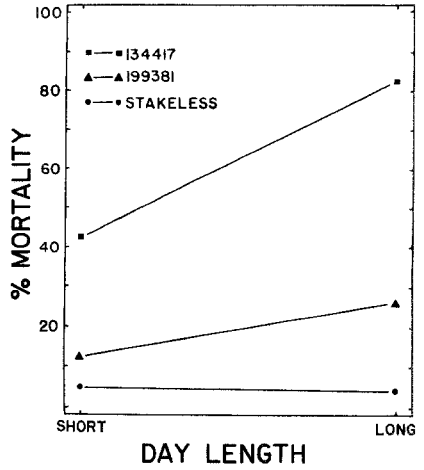

FIG. 1. Mortality of *M. sexta* larvae confined on excised foliage of three tomato lines grown under different light regimes. *L. hirsutum* f. *glabratum* = 134417 and 199381; *L. esculentum* = Stakeless. (day length–plant line interaction significant $P \leqslant 0.0001$).

Extract Studies. Bioassays revealed no mortality among larvae exposed to foliage extracts from PI 199381 even at the highest rate tested. The extract from PI 134417 was toxic to *M. sexta* larvae, but the day length–extract concentration interaction was highly significant (Figure 2; $P \leqslant 0.001$). This interaction was manifest in the more rapid increase in larval mortality with concentration observed with extract from plants grown under the long- than

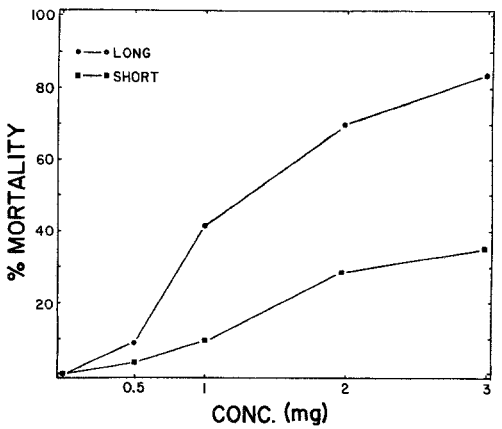

FIG. 2. Toxicity to *M. sexta* larvae of foliage extracts from PI 134417 plants grown under long- and short-day regimes (day length–extract concentration interaction significant $P \leqslant 0.001$).

TABLE 1. AMOUNT OF 2-TRIDECANONE IN SINGLE-LEAFLET EXTRACTS FROM PI 134417 PLANTS GROWN UNDER DIFFERENT LIGHT REGIMES[a]

Day length	Light intensity	Amount of 2-tridecanone (% foliage fresh wt.)	$\bar{X}$ percent larval mortality[b]
Long	High	0.36 a	91 a
Long	Low	0.43 a	72 a
Short	High	0.22 b	35 b
Short	Low	0.22 b	47 b

[a]Mean separation vertical $P \leqslant 0.05$.
[b]Results from excised leaflet bioassays with *M. sexta*.

under the short-day regime. None of the other testable interactions were statistically significant. In no case was any mortality observed in the controls.

The amount of 2-tridecanone in the foliage extracts of PI 134417 was significantly affected by day length, but not light intensity (Table 1). Greater quantities of 2-tridecanone were present in the extracts from plants grown under long- than under short-day regimes.

Trichome Density. Day length and light intensity interacted to significantly influence the density of glandular trichomes of PI 134417 foliage (Figure 3; $P \leqslant 0.001$). There was little difference in trichome density between plants grown under both short-day regimes and under the long day–low light intensity regime, but trichome density was far greater on plants grown under the long day–high light intensity regime.

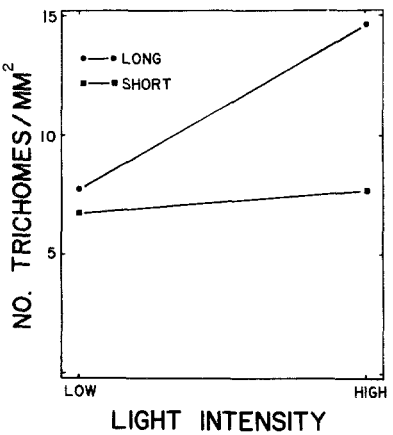

FIG. 3. Density of glandular trichomes on foliage of PI 134417 plants grown under different light regimes (day length–light intensity interaction significant $P \leqslant 0.001$).

DISCUSSION

As in previous studies, *L. hirsutum* f. *glabratum* PI 134417 was highly resistant to *M. sexta* larvae, while PI 199381 and *L. esculentum* were susceptible (Kennedy and Henderson, 1978; Kennedy and Yamamoto, 1979). Although mortality of larvae on PI 134417 foliage was greater than on the susceptible lines under all light regimes, the expression of resistance in PI 134417 was strongly influenced by day length, but not light intensity. Resistance was greatest in plants grown under the long-day regimes and was associated with high levels of 2-tridecanone in the foliage (Table 1). Previous studies (Williams et al., 1980) have shown that 2-tridecanone is toxic to *M. sexta*, as well as several other insect species, and is present in the foliage of PI 134417 plants grown under a long-day regime in sufficient quantity to account for the observed levels of resistance. The present observation that 2-tridecanone was about twice as abundant in the highly resistant plants grown under the long-day regimes than the less-resistant plants grown under the short-day regimes provides additional evidence for its role in the resistance of PI 134417 to *M. sexta*.

Repeated observations have shown that first instar *M. sexta* larvae placed on PI 134417 often die quickly without feeding, apparently because they receive a lethal dose of 2-tridecanone through either cuticular contact with the trichome exudate or vapor action, or both (Kennedy and Yamamoto, 1979; Williams et al., 1980). The observed effect of day length on resistance involved more than a simple effect on trichome density, since the densities under the various light regimes did not parallel either the 2-tridecanone levels or the resistance levels as measured in the bioassays. Apparently the availability of sufficient 2-tridecanone to cause the level of resistance observed in the plants (from the long-day regimes was related more to the amount present in the foliage than to the density of glandular trichomes. The occurrence of highly resistant plants with a high level of 2-tridecanone under the long day–low light regime, despite a trichome density similar to that of plants grown under the short-day regimes may have several possible explanations. Relative to plants grown under the short-day regimes, the plants grown under the long day–low light regime may produce a larger volume of exudate per trichome or they may produce a trichome exudate containing a higher percentage of 2-tridecanone. In either case, the amount of 2-tridecanone actually reaching the larvae via the trichomes would be greater. Alternatively, larvae may receive exposure to 2-tridecanone through avenues other than trichome exudate. Ingestion of 2-tridecanone by larvae is apparently of relatively little significance on plants grown under the long day–high light regime since little or no feeding occurs; however, it may be of major importance in the resistance of plants grown under the long day–low

light regime. The data necessary to assess the relative importance of these possible explanations are not presently available.

Although light intensity is not important in mediating expression of the 2-tridecanone-based resistance of PI 134417 to *M. sexta* larvae, its interaction with day length may be important in other *L. hirsutum* f. *glabratum* accessions in which resistance to spider mites is associated with the density of glandular trichomes on the foliage (Stoner 1968, 1970). In other plant species, light intensity significantly affects the expression of insect resistance. In wheat, where resistance to *Cephus cinctus* Norton is associated with stem solidness (Platt and Farstad, 1946; O'Keefe et al., 1960), Platt (1941) observed a loss of resistance when the wheat was grown in field cages or the greenhouse. Holmes et al. (1960) and Roberts and Tyrrell (1961) subsequently demonstrated that this loss of resistance was due to a reduction in the stem solidness of plants grown under low light intensities. Light intensity is also important in mediating the expression of resistance in sugar beet to *Myzus persicae* (Sulzer) (Lowe, 1974).

Day length, which was the most important factor influencing the expression of resistance to *M. sexta* in the present study by virtue of its effect on 2-tridecanone levels, has also been shown by Sinden et al. (1978) to interact with plant age to influence the resistance of PI 134417 foliage to *Leptinotarsa decemlineata* (Say). They reported that resistance, as measured by feeding rate of the beetles, was not affected by plant age under long days; but under short days, flowering and mature plants were more resistant than young plants. Further, they found that mature plants were more resistant when grown under short than under long days. These differences were attributed to higher levels of the glycoalkaloid tomatine, a potent feeding deterrent for *L. decemlineata* (Stürckow and Low, 1961), in the flowering and mature short-day plants than either the young short-day plants or the mature long-day plants. Tomatine levels, however, did not explain the greater level of resistance of PI 134417 than of *L. esculentum* when both were grown under a long-day regime (Sinden et al., 1978). It is entirely possible that the high levels of 2-tridecanone in the foliage of PI 134417 plants grown under long days play a role in resistance to *L. decemlineata*. Preliminary results indicate that 2-tridecanone is toxic to *L. decemlineata*.

Unfortunately, Sinden et al. (1978) obtained their day length treatments by varying the duration of the plants' exposure to photosynthetically active radiation. Thus, interpretation of their results is confounded by the fact that photosynthesis was less in the short day (8 hr) than the long day (16 hr) plants. In the present study, the effect of day length on the expression of resistance was not related to differences in the quantity of photosynthate produced under the long- and short-day regimes. Both treatments received 9 hr of photosynthetically active radiation (λ = 400–700 nm) per day. In the long-day

treatments, day lengths were kept effectively long by interrupting the dark period from 2300 to 0200 hr with 12 W/m^2 of photomorphogenic radiation (λ = 700–850 nm) from incandescent lamps.

Our results explain the observed seasonal changes in the level of *M. sexta* resistance of *L. hirsutum* f. *glabratum* PI 134417 plants as being a response to changes in day length. However, they do not explain the ecological significance of this response. Photoperiodic mediation of the production of 2-tridecanone may enable the plant to allocate a greater portion of its resources to production of 2-tridecanone for defense and whatever other functions it serves during that period of the year when it is of greatest value to the plant. During other periods of the year, the plant may allocate those resources to other functions (see Mooney and Chu 1974). A more complete understanding of its ecological significance must await: (1) a determination of how widespread the photoperiodic response is among *L. hirsutum* f. *glabratum* populations; (2) what the critical photoperiod(s) involved are; and (3) an understanding of the seasonal requirements for 2-tridecanone (e.g., defense) in the native habitats of *L. hirsutum* f. *glabratum*. PI 134417 was originally collected in Equador.

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CHEMICAL ECOLOGY OF *Reticulitermes flavipes* (KOLLAR) AND *R. virginicus* (BANKS) (RHINOTERMITIDAE):

Chemistry of the Soldier Cephalic Secretions

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Abstract—*Reticulitermes flavipes* and *R. virginicus* have been examined for the presence and possible defense functions of soldiers specific secretions. The cephalic extracts for soldiers of both species contained the identical two major sesquiterpenes which were absent from other castes. The sesquiterpenes have been identified as γ_1-cadinene (I) and the corresponding aldehyde (II) by high-resolution nuclear magnetic resonance spectrometry using homonuclear proton decoupling and by high-resolution mass spectrometry. When groups of termite soldiers were exposed to foraging parties of the sympatric native fire ant, *Solenopsis geminata*, the termites utilized only mechanical defenses. No evidence was obtained to indicate that the ants had been sprayed or coated with either an irritant or toxicant, and there was no evidence that an alarm had been promulgated.

Key Words—*Reticulitermes flavipes*, *R. virginicus*, cephalic extracts, γ_1-cadinene, γ_1-cadinene aldehyde, soldiers, Rhinotermitidae, Isoptera.

INTRODUCTION

Recent investigations on the chemical ecology of termites have particularly stressed the elucidation of the chemistry of the cephalic secretions of soldiers

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(Moore, 1974; Prestwich, 1979). It has been largely assumed that these compounds function as either alarm pheromones (Moore, 1969) or as defensive allomones (Prestwich, 1979).

Species in the termite family Rhinotermitidae have proven to be an especially rich source of diverse natural products. For example, the cephalic secretions of soldiers of *Coptotermes lacteus* (subfamily Coptotermitinae) contain a polyphasic mixture of water, mucopolysaccharides, and long-chain alkanes (Moore, 1968), whereas that of *Prorhinotermes simplex* (subfamily Prorhinotermitinae) largely consists of an α,β-unsaturated nitropentadecene (Vrkoč and Ubik, 1974). On the other hand, *Schedorhinotermes* spp. soldiers (subfamily Rhinotermitinae) produce a secretion that contains mixtures of aliphatic and vinyl alkyl ketones (Quennedy et al., 1973; Prestwich et al., 1975). In every case these chemicals arise from the frontal glands of the soldiers and are released via the fontanelle, a small hole in the head capsule which usually leads to a furrow or channel directed towards the labrum.

The subfamily Heterotermitinae is represented by two genera in North America, *Heterotermes* and *Reticulitermes*, both of which are of major economic importance. The soldiers of both genera have a head morphology consistent with the employment of chemical defenses (Prestwich 1979), but no behavioral data have been reported to suggest that the soldiers actually utilize such strategies. We have examined *Reticulitermes flavipes* and *R. virginicus* for the presence and possible defense functions of soldier-specific secretions, and herein report our results.

METHODS AND MATERIALS

Insects. Portions of two colonies each of *R. flavipes* and *R. virginicus* were collected from fallen logs in the DeSoto National Forest ca. 32 km north of Gulfport, Mississippi, during March and April of 1979. The termites were extracted from the logs and separated into workers (undifferentiated larvae beyond the 3rd stage), soldiers, nymphs (differentiated larvae beyond the 3rd stage possessing external wing pads), neotenic reproductives, and larvae. Soldiers were decapitated and their heads extracted with methylene chloride. The other castes were extracted with methylene chloride in toto.

A colony consisting of ca. 50,000 workers of the native fire ant, *Solenopsis geminata*, was obtained from Mr. Al Banks, USDA-SEA, Fire Ant Laboratory, Gulfport, Mississippi. The colony was housed in a plastic nest which opened up onto a large foraging arena.

Evaluation of Termite Soldier Defense Behaviors. A 15-cm-diameter open Petri dish, lined with filter paper and containing 10 *R. flavipes* soldiers, was carefully placed in the foraging arena of the fire ant colony. Visual observations were then recorded continuously until all 10 soldiers were dead

(ca. 1 hr). The experiment was repeated three times on three separate days using soldiers from three separate *R. flavipes* colonies collected in May 1979. Observations noted included orientation and attack behavior of the termites and behavioral responses of the ants which had been attacked by the termites. All ants which were killed by the soldiers on any given day ($N = 4$–6) were pooled, extracted in 100 μl of CS_2, and examined for the presence of soldier cephalic products by gas chromatography at maximum electrometer sensitivity. In addition, 10 *R. flavipes* soldier heads were individually crushed with jewelers forceps. Single *Solenopsis* foragers ($N = 10$) were then directly coated with the exuding soldier cephalic secretion and their behavior followed for 1 hr.

Quantitation of Soldier Secretions. Individual soldiers of *R. flavipes* from two colonies were decapitated and their heads individually crushed and suspended in 100 μl of CS_2. After 30 min, duplicate 3-μl GC injections were made onto a 1.83-m × 3-mm ID stainless-steel column packed with 3% (w/w) SP-2100 on 100/120 mesh Supelcoport in a Shimadzu GC-6AM equipped with dual flame-ionization detectors.[4] All GC analyses utilized temperature programing from 150 to 250° C at 5° C/min. Peak height values were compared to those produced by known amounts of two sesquiterpenoids of identical molecular composition and oxidation state (daucene for the hydrocarbon and 3-oxo-7β,10α-selina-4,11-diene for the aldehyde) as the termite sesquiterpenes. A total of 10 *R. flavipes* soldiers were assayed, five from each of the two colonies.

Collection of Secretions of Reticulitermes flavipes *and* R. virginicus *Soldier Heads.* Freshly excised soldier heads were suspended in methylene chloride. After standing in the freezer for approximately three months, the methylene chloride solutions were warmed to room temperature, decanted, and concentrated at room temperature under a stream of argon. Fresh methylene chloride was added to the soldier heads and after standing several days in the freezer, the above process was repeated. Measured amounts of dry methylene chloride were added to the residues for analysis by GC–mass spectrometry.

Mass Spectroscopic and Nuclear Magnetic Resonance Analyses. Mass spectra were measured using a Varian-MAT 112S mass spectrometer with a Varian SS200 data processing system. A Varian 3400 gas chromatograph was used for introduction of samples into the mass spectrometer. Analytical gas chromatography was done with a Hewlett-Packard model 402 gas chromatograph or a Varian model 2700 gas chromatograph; the latter was also used for preparative gas chromatography. A 4-mm × 2-m 3% OV-17 on Gas Chrom Q

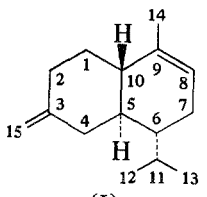
[4] Mention of trade names is solely to identify materials used and does not constitute an endorsement by the U.S. Department of Agriculture.

(100–120 mesh) U-shaped column was used in the Hewlett-Packard instrument while a 7-mm × 2-m 3% OV-17 on Gas Chrom Q (60–80 mesh) coiled column was used in the Varian instrument. Samples were collected in U-shaped glass capillary tubes cooled in dry ice–acetone. Nuclear magnetic resonance spectra were recorded with a Bruker WH-250 and a Bruker WH-400 spectrometer with an ASPECT-2000 data system. Samples were run in a 5-mm tube using $CDCl_3$ as solvent. Optical rotatory dispersion curves were measured with a JASCO ORD/UV 5 spectrophotometer using a 1-cm quartz cell with samples dissolved in $CHCl_3$.

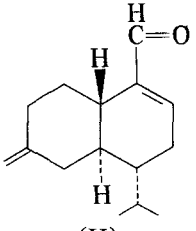
RESULTS AND DISCUSSION

Gas chromatographic analyses at 160° indicated that cephalic extracts for soldiers of both *R. flavipes* and *R. virginicus* contained two major constituents with retention times (R_t) of 2.1 and 6.7 min, respectively. When extracts from other castes were analyzed, the peaks corresponding to these components were not seen. The two compounds in each of the two species were shown to be identical by mixed injection in the gas chromatograph and by their having identical mass spectral fragmentation patterns. Each compound was collected by preparative gas chromatography and shown to be homogeneous by comparison of the mass spectral fragmentation patterns of the front, top, and back of the peaks. The homogeneity was also confirmed by the high-field NMR spectra.

The compound giving the peak of lower retention time (2.1 min) has been identified as γ_1-cadinene (I), while the compound with R_t = 6.7 min has been identified as the previously unreported aldehyde II as follows:



(I)



(II)

The mass spectrum of the lower R_t compound showed a molecular ion at m/z 204 with a base peak (100%) at 161 (Figure 1). A computer comparison was made of the mass spectrum of this material with the EPA/NIH Mass Spectral Data Base (EPA/NIH, 1978) according to the method of Hertz et al. (1971). By this method, similarity indices of 0.729, 0.702, and 0.665 were obtained for (+)-γ-cadinene (III), (+)-γ-murrolene (IV), and (+)-ϵ-murrolene (V), respectively. Thus, a carbon skeleton of the type illustrated by compounds

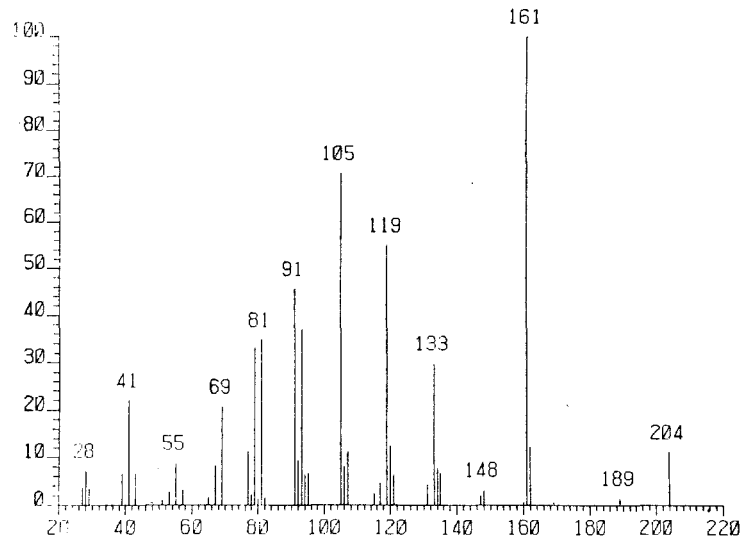
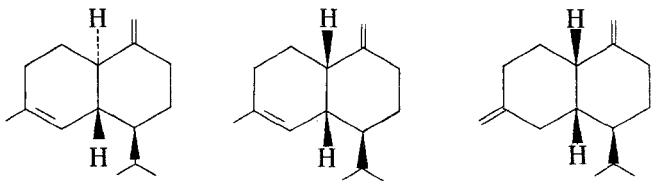


FIG. 1. Mass spectrum of sesquiterpene hydrocarbon.



III–V was strongly suggested for compound I. A 250-MHz [^{1}H]NMR spectrum clearly showed the presence of isopropyl methyl groups (δ 0.75, δ 0.92, d, J = 6.9 Hz), a methyl group on a double bond (δ 1.69, s), an endocyclic olefinic proton (δ 5.56, bs) and two exocyclic olefinic protons (δ 4.53, δ 4.65, d, J = 2 Hz). Thus, a structure such as I, III, or IV (disregarding absolute configuration) was clearly indicated. The compound of longer R_t (6.7 min) showed a molecular ion at m/z 218 (Figure 2), and the exact masses of several fragments (m/z 175, 148, 133) clearly showed the presence of an oxygen atom in the molecule (see Table 1 for mass spectral data of both compounds). That this compound was an aldehyde skeletally related to the above-mentioned sesquiterpenoid hydrocarbon was clear from the [^{1}H]NMR spectrum which showed the isopropyl methyl groups (δ 0.82, δ 0.99, d, J = 6.9 Hz), but the methyl group on the double bond of the hydrocarbon was missing and an aldehyde proton appeared at δ 9.48. The endocyclic double bond was conjugated with this aldehyde (δ 6.90). Two exocyclic olefinic protons remained as in the hydrocarbon (δ 4.62, d, J = 1 Hz and δ 4.74, d, J = 1 Hz).

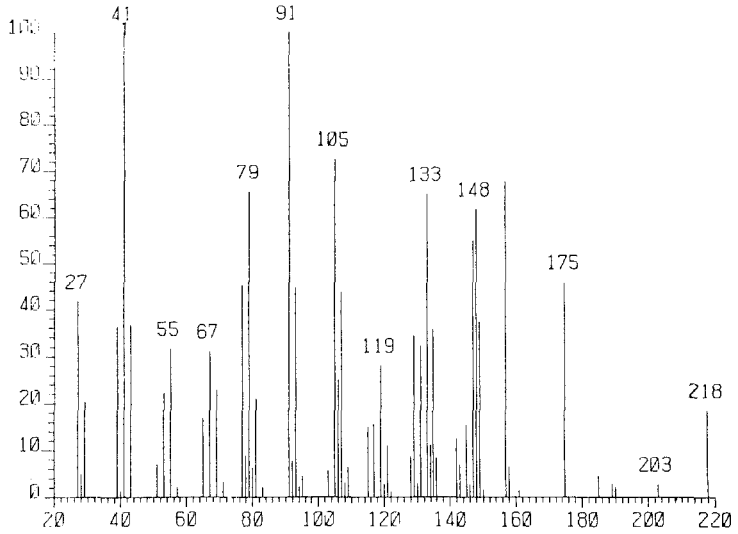


FIG. 2. Mass spectrum of sesquiterpene aldehyde.

Unequivocal proof of the structures of the sesquiterpenoid hydrocarbon and aldehyde was arrived at by a series of homonuclear proton decoupling experiments which were undertaken at 400 MHz and which are outlined in Tables 2 and 3. As a result of these experiments, almost all the protons in the hydrocarbon and aldehyde could be assigned chemical shift positions as shown in Tables 4 and 5. These assignments are only consistent with the structures, including relative stereochemistry, as in I and II.

In the 400-MHz [^{1}H]NMR spectrum of the hydrocarbon (Figure 3) a characteristic multiplet appeared at δ 1.12 as a doublet of quartets with $J = 4$ and 13 Hz, respectively. A similar pattern appeared in the spectrum of the aldehyde (Figure 4) (δ 1.21, d, $J = 4$ Hz, q, $J = 13$ Hz). These signals can be assigned to the axial C-5 hydrogens (H_{5a}) of I and II in the conformations and relative configurations indicated in VI and VII. Thus, the three transaxial protons (H_{4a}, H_{6a}, and H_{10a}) would be expected to give rise to a quartet with a large coupling constant, in this case 13 Hz, and this quartet would in turn be split into a doublet with a small coupling constant, 4 Hz in this case, by the equatorial hydrogen on C-4 (H_{4e}). Irradiation at δ 2.38 in the hydrocarbon and δ 2.44 in the aldehyde resulted in the collapse of the doublet of quartets at H_{5a} to quartets, in each case, with $J = 12.9$ Hz (13 Hz for the aldehyde), thus leading to the assignments of these chemical shifts to H_{4e}. The signals for H_{4e} in each spectrum appeared as doublets of triplets (d 13 Hz, t 3 Hz), with the large value arising from geminal coupling with H_{4a} and the smaller value arising from the previously mentioned H_{5a} and by "W" coupling from H_{2a}. The chemical shift position of H_{4a} in each case could be assigned by noting the

TABLE 1. MASS SPECTRA

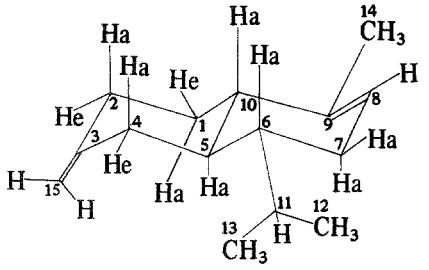
Compound I		Compound II			
m/e	%	*m/e*	%	Calculated for	Observed
41	22	41	100		
69	20	43	37		
79	33	53	22		
81	35	55	32		
91	46	67	31		
93	37	69	23		
105	71	77	45		
119	55	79	65		
120	12	81	21		
133	30	91	100		
161	100	93	45		
162	12	105	73		
204	11	106	25		
		107	44		
		119	28		
		129	34		
		131	32		
		133	65	C_9H_9O 133.0653	133.0761
		134	11		
		135	36		
		142	12		
		145	15		
		147	55		
		148	62	$C_{10}H_{12}O$ 148.0887	148.1001
		149	37		
		157	68	$C_{12}H_{13}$ 157.1017	157.0910
		175	46	$C_{12}H_{15}O$ 175.1123	175.1256
		218	18		

changes occurring on irradiation of H_{4e} and H_{5a}, respectively. The chemical shift of H_{11} could be assigned, in each case, because of its complex multiplicity (d, sep.). Irradiation of H_{11} not only led to the collapse of the C-12 and C-13 methyl doublets to singlets, but it allowed us to assign a chemical shift position to H_{6a}. This was verified by irradiating the signal assigned to H_{6a} which resulted in the expected changes at H_{11} and H_{5a} and also led to assignment of H_{7e} and H_{7a}. In a similar manner, irradiation at H_{5a} led to the chemical shift position of H_{10a} as well as the previously mentioned H_{4e}, H_{4a}, and H_{6a}. Irradiation of H_{10a} led to the chemical shift assignment of H_{1a}, and irradition of the latter led to the assignments of H_{1e}, H_{2a}, and H_{2e}. Thus, almost all of the protons in each compound could be assigned chemical shift positions and the

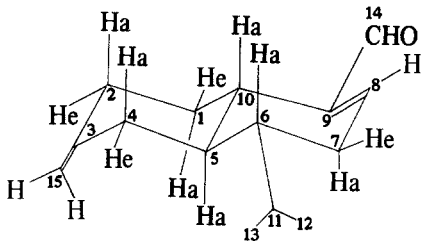
TABLE 2. HOMONUCLEAR PROTON DECOUPLING EXPERIMENTS FOR SESQUITERPENOID HYDROCARBON

Position of irradiation (δ ppm)	Observed changes	
	Position (δ)	Change
1.12	1.21	bt → m
	1.76	m → m
	2.04	m → m
	2.38	d,t → d (12.9), d (3.2)
1.23	2.18	d, sep → sep (6.9)
1.63	1.21	bt → m
	1.76	m → m
1.76	1.12	dq → m
	1.21	bt → m
	1.48	dq → m
	1.63	m → m
	1.94	m → d (13.9), d (6.4)
	2.03	m → m
1.94	1.48	dq → t (12.6), d (6.9)
	1.76 ?	m → m ?
	2.04	m → m
2.04	1.12	dq → m
	1.48	dq → q (12.6)
	1.94	m → m
	2.38	dt → m
2.18	0.75	d → s
	0.92	d → s
	1.21	bt → t (12.6), d (2.1)
2.38	1.12	dq → q (12.9)
	2.04	m → m

magnitude of the coupling constants led to the relative configurations and conformational assignments illustrated in VI and VII.



(VI)



(VII)

We have assigned the absolute configuration of the hydrocarbon as illustrated in VI because it showed a plain positive ORD curve (see Table 6), similar to that previously reported by Andersen et al. (1977) for (+)-γ_1-cadinene isolated from the plant *Scapania undulata*. The magnitude of our rotations are considerably larger than those reported by them. We cannot account for this discrepancy at this time, since we have been unable to attain an authentic sample of *S. undulata* γ_1-cadinene. The enantiomeric (−)-γ_1-cadinene has been reported (Irie et al., 1964; Herout and Sykora, 1958), and we can only conclude from the sign of rotation and our NMR studies that

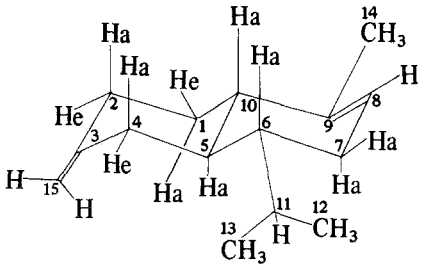
TABLE 3. HOMONUCLEAR PROTON DECOUPLING EXPERIMENTS FOR SESQUITERPENOID ALDEHYDE

Position of irradiation (δ ppm)	Observed changes	
	Position (δ)	Change
1.21	1.42	m → m
	1.87	m → m
	2.08	m → m
	2.44	dt → d(13), d(3)
1.42	1.21	dq → bt
	1.87	m → m
	2.08	m → m
	2.26	d sep → sep (6.9)
1.49	1.87	m → m
	1.92	m → m
	2.14	m → m
	2.50	m → m
1.87	1.21	dq → d(4), t(13)
	1.49	m → m
	2.08	m → m
	2.44	dt → d(13), d(4)
2.08 (2.14)	1.21	dq → m (q?)
	(1.49)	m → m
	1.87	m → m
	(1.92)	m → m
	2.44	dt → m
	2.50	m → m
	1.44	m → m
2.26	0.82	d → s
	0.99	d → s
	1.44	dt → m
2.44	1.21	dq → q(13)
	2.08	m → m
2.50	1.49	m → m
	1.92	?
	2.14	m → m

TABLE 4. NMR ASSIGNMENT FOR SESQUITERPENOID HYDROCARBON[a]

Position	Configuration	δ	J
1	e	1.76 ?	
	a	1.48	d 6.4, q 13
2	a	1.94	m
	e	2.04	m
4	a	2.04	m
	e	2.38	d 13, t 3
5	a	1.12	d 4, q 13
6	a	1.21	bt
7	e	1.63 (1.75)	m
	a	1.75 (1.63)	m
8		5.56	bs
10	a	1.76	m
11		2.18	d 3.2, sep. 6.9
12		0.92 (0.75)	d 6.9
13		0.75 (0.92)	d 6.9
14		1.69	s
15		4.53	d2
		4.65	d2

[a]



(VI)

structure VI correctly represents the isolated hydrocarbon. The aldehyde showed an ORD curve similar to that of the hydrocarbon with a superimposed positive Cotton effect. Therefore, we conclude that the aldehyde has the related absolute configuration illustrated in VII.

Individual soldiers of *R. flavipes* were found to contain relatively large quantities of the cadinene I (2.64 ± 0.57 μg/termite, $X \pm$ SD, $N = 10$) and aldehyde II (2.58 ± 0.87 μg/termite). The average live biomass of an *R. flavipes* soldier is 3.5 mg (Howard, unpublished observation), so that the combined average mass of secretion (5.22 μg) represents 0.15% of the soldier's biomass. Clearly this represents a significant portion of the energy budget of

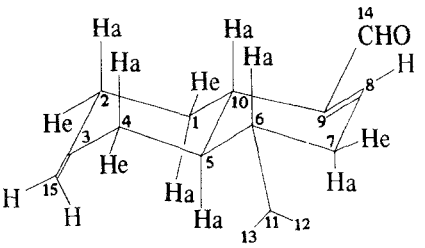
these animals, and hence is likely to be of major ecological significance to the species.

Since these chemicals are found only in the soldier caste, a reasonable hypothesis is that they function as defensive chemicals. The major predators of *Reticulitermes*, as of other termites, are ants. To test the hypothesis that *R. flavipes* soldiers can utilize I and II as chemical defense agents against an aggressive ant predator, we exposed groups of termite soldiers to foraging parties of the sympatric native fire ant, *Solenopsis geminata*. Extended behavioral observations demonstrated that the termites utilized only mechanical defenses against their formicid aggressors. Upon being contacted by an ant (the termites are blind), the soldier would set its legs firmly, rock

TABLE 5. NMR ASSIGNMENTS FOR SESQUITERPENOID ALDEHYDE[a]

Position	Configuration	δ	J
1	e	1.92	m
	a	1.49	m
2	a	2.14	m
	e	2.50	bd 16
4	a	2.08	m
	e	2.44	d 13, t 3
5	a	1.21	d 4, q 13
6	a	1.42	t 12.7, t 3.4
7	e	2.08	m
	a	1.87	m
8		6.90	bs
10	a	1.87	m
11		2.26	d 3.4, sep. 6.9
12		0.99 (0.82)	d 6.9
13		0.82 (0.99)	d 6.9
14		9.48	s
15		4.62	d 1
		4.74	d 1

[a]



(VII)

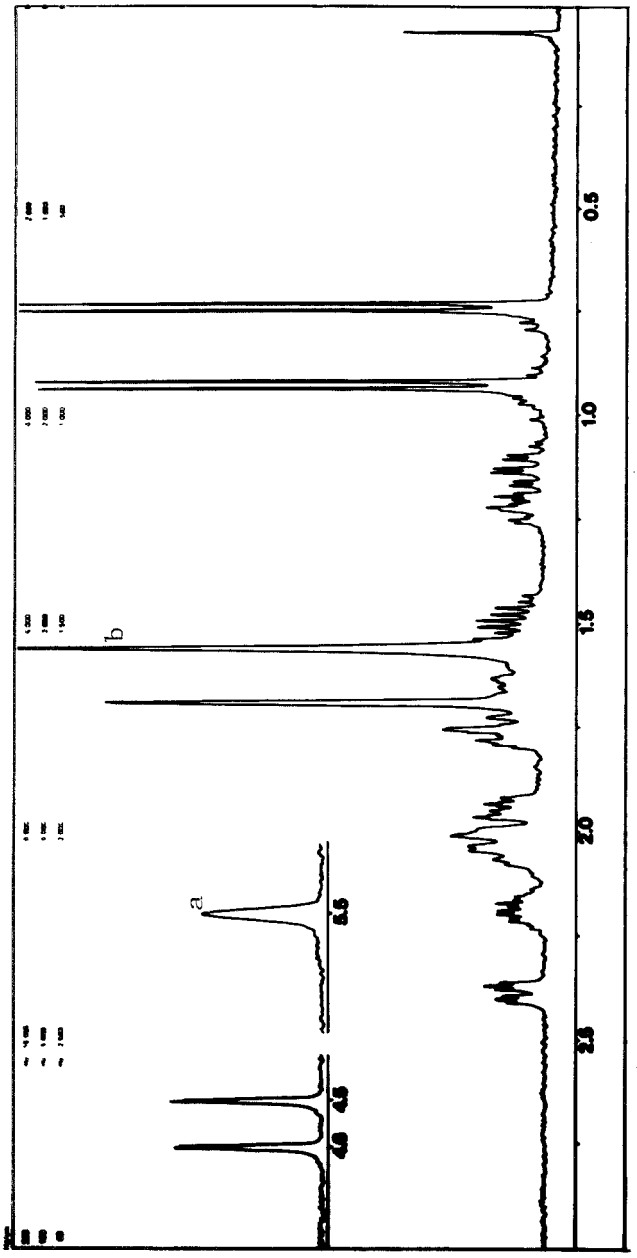


FIG. 3. 400 MHz ^{1}H NMR of the sesquiterpene hydrocarbon. a) Expanded to twice the normal scale. b) H_2O, as determined by the addition of D_2O.

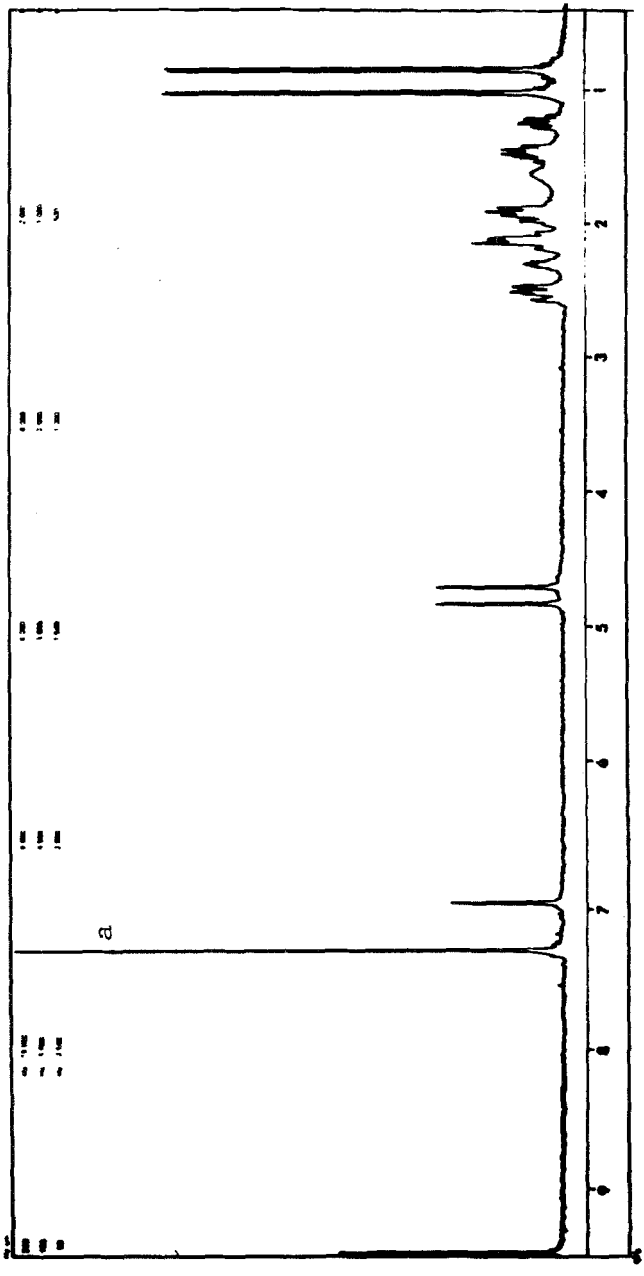


FIG. 4. 400 MHz ^{1}H NMR of the sesquiterpene aldehyde. a) $CHCl_3$.

TABLE 6. OPTICAL ROTATORY DISPERSION DATA

Compound	Concentration[a]	λ (nm)	[α] (degrees)
VI	0.01 g/100 ml in $CHCl_3$	300	1000
		365	700
		400	500
		589	250
VII	0.1 g/100 ml in $CHCl_3$	307	541
		343	1773
		352	1667
		400	729
		589	167

[a]Concentrations were determined indirectly by GLC peak area comparisons with weighed samples of daucene for the hydrocarbon and 3-oxo-7β,10α-selina-4,11-diene aldehyde.

backwards slightly, and then lunge forward rapidly with its body while snapping its open mandibles shut. If the ant was struck by the soldier's mandibles (a rare event), it would stagger away, often dragging its body with only 1 or 2 pairs of legs. Never was any evidence obtained indicating that the ants had been sprayed or coated with either an irritant or toxicant. None of the other termite soldiers were attracted to the attack site, nor did they show any evidence of perceiving an alarm pheromone. The wounded ants showed no evidence of grooming behavior. As they died, they did not display any motor patterns suggestive of chemical poisoning. Chemical examination of the pooled dead ants at the maximum sensitivity of our gas chromatograph (minimal detectable amount of either I or II being ca. 100 pg) failed to provide any evidence for the presence of either I or II on the bodies of the ants.

Finally, ants which were deliberately smeared with cephalic secretions of crushed soldier heads evinced only momentary grooming behavior (10–30 sec) and no evidence of irritability or toxicity over a 1-hr period. We therefore conclude that *R. flavipes* is not using the sesquiterpenes as defensive agents. Indeed, since the soldier caste makes up only ca. 2% of the colony (Howard and Haverty, 1980), it is difficult to conceive how the colony could depend upon such a defensive strategy. Rather, it has been our experience that the worker caste (which makes up ca. 85% of the colony) is the primary defense guild. Other termite species (primarily in the family Termitidae) do depend on their soldiers for colony defense, and the cephalic secretions of these soldiers appear to function at least in part as defensive allomones (Eisner et al., 1976; Prestwich et al., 1977; Longhurst, 1978).

The ecological functions of I and II thus remain unexplained. Experiments are in progress in our laboratories evaluating other possible roles of

these compounds in rhinotermitid biology. In addition, we are studying the chemistry of the frontal gland secretions of additional members of the Rhinotermitidae, the genera of which have already been demonstrated to produce a wide variety of natural products.

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RELATIVE EFFICIENCY OF STICKY AND CYLINDRICAL ELECTRICAL GRID TRAPS IN CAPTURING *Spodoptera exigua*[1,2]

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Abstract—The relative efficiency of sticky traps and cylindrical electrical grid traps for capturing male beet armyworm moths, *Spodoptera exigua* (Hübner), was studied using night vision equipment. The sticky and grid traps captured 34 and 40%, respectively, of the insects which oriented to them. The grid trap's capacity was limited only by the size of the receptacle (3.8-liter bucket) at the base of the funnel. The maximum capacity of the sticky trap was ca. 110 insects, and the efficiency of this trap declined rapidly when captures exceeded 50% capacity. This decline was caused by the physical blockage of the sticky surface by the captured insects. There was no evidence to indicate that captured males had any repelling effect on other males subsequently attracted to the sticky trap.

Key Words—Beet armyworm, *Spodoptera exigua*, Lepidoptera, Noctuidae, pheromone trap, trap efficiency, Pherocon 1C trap, population monitoring.

INTRODUCTION

When traps are utilized to monitor population levels, different types of traps placed in the same area often will capture different numbers of insects. A

[1]Lepidoptera: Noctuidae.
[2]Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the USDA.
[5]Research supported under a cooperative agreement with AR/SEA, USDA. Present address: Yale University, Department of Epidemiology and Public Health, New Haven, Connecticut 06510.

variety of factors, including trap design and placement, are significant in influencing trap captures (Lewis and Macaulay, 1976). Due to economic considerations, a choice often must be made between relying upon a few expensive traps or a greater number of inexpensive ones for population monitoring. To compare the merits of such alternative strategies, the efficiency of a relatively cheap, disposable trap (Pherocon 1C®) was examined and compared to that of a cylindrical electric grid trap for capturing the beet armyworm, *Spodoptera exigua* (Hübner).

Trap captures and direct observation of insect responses to traps have been utilized to evaluate trap efficacy for diurnal moths (Sharp et al., 1976). Until recently, trap captures of nocturnal insects provided the only basis for evaluating trap efficiency. The develpment of a technology specifically designed to enhance night vision has meant that behavioral responses of nocturnal insects to traps can be studied directly (Lingren et al., 1978). This approach, in conjunction with trap captures, was used to assess the trap efficiency of the Pherocon 1C and electric grid traps.

METHODS AND MATERIALS

Beet armyworms (BAW) in laboratory culture were reared on artificial diet (Burton, 1970). Insects were sexed in the pupal stage (Butt and Cantu, 1962) and placed into separate screen holding cages (35 × 46 × 37 cm) until eclosion. Daily sequential tranfers of pupae were made to uninhabitated cages so each holding cage contained insects of a single age and sex. Females were maintained under a reverse light cycle of 14:10 light–dark (65% relative humidity, 24° C). A 25-W incandescent bulb, regulated with a rheostat to an intensity of 0.2 lux at 1 m, served as a night light.

Pheromone Extraction. Pheromone extractions were begun 7 hr into the scotophase. Calling females (2–6 days old) were grasped on the abdomen and gently squeezed to extrude the 8th and 9th abdominal segments. These segments were removed with forceps and placed into hexane. Light for this procedure was provided by a battery-powered head lamp masked with red cellophane.

After a 30-min soak in hexane, each day's collection was filtered using Whatman® No. 1 phase-separating paper to remove particulate matter. The filtrates were pooled and stored at −60° C until bioassay.

Trap Location. From May to September 1978 bioassays were perfomed at various sites in Alachua County, Florida, where heavy concentrations of pigweed, *Amaranthus* spp., were present. Pherocon 1C sticky traps (Figure 1) located ca. 1.1 m above ground level on metal stakes were utilized in trapping and observational studies. These traps were located 20 m apart with baits assigned in a randomized block design, and treatments were rotated daily.

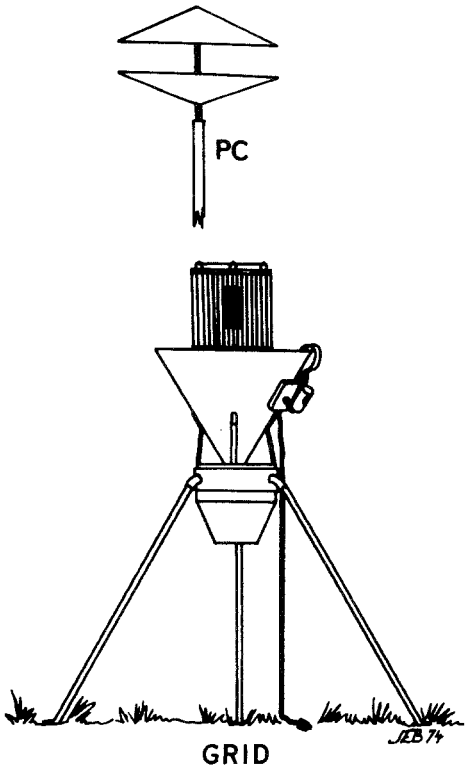

FIG. 1. Pheromone traps evaluated for capture of beet armyworm males: Electrocutor grid and Pherocon 1C (PC).

Daily counts of male captures were made, and the sticky trap bottoms were replaced as necessary. A cylindrical electric grid trap (Figure 1), located 20 m from the nearest Pherocon trap, was used for the observational study. The grid was powered from a 12-V DC power source (heavy-duty automobile battery) (Mitchell et al., 1973).

Depending upon the experiment, traps were baited with either 3 live virgin BAW females enclosed in small plastic cylindrical cages (4 × 6 cm) with screen ends, or 5 female equivalents (FE) of hexane extracts from abdominal tips on Whatman No. 1 filter paper disks. The bait was suspended inside the traps with wire. Females in the field were replaced every third day. Hexane extracts were renewed nightly about 0230 hr, at the start of the peak period of BAW mating activity (Tingle and Mitchell, 1975). Extracts remained attractive for over 2 hr.

Trap Efficiency. The maximum capacity of the Pherocon trap was determined by leaving 7 traps in the field with their trapping surfaces

untended until no more captures were recorded. The trapping surfaces of the 7 control traps were changed daily. Male captures were recorded daily. Both control and experimental traps were baited every third day with three 2- to 3-day-old virgin females.

Since a decline in trap efficiency through time was observed in the previous experiment, two tests were performed to determine if this was due to physical obstruction or was the result of some chemical signal emanating from the trap. Fifteen modified Pherocon traps were assembled with sticky panels as both the top and bottom. To investigate the possibility that captured males released a chemical which repelled other BAW males, panels placed in the top position had been previously used as sticky bottoms in other traps and had variable numbers of feral males adhering to them. Treatment traps and 15 matched control traps contained new sticky panels in the bottom position. Tests were run on 8 nights with each matched pair serving as one replication.

An experiment was performed to determine if the decline in trap efficiency was attributable to a reduction in the effective trap area. Effective trap area was considered as the sticky surface not covered by insect bodies or scales. Sticky bottoms which had each captured 70 ± 5 feral BAW males were matched in pairs and then placed in the field adjacent to each other. One member of each pair had all captured insects removed, while males captured in the other trap were left intact. Test traps and controls with new sticky bottoms were baited with 3 virgin females. Replications, each consisting of a matched pair of treatment and control traps, were performed on 5 nights.

The Pherocon trap consisted of two cardboard panels, the lower one covered with a sticky adhesive mounted on a wire frame (Figure 1). It can be constructed with varying degrees of openness on its sides and ends. Four modifications of trap form were evaluated to determine their influence on capture of BAW males: fully open; 1/4 compressed; 3/4 compressed; and fully compressed with only the ends open. Each trap was baited with 3 virgin laboratory-reared females. Fifteen replications, each consisting of a test of the four treatment traps on a single night, were performed.

Observations of male BAW responses to pheromonal stimuli emanating from Pherocon and electric grid traps were made using night vision goggles (Varo model 9682, Varo, Inc., Garland, Texas). Traps were baited with either 2-day-old virgin BAW females or hexane extracts (5 FE). Insect movement within a 2-m radius of these traps was scored as follows: (1) insect attraction, consisting of insect flight of any origin within a 2-m radius of a trap; (2) insect orientation to the trap, typically consisting of hovering within a 1 m of the trap; and (3) insect capture by the trap. To facilitate analysis, insects were scored on the basis of their ultimate behavioral response, i.e., a capture was not scored as attraction or orientation. Insect responses were not scored with regard to wind direction. Observations were made for 68 ten-min periods,

each of which served as a replicate, beginning at 0200 hr on 9 nights during August and September 1978. Using the data from these observations, calculations were made to determine trap capture efficiences. These percentages were determined as follows:

$$\frac{\text{Insects captured}}{\text{Insects oriented + insects captured}} \times 100 = \text{Trap capture efficiency}$$

RESULTS AND DISCUSSION

The maximum trap capacity of the Pherocon trap was ca. 110 BAW males. Depending upon the population levels, the time required to reach trap capacity ranged from 5 to 16 days ($\bar{X}$ = 11.3 days). Using male captures as a measure of trap efficiency, the Pherocon traps declined after the traps became half full; the time to 50% trap capacity ranged from 2 to 7 days ($\bar{X}$ = 4 days). It took from 2 to 8 days, with a mean of 4.2, for control trap catches to exceed paired experimental catches by 10%.

Experiments designed to determine the cause of the reduction of trap efficiency (Table 1) revealed that traps constructed with sticky tops containing dead males captured a mean of 17.1 BAW males/night; control traps attained catches averaging 22.0 BAW males/night. There was no significant difference in trap catch between experimental and control traps, regardless of the number of insects adhering to the sticky top, indicating that BAW males were not repelled by odors emanating from the captured (dead) insects. Traps that had captured insects removed from their sticky surface did not differ significantly from traps that retained insects (Table 2). Control traps, which captured the highest mean number of insects, differed significantly in trap

TABLE 1. MEAN CAPTURE OF WILD *S. exigua* MALES IN PHEROCON 1C TRAPS[a]

No. of males on sticky top panel	No. of trap nights	$\bar{X}$ capture/trap night[b] (± SE)
0	15	22.0 ± 3.2
1–30	7	11.7 ± 2.2
31–51	8	21.7 ± 6.7
1–51	15	17.1 ± 3.8

[a] Sticky panels retained various numbers of males mounted in the upper position. Traps baited with three 2-day-old virgin females.

[b] Each treatment trap was compared to a control trap having no males on sticky top panel. Differences between means are not statistically significant at the 5% level, Student's *t* test.

TABLE 2. MEAN CAPTURE OF WILD *S. exigua* MALES IN VIRGIN-FEMALE-BAITED NEW AND USED PHEROCON 1C TRAPS

Trap	$\bar{X}$ capture/trap night (± SE)[a]
Control (new)	18.0 ± 3.4 a
Insects retained (70 ± 5)	5.0 ± 2.2 b
Insects removed (70 ± 5)	7.8 ± 3.3 b

[a] Five replicates/treatment. Means followed by the same letter are not significantly different at the 5% level, Duncan's multiple-range test.

catch from both of the experimental traps. The difference in captures between the new trap and the used traps (with and without insects) indicates that the adhesive material in used traps was insufficient for capturing the responding insects. In case of the used trap with insects, the sticky surface apparently was partially blocked by the presence of the dead carcasses. In the trap from which the captured BAW had been removed, much of the adhesive was also removed, hence, the reduced captures.

Physical obstruction of the sticky areas of the trap by insect scales and debris determined the maximum trap capacity of 110 insects. Captured insects did not influence subsequent captures except by blocking areas of the trap, indicating they did not actively repel other insects. Since trap efficiency declined after the Pherocon trap was ca. 50% full, Pherocon 1C traps used to monitor adult BAW populations should be changed when ca. 55 insects are captured, or when the traps become cluttered with dirt, leaves, and other debris.

When structural modifications of the Pherocon 1C trap were tested, the nightly mean capture of the fully open form (Table 3, trap A) was 25.6 males. All other traps captured fewer BAW males, but traps A, B, and C did not differ significantly in effectiveness. Only trap D, the most compressed trap form, captured significantly fewer males than did the other traps. It seems probable that these results are due to the nature of the pheromone plume. Evaluating a variety of trap designs, including the Pherocon 1C trap, Lewis and Macaulay (1976) determined that plume shape was a critical factor influencing trap catches and suggested that traps which generated elongated plumes be used for monitoring purposes. As a wind-blown chemical trail, the pheromone plume is very susceptible to disruption by physical barriers. Once disrupted it is difficult, if not impossible, for males to follow it to females (Kennedy, 1977). Trap D, with its closed sides, restricted and altered wind flow thereby disturbing the pheromone plume. Practically, this means that the Pherocon 1C trap should always be assembled with openings on the sides and ends. In order to prevent dust and debris from entering the trap and covering

TABLE 3. MEAN MALE *S. exigua* CAPTURED IN MODIFIED PHEROCON 1C STICKY-TRAPS BAITED WITH 3 VIRGIN LABORATORY-REARED FEMALES

Trap	Description	Opening (cm)		Mean capture/trap night (± SE)[a]
		Side	End	
A	Fully open	3.5	6	25.6 ± 4.3 a
B	1/4 compressed	2.5	4	20.7 ± 4.3 a
C	3/4 compressed	1.2	2	20.0 ± 4.4 a
D	Fully compressed	0	1	4.4 ± 1.6 b

[a] Means followed by the same letter are not significantly different at 5% level, Duncan's multiple-range test. Fifteen replicates per treatment.

its sticky surface, the trap should be constructed in a partially compressed form.

When the Pherocon trap was baited with 3 virgin females, insect activity (including attraction, orientation, and capture) within the observation area (2-m radius around the trap) resulted in capture 12.1% of the time (Table 4). When baited with 5 FE of hexane abdominal tip extract, captures were recorded only 4% of the time. The electric grid trap baited with live females caught 10.4% of the insects observed.

As scored in the observations made in this study, insect flight past the trap is a general measure of nocturnal insect activity. In contrast, insect orientation and insect capture are more precise indices of BAW activity because the pheromone bait is specifically attractive. In order to evaluate trap efficiency solely with regard to BAWs, calculations were made to determine what percentage of insects that oriented to the trap were actually captured. Error exists in these calculations if other unrecognized species of moths oriented to the trap. Only BAWs were captured.

TABLE 4. PERCENTAGE OF EACH TYPE OF ADULT *S. exigua* ACTIVITY IN A 2-m RADIUS OF TRAP, BASED ON OBSERVATIONS WITH NIGHT VISION GOGGLES

Bait	No. of replicates	Insect					
		Attraction		Orientation		Capture	
		No.	%	No.	%	No.	%
Pherocon 1C							
Virgin females (3)	28	133	64.4	49	23.5	25	12.1
Hexane extract (5 FE)	19	130	87.4	13	8.6	6	4.0
Electric grid							
Virgin females (3)	21	178	74.6	36	15.0	25	10.4

The Pherocon sticky trap baited with 3 females and hexane extracts (5 FE) captured 34.0 and 31.8%, respectively, of the insects which oriented to them. The electric grid trap baited with live females provided the most effective combination of trap and bait, capturing 40.9% of the insects which oriented to it. These results suggest that both types of traps are relatively inefficient in capturing the BAWs which are attracted and orient to them. In no case did a trap capture more than 40% of the insects attracted to it. There are several factors with the potential to cause low efficiency.

Low efficiency may have been an artifact of the scoring system. For example, if species other than the BAW were attracted to the trap, but were insufficiently stimulated by the sex pheromone to enter the trap, then trap efficiency with regard to BAW captures would be artificially low. Since all pheromone baits were approximately equal in attractiveness, a uniform depression of trap efficiency would therefore be expected for every combination of bait and trap. Consequently, only relative measures of trap efficiency could be determined.

The inherent ability of a trap to capture insects influences efficiency. The Pherocon trap baited with either hexane extracts or live females captured a fairly consistent percentage of the attracted insects. The more costly electric grid trap was more effective than the pheromone trap, suggesting that this trap might be more wisely used when the threshold of economic damage is low and BAW population must be detected and destroyed early.

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AUTOINTOXICATION MECHANISM OF *Oryza sativa* III. Effect of Temperature on Phytotoxin Production During Rice Straw Decomposition in Soil[1]

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Abstract—The phytotoxicity produced during decomposition of rice straw in soil was evaluated under both constant and changing temperature conditions. Bioassay tests showed that the aqueous extract from a soil–straw mixture after incubation at constant temperature was more than twice as phytotoxic as the extract from soil incubated alone. The phytotoxicity was highest at 20–25°C. Temperatures above 25°C enhanced rice straw decomposition and also degraded the phytotoxic substances more rapidly. After incubation of soil mixtures under changing temperature regimes in a phytotron, the phytotoxicy of the soil aqueous extracts increased in the following order: soil alone < soil + fertilizer < soil + straw < soil + straw + fertilizer. Growth inhibition of lettuce or rice seedlings was also at the highest at the temperature range of 25–30°C irrespective of the direction of temperature changes from either low to high or vice versa. Five phytotoxic phenolics, *p*-hydroxybenzoic, vanillic, *p*-coumaric, syringic, and ferulic acids, were obtained from both the aqueous extract and residue of the incubated soil samples and were quantitatively estimated by chromatography. The amount of phytotoxins found in various soil mixtures followed the same increasing order as that found by the seed bioassay test. Although no definite distribution pattern of the phenolics in the incubated soil samples can be attributed to temperature variations, the amount of the phenolics was likely higher in the samples incubated at 25°C

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than at either 15°C or 35°C. The quantity of toxins released during decomposition of rice straw in soil reached highest levels six weeks after incubation and gradually disappeared after twelve weeks.

Key Words—Autointoxication, allelopathy, *Oryza sativa*, phytotoxins, phytotoxic phenolics, phytotoxicity, temperature effect, rice straw decomposition.

INTRODUCTION

Severe reduction of vegetation productivity has been noted under continuous monoculturing regimes (Muller, 1974; Patrick, 1965; Rice, 1974, 1979). This phenomenon is particularly evident in crop plants in which the unharvested portion of the plant residues is either left on the soil surface or incorporated into the soil. When the plant residues decompose in the soil, phytotoxic substances are released which affect the productivity of subsequent crops (Chandramohan et al., 1973; Chandransekaran and Yoshida, 1973; Chou et al., 1976; Kuwatsuka and Shindo, 1973; McCalla, 1971; Patrick, 1965; Wang et al., 1967). The phytotoxins that have been identified include plant phenolic acids, such as *p*-hydroxybenzoic, *p*-coumaric, ferulic, vanillic, syringic, and *o*-hydroxyphenylacetic acids (Chou and Lin, 1976) and a number of other phenolics (Chou and Patrick, 1976).

In the subtropical climate of Taiwan, two crops of rice (*Oryza sativa*) can be produced in one growing season. However, under these conditions, the effect of monoculturing is accentuated and the yield of the second crop is generally 25% lower than that of the first crop (Wu, 1979). The cause of the rice yield reduction in some areas has been attributed to the autotoxic effects of the crop residues from the first crop (Chou et al., 1976; Houng and Lin, 1977). Phytotoxin production during the decomposition of rice residues in soil can be affected by such factors as temperature and unfavorable water drainage. The water drainage problem may be overcome by improved management practices, but temperature is less manageable and affects the two crops differently. During the first crop season, from early March to mid-July, the average daytime temperature gradually increases from 15°C to 30°C, whereas for the second crop, from early August to mid-December, it reaches 35°C in the second month after rice transplanting and then decreases gradually to 15°C toward the end of the season. In this study, we have attempted to characterize the effects of temperatures on phytotoxin production during the decomposition of rice straw in soil under controlled environmental conditions, simulating the temperature changes during the two crop seasons.

MATERIALS AND METHODS

Materials. Rice straw was obtained from the field after rice (Taichung 65 variety) was harvested at Nankang, Taiwan, in June 1976 and 1977. The rice straw was cut into pieces about 3 mm long. Soil samples from the surface to 20-cm depth were taken from the same rice fields and air-dried. All plant residues including root fragments were removed from the air-dried soil by passing the soil through a 60-mesh sieve.

Experiment I. The effect of temperature on phytotoxin production in soil–straw mixtures was evaluated first under constant temperature conditions. To simulate the soil–straw ratio in paddy fields, 40 g of air-dried soil was mixed thoroughly with 1.2 g of rice straw and submerged in 40 ml of distilled water in a 250-ml wide-mouth plastic centrifuge tube which was then sealed with a cap. Sets of these tubes were placed in constant-temperature incubators set at 15, 20, 25, 30, or 35° C (± 1° C). At two-, four-, and six-week intervals, four tubes from each treatment were examined for phytotoxicity and for phytotoxin determinations. In the controls, soil samples were incubated at the same temperatures but without the rice straw.

Experiment II. The effect of soil treatments and temperature changes on soil phytotoxicity was then evaluated under controlled environmental conditions. The soil treatments were as follows: (1) soil alone as control, (2) soil + straw, (3) soil + fertilizer, and (4) soil + straw + fertilizer. For each treatment, 40 g of air-dried soil was placed in a plastic centrifuge tube and mixed thoroughly with or without 0.4 g of rice straw powder and with or without 0.054 g of ammonium sulfate. The mixture was then submerged in 40 ml of distilled water and the tube was sealed with a cap. Sets of these tubes were placed in controlled environemental chambers maintained at temperatures of 15, 20, 25, or 30° C (± 1° C) in a phytotron, located at the National Taiwan University, Taipei. At three-week intervals four tubes from each set were sampled for analysis and the remainder shifted to the next temperature chamber in the following sequences: A, 15° C → 20° C → 25° C → 30° C; B, 20° C → 25° C → 30° C → 25° C; C, 25° C → 30° C → 25° C → 20° C, and D, 30° C → 25° C → 20° C → 15° C. Samples were taken at each three-week interval and were assayed for phytotoxicity and analyzed for phytotoxin production.

Determination of Phytotoxicity of Soil Aqueous Extracts. Each sampled tube was centrifuged at 3000 rpm for 20 min. The supernatant of the tube contents was then filtered through a Whatman No. 42 filter paper and the filtrate collected. An aliquot of the filtrate was bioassayed to determine its phytotoxicity by using two bioassay techniques (Chou, 1976; Chou and Lin, 1976), with seeds of lettuce (*Lactuca sativa* var. Great Lakes) and rice (*Oryza sativa* Taichung 65) as test materials. After incubating the seeds with soil extract or with distilled water as control at 25° C for 72 hr, the growth of

seedling radicles was measured. The phytotoxicity of the soil extract was expressed as percent inhibition of radicle growth by comparing the length of radicle grown in soil extract with that grown in the distilled water control. The phytotoxicity was determined in three replications.

Determination of pH and Osmotic Concentration of Soil Extracts. Each soil aqueous extract obtained from the aforementioned treatments was evaluated for pH and osmotic concentration, using a pH/temperature meter (Jenco model 671) and an osmometer (Fiske G-66), respectively.

Identification of Phytotoxic Phenolics in Treated Soils. Another aliquot of the aqueous extract of the incubated soil sample was extracted with ethyl ether following the procedures described by Chou and Lin (1976). The residue of soil mixture after removal of the aqueous supernatant was subjected to alkaline alcohol extraction employing the techniques described by Wang et al. (1967) and Chou et al. (1977). These two extracts were chromatographed, and the phytotoxic phenolics separated were identified by comparing the chromatographic spots with standard reference compounds. In addition, the phytotoxic compounds on paper chromatograms were eluted with 95% ethanol and then measured at 700 nm by using a Shimadzu Spectronic-20, according to Chou et al. (1977), which was a modified method of Kuwatsuka and Shindo (1973). The estimation of each phenolic phytotoxin was based on three determinations in three replications.

RESULTS AND DISCUSSION

Effect of Temperature and Fertilizer on Soil Phytotoxicity. Bioassay of the aqueous extracts from the soils of Experiment I with seeds of lettuce and rice indicated that soil–straw extracts exhibited a greater inhibition than the control soil extract (Figure 1). In the lettuce bioassay, the phytotoxicity of the soil–straw extracts ranged from 75% to 100% inhibition, whereas that of control soil extracts was below 30%. In the rice bioassay, the soil–straw extract was also more phytotoxic than the control soil extract, but generally lower than that exhibited by the lettuce bioassay. Phytotoxicity, as measured by growth inhibition, was highest at 20–25° C and decreased at both lower and higher temperatures. Whereas the soil phytotoxicity persisted for the duration of the experiment based on the lettuce bioassay, its effect on rice seedling growth had virtually disappeared after six weeks of anaerobic incubation of the soil–straw mixtures. The decrease of soil phytotoxicity with time was more noticeable at temperatures above 30° C. It appears that at such temperatures the decomposition of rice straw in soil would be enhanced but the resulting phytotoxicity to soil was of shorter duration.

Bioassay of the aqueous extracts of the treated soil samples from Experiment II showed that lettuce was also more sensitive to soil phyto-

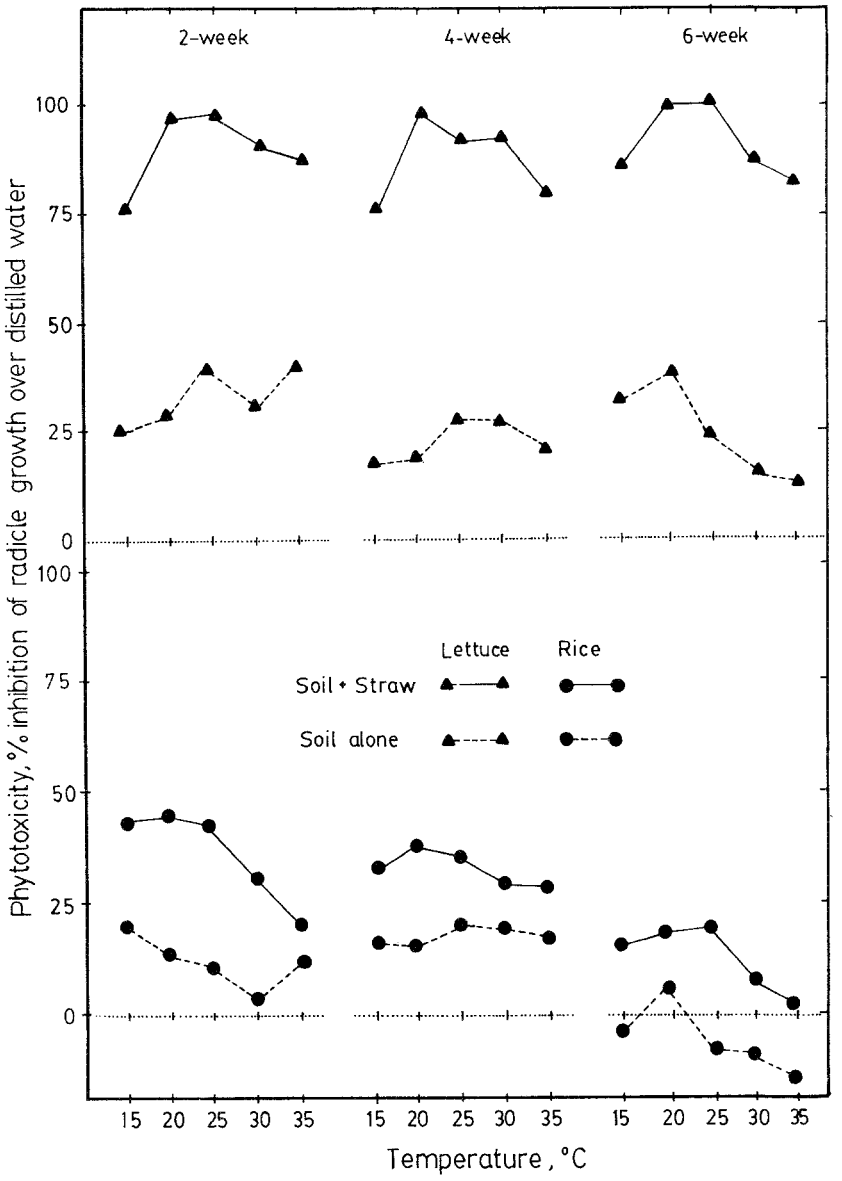

FIG. 1. Effect of temperature on phytotoxicity produced during rice straw decomposition in soil. The phytotoxicity is expressed as percent inhibition of growth of lettuce or rice radicles in various soil extracts as compared with distilled water controls.

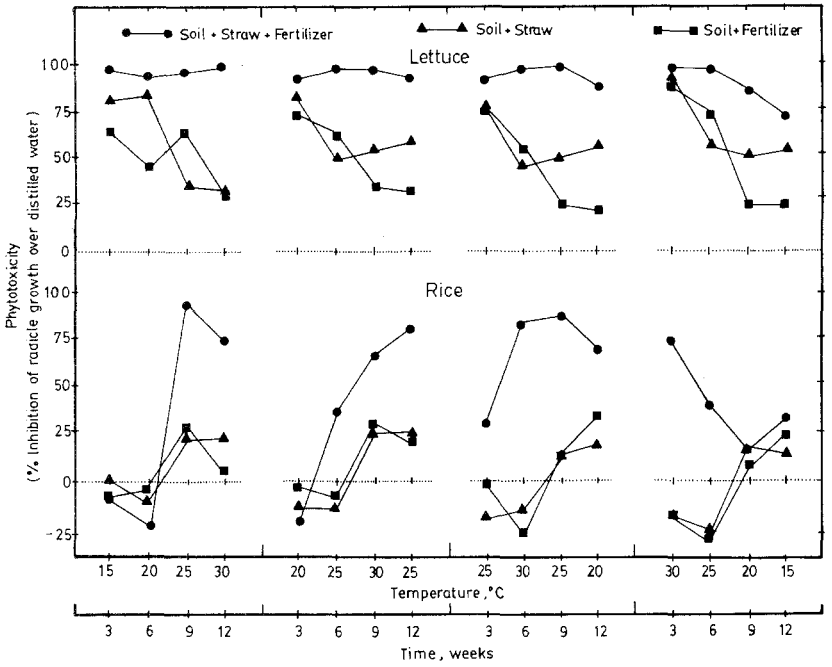

FIG. 2. Effect of temperature and ammonium sulfate fertilizer on phytotoxicity produced during rice straw decomposition in soil. The phytotoxicity is expressed as percent inhibition of growth of lettuce or rice radicles in soil extracts as compared with distilled water controls.

toxicity than rice (Figure 2). The phytotoxicity of aqueous extracts obtained from the soil-straw-fertilizer mixture was greater than from soil-straw and soil-fertilizer mixtures. Practically no phytotoxicity was found by bioassay of the extracts from the control soil; therefore the data were not graphed. Growth inhibition was found to be always greater in extracts from the treated soil at 25° C and 30° C regardless of whether the temperature change was from low to high or vice versa. The inhibition of rice seedling growth in aqueous extracts from soil-straw and soil-fertilizer mixtures was only slight and appeared only after nine weeks of incubation. On the other hand, the inhibition of lettuce growth in extracts from these two treated soils was high initially, but decreased with time, regardless of the temperature regime. However, the incorporation of nitrogen fertilizer into the rice-straw mixture greatly increased the soil toxicity, indicating that fertilizer addition probably

favored the growth of decomposer microorganisms and enhanced the rate of straw decomposition, resulting in the release of more phytotoxic substances. The results from Experiment II support those from Experiment I even though there was a difference in the temperature regimes imposed and in the amount of rice straw incorporated into soil, as the amount of straw added was three times more in the first than in the second experiment.

During the decomposition of rice residue in soil, the pH value of soil aqueous extracts ranged from 5.5 to 7.8 and osmotic concentration was below 15 mosmol. In such ranges both the pH and the osmotic concentration were unable to cause significant inhibition based on lettuce and rice bioassays (Chou et al., 1979 unpublished data).

Effects of Temperature and Fertilizer on Phytotoxin Production. Seven phytotoxic phenolics have been found in the extracts of decomposing rice residues in soil by Chou et al. (1976, 1977, 1979). These were *trans-p*-coumaric, *cis-p*-coumaric, ferulic, vanillic, syringic, *p*-hydroxybenzoic, and *o*-hydroxyphenylacetic acids. Except for *o*-hydroxyphenylacetic acid, the other six compounds are ubiquitously distributed in agricultural soils of Taiwan (Wang et al., 1967). However, the quantity of these compounds found was often variable in different soils and under different crops. In Experiment I, none of the phytotoxic phenolic compounds was found in the aqueous extracts of the control soil, but small amounts were found in the alkaline alcohol extracts of the residues of control soil (data not shown). However, these compounds were found in higher concentrations in both the aqueous and the alcoholic extracts of the soil–straw mixtures (Figure 3). The amount of these compounds found varied with the temperature and duration of incubation. Although it would be difficult to conclude that the phytotoxin distribution followed any definite pattern in relation to temperature variation, the concentration of the compounds isolated tended to be higher in the extracts from treated soil incubated at 20–30° C, and lower in the extracts from soils incubated at either 15° C or 35° C. This pattern was most evident in the contents of ferulic, syringic, vanillic, and *p*-hydroxybenzoic acids obtained from the alcohol extraction. No decrease in the level of phytotoxin accumulation in the treated soil was apparent after six weeks of incubation.

The phenolics obtained from alcohol extract of the incubated soil mixtures from Experiment II are shown in Figure 4. The estimated amounts of these compounds present in the extracts followed the same increasing order of magnitude as those shown by the phytotoxicity tests: soil + fertilizer < soil + straw < soil + straw + fertilizer. Only negligible amounts of phytotoxins were found in the control soil, without straw or fertilizer. There was no definite pattern of accumulation or disappearance of the individual phenolics that could be related to temperature difference. However, ferulic acid was absent in the absence of straw, and syringic acid decomposed rapidly regardless of the sequence of temperature change. *cis-p*-Coumaric acid was

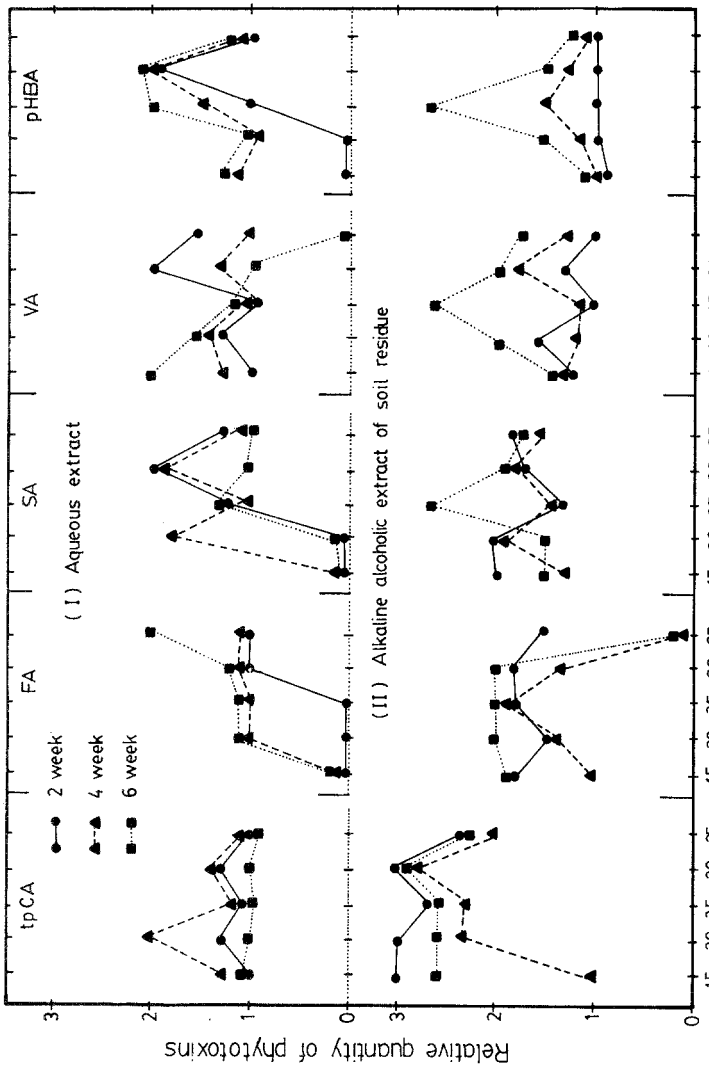

FIG. 3. Relative quantity (arbitrary scale) of phytotoxic phenolics produced during the decomposition of rice straw in soil. The phytotoxins were obtained by (I) ether extract of aqueous extract, and (II) alkaline alcoholic extraction of the residue of each incubated soil–straw mixture. The abbreviations: tpCA = *trans*-*p*-coumaric acid, FA = ferulic acid, SA = syringic acid, VA = vanillic acid, and pHBA = *p*-hydroxybenzoic acid.

produced slowly and both forms of *p*-coumaric acid were quite persistent.

The finding that the extract from the soil–straw–fertilizer mixture caused a greater growth inhibition and contained a higher concentration of phytotoxins than the extracts from other soil mixtures was contradictory to the findings of Chandrasekaran and Yoshida (1973), who reported that ammonium sulfate effectively eliminated the injury to rice plant growth caused by organic acids which were produced in soil under submerged conditions. In the present study, the high toxicity from the soil–straw–fertilizer mixture was unlikely to be from ammonium sulfate because the extracts from soil–fertilizer mixtures without straw caused the lowest inhibition whereas the extracts from soil–straw mixtures also showed significant toxicity. The addition of ammonium sulfate probably accelerated the decomposition of rice straw in soil and the production of phytotoxic substances, both of which were also affected by temperature and duration of incubation.

The high accumulation of phytotoxins at 20–25° C during the 6 to 9-week interval of incubation could have a severe impact on crop production. At the early stages of the second crop season, the daytime temperature is usually above 30° C, which could expedite the decomposition of rice stubble remaining in soil, resulting in the release of large amounts of phytotoxins. As the crop season progresses, the daytime temperature gradually drops below 15° C, which would slow down the decomposition of rice residues. Phytotoxicity in paddy soil under natural conditions during the second crop season has been observed to persist for over 4 months (Chou et al., 1976), thus both the tillering and the panicling of rice plants could be significantly retarded, resulting in a reduction of rice yield (Chou et al., 1979).

Harborne (1977) indicated that aquatic plants may produce shikimic acid as an adaptation to avoid the accumulation of toxic intermediates such as ethanol. If this is the case for rice plants, a substantial quantity of shikimic acid could be accumulated in the paddy soil. However, shikimic acid is a key precursor for synthesizing phytotoxic phenolics, such as *p*-hydroxybenzoic, *p*-coumaric, vanillic, and ferulic acids, as well as for synthesizing plant growth stimulators, such as indole 3-acetic acid (IAA) (Kefeli, 1971). It is possible that shikimic acid could be favored to form growth inhibitors instead of stimulators when the paddy soil is under poor water drainage and unfavorable temperature conditions. This possibility needs to be examined further in field experiments.

Acknowledgments—We wish to express our thanks to the National Science Council, Republic of China, and the National Science Foundation, U.S.A., for their financial assistance, and to the College of Agriculture, National Taiwan University, for the use of the phytotron facility.

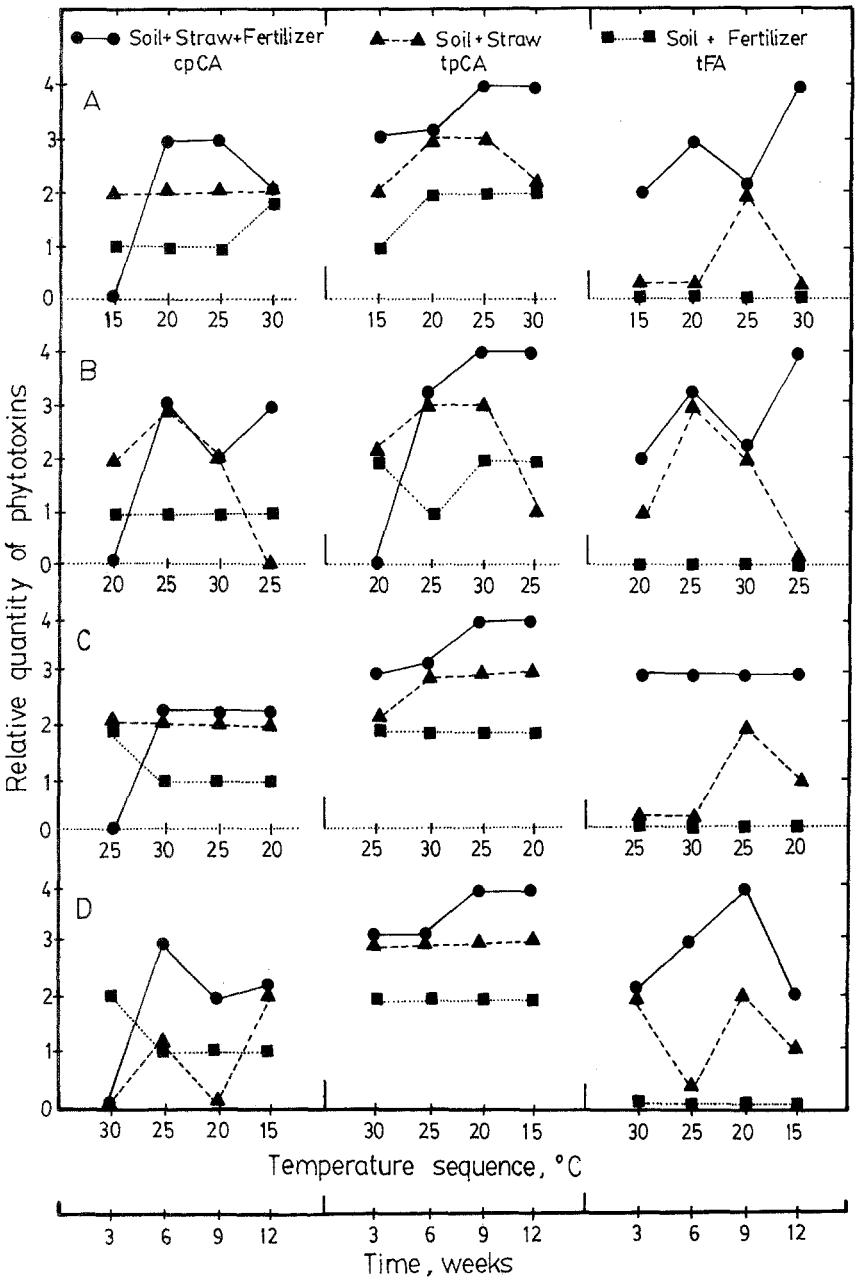

FIG. 4. Relative quantity (arbitrary scale) of phytotoxic phenolics produced during the decomposition of rice straw in soil mixtures and obtained by alkaline alcoholic

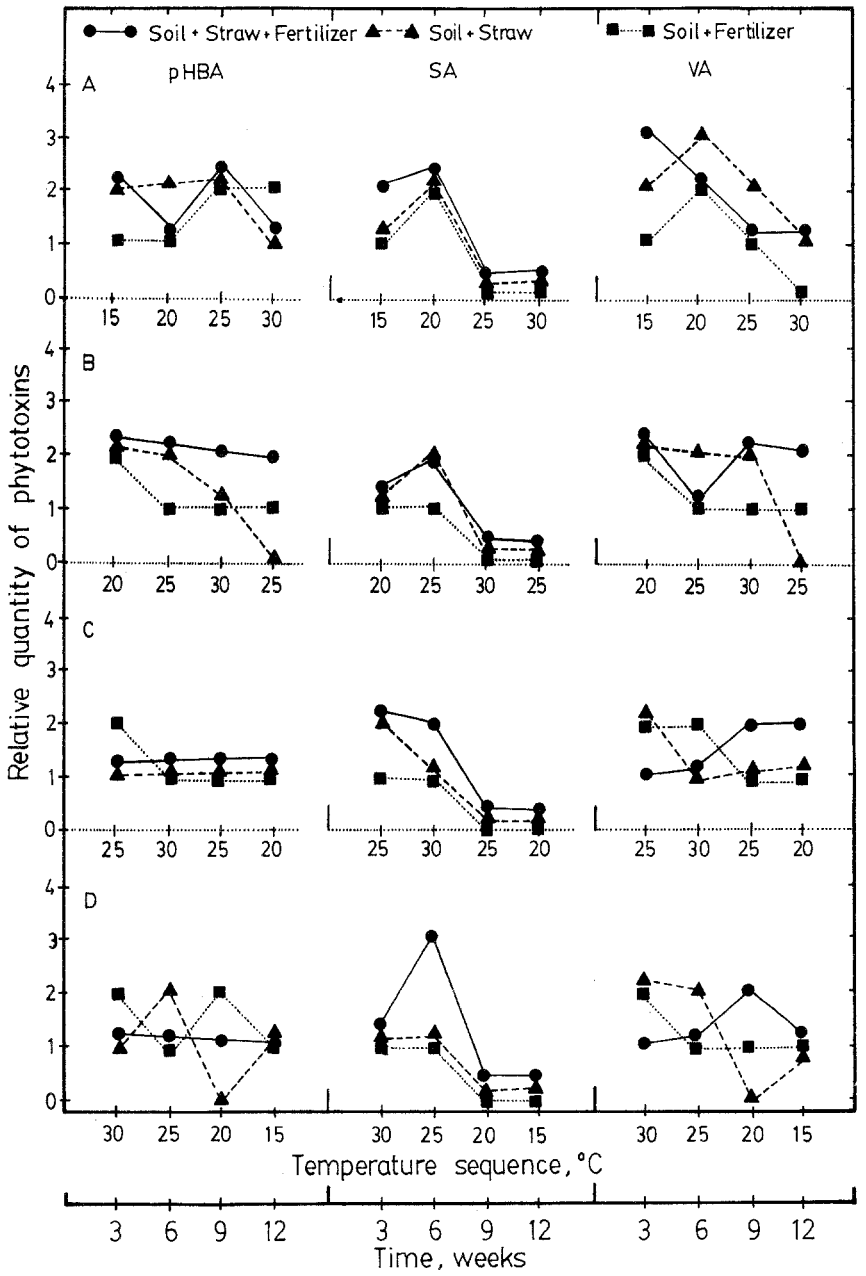

FIG. 4. Continued.

extraction. The abbreviations: cpCA = *cis-p*-coumaric acid, tFA = *trans*-ferulic acid, others as in Figure 3.

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GROWTH INHIBITORS IN TOMATO (*Lycopersicon*) to TOMATO FRUITWORM (*Heliothis zea*)[1]

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Abstract—Several compounds that retard the larval growth of the tomato fruitworm, *Heliothis zea* (Boddie) have been isolated and identified from tomato leaves, *Lycopersicon esculentum* Mill. The major allelochemics are α-tomatine (I), chlorogenic acid (II), rutin (III), and a new caffeyl derivative of an aldaric acid (IV). The isolation, analyses, and toxicity of these compounds to *H. zea* are presented.

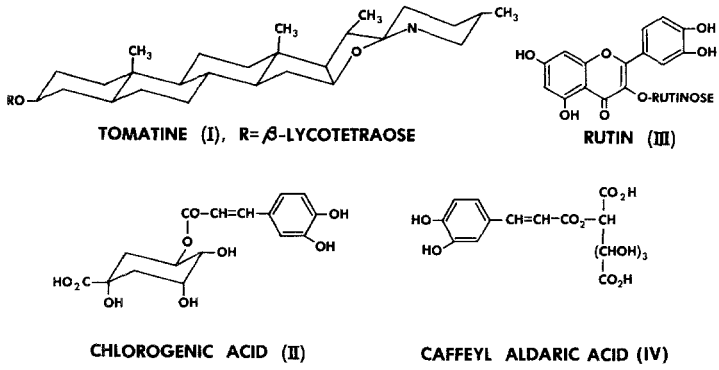
Key Words—*Lycopersicon esculentum*, *Lycopersicon hirsutum f. glabratum Heliothis zea*, Lepidoptera, Noctuidae, α-tomatine, rutin, chlorogenic acid, caffeyl aldaric acid, allelochemics, tomato, tomato fruitworm.

INTRODUCTION

Tomato fruitworm, *Heliothis zea*, is an important insect pest of tomato, *Lycopersicon esculentum*, particularly in the southeastern U.S. Efforts to establish the nature of resistance and to improve tomato's resistance against this insect through breeding have been made by a number of researchers. Fery and Cuthbert (1975) reported varying degrees of antibiosis among excised foliage of tomato species. They further showed that an ethanolic extract from tomato leaves was responsible for reducing survival rates of *H. zea* larvae. Both antixenosis and antibiosis against tomato fruitworm in commercial tomatoes were observed by Cosenza and Green (1979). More recently 2-tridecanone, a toxicant to several tomato insects, was isolated from the wild

[1]Reference to a company and/or product named by the department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

tomato *L. hirsutum f. glabratum* C.H. Mull. (Williams et al., 1980). This paper reports the isolation procedures, biological activities, and analyses of antigrowth factors of α-tomatine (I), cholorogenic acid (II), rutin (III), and caffeyl aldarate (IV) (Scheme 1) in the "fresh market" tomato cultivar Ace,



SCHEME 1

"processing" tomato cultivar Campbell 29, and wild tomato *L. hirsutum f. glabratum* (P.I. 134417).

METHODS AND MATERIALS

Tomato cultivar Ace and *L. hirsutum f. glabratum* (P.I. 134417) were field grown in Albany, California, and Campbell 29 was field grown in Davis, California. Young fully expanded leaves of these varieties were collected during the middle of July 1979 (daylength ca. 15 hr).

Extraction and Purification. In the typical extraction and purification procedure, 100 g of freeze-dried tomato leaves (cultivar Ace, dried to 17.6% of fresh weight) were ground with 800 ml of acetone in a 2-liter beaker for 5 min using a Polytron grinder at maximum speed. The mixture was filtered through a sintered glass funnel, and the residue was extracted again with three more portions of acetone. The combined extract was diluted to 2.5 liters and kept in the refrigerator at 5° C. The extracted residue was further extracted with four 800-ml portions of methanol and water, respectively. The weights of materials from the solvent extraction were 4.4 g from acetone, 9.0 g from methanol, 22.5 g from water, and 64 g of residue. Aliquots of extracts were tested for antigrowth activity against first instar *H. zea.* larvae (Figure 1).

Methanol extract, equivalent to 30 g of dried leaves, was purified through 500 ml of Amberlite XAD-2 by a previously reported procedure of Waiss et al. (1979), and the methanol eluent was chromatographed through a 4 × 110-cm Sephadex LH-20 column. α-Tomatine (I), chlorogenic acid (II), and rutin (III) were separated.

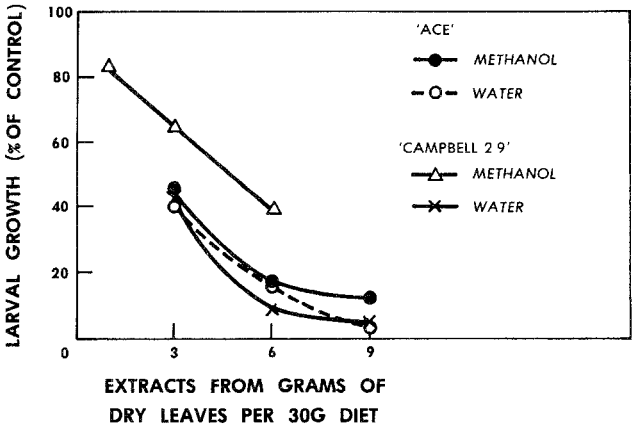


FIG. 1. Growth inhibition of *H. zea* larvae by tomato leaf extracts.

Bioassays. Bioassays for antigrowth activity in tomato leaves, their extracts, and purified compounds were performed according to our earlier reported procedure (Chan et al., 1978). Tests were conducted on samples of ten larvae per level for crude extracts and 20 larvae per level for the pure compounds examined. Standard deviations from the mean were typically about ±15% for controls and became much larger when growth suppression took place.

Quantitative Analysis of α-Tomatine. Two grams of freeze-dried leaf were ground with methanol (3 × 50 ml) using a Polytron grinder for 1 min in a 100-ml beaker. The extracted solution was concentrated to about 2 ml in a vacuum rotatory evaporator at 35° C. For the removal of phenolic compounds, the sample solution was pretreated by passage through a short AG 1 × 8 (OH-form) anion-exchange column (1.3 × 7 cm) followed by elution with an additional 2 ml of methanol. Effluent was collected in a 5-ml volumetric flask and diluted to volume for high-performance liquid chromatographic (HPLC) analysis. α-Tomatine standard solution was prepared similarily.

The analysis was carried out under isocratic condition on a μ-Bondapak--NH_2 column (3.9 × 300 mm) in reverse-phase mode (20% 0.01 M $(NH_4)_2HPO_4$ in acetonitrile), and monitored with a differential refractometer.

Quantitative Analysis of Chlorogenic Acid and Rutin. One gram of freeze-dried leaf was ground for 1 min with 10 ml of $CHCL_3$–MeOH–H_2O (6:4:1) containing 10 mg of quercitrin (as internal standard) using a Polytron grinder. The residue was separated by filtration, and the filtrate was shaken with 5 ml of water. After centrifugation to separate the phases, the upper aqueous layer was analyzed by HPLC (Ultrasphere octyl column, 4.6 × 250 mm 0.5% aq. HCO_2H–MeOH, 60:40).

Isolation of Caffeyl Aldarate (IV). The aqueous extract from the equivalent of 30 g of dried Ace tomato leaves was purified through a 4 × 80-cm column of Amberlite XAD-2 and separated by Sephadex LH20 as previously reported (Waiss et al., 1979). Caffeyl aldarate isomers were isolated at approximately 1% of dried leaf weight.

RESULTS AND DISCUSSION

Using Ace tomato leaves, we found that, while the acetone extract showed no antigrowth activity, sequential extraction of tomato leaf with solvents of increasing polarity showed incorporation of methanol and water extracts deterred appreciably the growth of *H. zea* larvae (Figure 1). Similar results were obtained from leaf extracts of Campbell 29.

Purification of the methanolic extract through absorption on XAD-2 followed by chromatographic separation with LH20 afforded three *H. zea* growth inhibitors, namely, α-tomatine, chlorogenic acid, and rutin (Table 1). These allelochemics are well-known phytochemicals, and their identities were confirmed by comparing spectroscopic (UV and NMR) and chromatographic patterns with those of authentic, commercially available compounds. Tomatine was first isolated from tomato by Fontaine and coworkers (1948). While the toxicity of α-tomatine to fungi, potato insects, and mammals has been reviewed (Roddick, 1974), its antibiotic activity toward *H. zea* has not been well documented.

Chlorogenic acid and rutin are ubiquitous in higher plants. Rutin has been isolated as the coloring matter of tomato stems (Blout, 1933) and its growth inhibitory effect to *Heliothis* species in cotton has been reported (Chan et al., 1978b). The presence of chlorogenic acid in tomato and *Solanaceae* has been reported by Politis (1948), but its antibiotic effect toward

TABLE 1. CONCENTRATION AND ED_{50} OF α-TOMATINE, CHLOROGENIC ACID, AND RUTIN IN TOMATO FOLIAGE[a]

	α-Tomatine	Chlorogenic acid	Rutin
L. esculentum			
Ace	0.76	1.2	2.3
Campbell 29	0.61	0.8	1.3
L. hirsutum f. glabratum P.I. 134417	2.45	0.3	Not detectable
ED_{50}	0.40	2.5	2.4

[a] Milligrams per gram fresh weight.

tomato fruitworm has not been recorded. Chlorogenic acid is toxic to greenbug (Todd et al., 1971).

The effective dosage to reduce the larval growth of tomato fruitworm (bioassayed at first instar stage) to 50% of growth attained on synthetic diet (ED_{50}) for the allelochemics I, II, and III were estimated through bioassays of these compounds at several concentrations (Table 1).

The water extract of tomato leaves, after extraction with methanol, gave a new compound which was identified as a caffeyl aldaric acid (IV) through chemical degradation and derivatization, UV, IR, [^{1}H]NMR, [^{13}C]NMR, and mass spectroscopy. Details of structural determination will be reported elsewhere. The compound occurs as a mixture of isomeric esters and lactones and is present as approximately 1% of the dry tomato (Ace) leaf. The antibiotic activity of this new allelochemic (ED_{50}) is similar to that of chlorogenic acid. This observation is not unexpected since we have observed that the growth inhibitory activity is derived from the catechol moiety of the molecule (Elliger et al., 1980).

It appears from the results presented above that the chemically based growth inhibiting properties of *L. esculentum* toward *H. zea* are derived from the combination of α-tomatine and the three phenolic allelochemics II, III, and IV. It is worthwhile to note that the presence of 2-tridecanone in *L. hirsutum f. glabratum* as reported by Williams and coworkers (1980) may explain in part the high level of resistance of this wild tomato (Fery and Cuthbert, 1975), but the high level of α-tomatine in the foliage and its importance in pest resistance should not be minimized.

The presence and diversity of the allelochemics in the foliage of tomato present an opportunity for plant breeders to manipulate the concentration of one or all of these resitance factors tailored to a specific feeding site and time for the protection of the plant. We hope that the analytical procedures presented in this paper facilitate this endeavor.

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DOMINICALURE 1 AND 2: Components of Aggregation Pheromone from Male Lesser Grain Borer *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae)[1,2]

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Abstract—Volatiles from lesser grain borers, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), were collected on Porapak Q, and those from the male beetles were shown to contain two compounds, that were attractive individually and in combination to both sexes. These compounds were identified as (*S*)-(+)-1-methylbutyl (*E*)-2-methyl-2-pentenoate and (*S*)-(+)-1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate by spectrometry and comparison with synthesized compounds. The two compounds have been assigned the trivial names dominicalure 1 and dominicalure 2, respectively. Synthesized samples of these compounds, individually and in combination, were effective in trapping both sexes in field studies.

Key Words—Pheromone, *Rhyzopertha dominica*, lesser grain borer, Coleoptera, Bostrichidae, unsaturated ester, dominicalure, 1-methylbutyl (*E*)-2-methyl-2-pentenoate, and 1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate.

[1]Presented in part at the Northeastern Regional Meeting of the American Chemical Society, June 25–28, 1978, Boston.
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INTRODUCTION

The lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), is a worldwide primary pest of stored products. Both larvae and adults use their strong mandibles to attack whole, sound grains, causing extensive damage. Because adults are strong fliers, infestations spread easily; the beetles (3 mm in length) are difficult to detect before damage has occurred. The male beetles produce an aggregation pheromone that attracts both sexes (Khorramshahi and Burkholder, 1981), and this pheromone may prove useful for early detection of an attack. In this paper we report the isolation, identification, synthesis, and preliminary field testing of two components of this pheromone.

METHODS AND MATERIALS

Pheromone Collection

Rhyzopertha dominica beetles were reared at the Stored Product and Household Insects Laboratory, AR, SEA, USDA, University of Wisconsin, Madison, Wisconsin. Volatiles were collected by passing charcoal-filtered air at 2 liters/min through glass chambers containing insects of both sexes into glass collection tubes packed with purified Porapak Q (Cross et al., 1976).[7] Samples of food alone and of segregated males and females were aerated, and volatiles were collected in the same manner. Aeration periods were as long as 16 days. The Porapak Q samples were extracted with pentane (Mallinkrodt nanograde, redistilled) in a Soxhlet extractor for 24 hr,[8] and the extracts were concentrated to 1–2 ml by distillation through a short packed column.

Isolation and Identification

The concentrated samples were fractionated by GC on a modified Varian 1700 chromatograph on a 2.43-m × 4-mm ID glass column packed with 5% OV-101 on Chromosorb G, 70/80 mesh, AW/HMDS, helium flow 60 ml/min. The instrument was equipped with a flame ionization detector, a 99:1 effluent splitter, and a thermal gradient collector (Brownlee and Silverstein, 1968). Fractions were collected in 30.5-cm × 1.3-mm OD glass capillary tubes that were flame-sealed and stored at −30° until used. Mass spectra were recorded on a Hitachi RMU-6 spectrometer with an inlet modified to accept the glass capillary tubes. [^{1}H]NMR spectra were obtained

[7] An improved purification procedure for Porapak Q consists of stirring with 3 changes of methylene chloride, each for 2 hr at room temperature, then drying in a nitrogen stream.

[8] Absorbed compounds can be removed directly from the Porapak Q column by eluting with 20% ether–80% pentane.

on a Varian XL-100 FT NMR instrument using 5-mm tubes with "100%" $CDCl_3$ as solvent. IR spectra were recorded on a Perkin-Elmer 337 instrument on dilute $CDCl_3$ solutions contained in 10 μl cavity cells. Shift reagent studies were performed with $Eu(hfc)_3$ (Aldrich) at the molar ratio specified in benzene-d_6–CCl_4, 1 : 1, using 5-mm tubes. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter on samples in ether or absolute ethanol solution contained in a 1-ml microcell equipped with a 25° C thermostated water jacket.

Bioassays

Fractions for biological testing were sent to the Stored Product and Household Insects Laboratory, AR, USDA, University of Wisconsin, Madison, Wisconsin, and were tested in a differential arena olfactometer (Burkholder, 1970). Field trapping studies were conducted under simulated warehouse conditions in four rooms of a farmhouse near Madison. The rooms were each approximately 12 m^2 in area and were 2.42 m high. The room windows were covered with plywood, and a 60-W light bulb was located near the center of the ceiling. Throughout the tests, rooms were under environmental conditions at 28° ± 2° C, 40% ± 12% relative humidity, and 16:8 light–dark photocycle. Beetle sex ratios were approximately 1 : 1.

In test A, designed to measure the effect of pheromone concentration on trapping efficiency, rubber septa were impregnated with either 0, 44, 88, or 176 mg of synthetic dominicalure 2 (75% pure, 25% other isomers of the same compound). A Sectar I trap containing one of the pheromone-treated septa or the untreated control was placed 1.5 m above the floor near the center of each of the four test rooms, the four concentrations being set up in a Latin square design. Each concetration was tested in each room once during the four day test period.

Two hundred 1 to 2-month-old, mixed sex *R. dominica* beetles were released evenly on the floor around each room. The test was usually begun between 10:00 and 11:00 AM, and the results were recorded 24 hr later.

Test B was conducted in a similar manner except that the other synthetic component, dominicalure 1, which was 85% pure, was used in addition to a purer synthetic dominicalure 2 (92%). Four traps were placed in each of the four rooms 1.8 m above the floor, spaced equally 1 m from the center. The traps contained, respectively, 5 mg of dominicalure 1, 5 mg of dominicalure 2, 5 mg of a 1 : 1 mixture of the two, or nothing. *R. dominica* beetles (200 mixed males and females) were released in the center of the floor.

Test C was conducted in the same facility. Each trap consisted of four layers of 9-cm^2 corrugated paper (Burkholder, 1976), and the traps were equally spaced on the floor 1 m from the center. Dichlorvos was placed in the traps to kill the attracted insects. The four traps in each of the four rooms

contained, respectively, 5 mg dominicalure 1, 5 mg dominicalure 2, 5 mg of 1:1 dominicalure 1:dominicalure 2, or nothing. *R. dominica* beetles (200 mixed males and females) were released in the center of the floor. Experimental data were examined statistically by using analysis of variance and Duncan's new multiple-range test (Steel and Torrie, 1960). Differences at the 5% level ($P \leqslant 0.05$) were accepted as statistically significant.

Synthesis

(E)-2-Methyl-2-pentenoic Acid (1). This was prepared from the cyanohydrin of 75 g 2-pentanone by the procedures of Lucas and Prater (1937) and Chan et al. (1968) in 51% overall yield, bp 105–110° at 10 mm [Lucas and Prater (1937) report bp 106.5° at 10 mm], 75% *E* by GC analysis. In this case and in the synthesis of IV below, the product was contaminated with the *Z* isomer and the α-methylene isomer because of incomplete separation during distillation. Purity was determined by GC analysis of the pheromones produced from these acids as described below. NMR spectra of mixtures of these isomers prepared by preparative GC showed olefinic proton signals consistent with their assigned structures (Chan et al, 1968).

(E)-2,4-Dimethyl-2-pentenoic Acid (4). This was prepared in the same manner as 3 from 150 g of 4-methyl-2-pentanone in 35% overall yeld, bp 113° at 10 mm [Bartrakov and Bergelson (1964) report bp 108–109° at 10 mm], 92% *E* by GC analysis.

1-Methylbutyl (E)-2-Methyl-2-pentenoate (1). Method 1: One gram of 3 mixed with 1 g thionyl chloride was heated gently with a heat gun for 15 min, rotary-evaporated, and mixed cautiously with excess 2-pentanol. After a further 15 min period of heating, the mixture was shown by GC analysis to have one large peak (75% of total) with a retention time on the OV-101 column previously described, operated isothermally at 130°, of 5.3 min, identical to that of natural I. A minor, partially resolved peak at 4.6 min represented the *Z* and α-methylene isomers, which on bioassay were also found to be biologically active. Method 2: A solution of 22.8 g (0.2 mol) 3 in 25 ml dry benzene was treated with 30.4 g (0.22 mol) oxalyl chloride and allowed to stand overnight. The mixture was refluxed for 1 hr and rotary-evaporated. The resulting oil was treated with 19.4 g (0.22 mol) of 2-pentanol with gentle heating for 1 hr. Distillation (caution, bumps easily) gave 29 g (79%) of 1, bp 92° at 10 mm.

1-Methylbutyl (E)-2,4-dimethyl-2-pentenoate 2. This was prepared by method 2 from 12.8 g (0.1 mol) 4 in 13.8 g (70%) yield, bp 94° at 10 mm (caution, bumps easily). GC analysis as before at 130° showed 92% of product to have a retention time of 7.5 min, identical to the second pheromone peak, and 8% of the 4.6-min, partially resolved peak of the α-methylene and *Z* isomers.

(R)-(−)-1,4-Pentanediol (5). (S)-(+)-γ-Tosyloxymethyl-γ-butyrolactone (3.5 g, 0.014 mol), prepared by the method of Ravid, et al. (1978), was converted to the iodide by the method of Mori (1975), and the resulting oil was added over a 30-min period to 1.1 g (0.028 mol) of lithium aluminum hydride in 20 ml of dry THF at room temperature. The mixture was stirred under nitrogen overnight, treated slowly with 1 ml water, 1 ml 10% potassium hydroxide, and 2 ml water, stirred for an additional hour, filtered, and dried over sodium sulfate. Evaporation and short-path distillation gave 1.0 g (70%) diol, bp 120–130° at 10 mm $[\alpha]_D^{25} = -10.0$ ($c = 0.31$ in EtOH).

(S)-(+)-1,4-Pentanediol. This was prepared in the same manner in 55% yield from 2.1 g enantiomeric tosylate. $[\alpha]_D^{25} = +9.8°$ ($c = 0.52$ in EtOH).

(R)-(−)-2-Pentanol (6). One gram (0.0096 mol) (S)-(−)-5 was dissolved in 10 ml dry pyridine at 0° and 1.8 g (0.0096 mol) *p*-toluenesulfonyl chloride was added over 30 min with stirring. The flask was stoppered and allowed to stand at 0° overnight. The solution was filtered, poured into cold 10% $NaHCO_3$ (20 ml), extracted with 3 × 10 ml ethyl acetate, and the combined extract was washed with brine, dried over sodium sulfate, and evaporated. The residual yellow oil was dissolved in 5 ml dry THF and added slowly at 0° to a slurry of 0.36 g (0.0096 mol) lithium aluminum hydride in 10 ml of dry THF. The mixture was stirred overnight at 25°, treated slowly with 0.4 ml water, 0.4 ml 10% potassium hydroxide, and 0.89 ml water, then dried over sodium sulfate. Removal of solvent by distillation through a short column followed by preparative gas chromatography (2 m × 4 mm ID, 5% Carbowax 20 M on Chromosorb G) gave 0.24 g (28%) of (S)-(−)-pentanol, $[\alpha]_D^{25} = -13.2°$ ($c = 0.72$, EtOH). (S)-(+)-2-Pentanol was prepared in the same manner from the enantiomeric diol in 19% yield on a 0.6 g scale, $[\alpha]_D^{25} = +13.1°$ ($c = 0.55$, EtOH).

(S)-(+)-1 was prepared from 3 and (S)-(+)-6 by method 1, and purified by GC, $[\alpha]_D^{25} = +13.4°$ ($c = 0.175$, ether).

(R)-(−)-1 was prepared in the same manner from (R)-(−)-6, $[\alpha]_D^{25} = -29.7°$ ($c = 0.101$, ether).

(S)-(+)-5 was prepared in the same manner from (S)-(+)-6 and 4, $[\alpha]_D^{25} = -31.8°$ ($c = 0.210$, ether).

(R)-(−)-2 was prepared in the same manner from (R)-(−)-6, $[\alpha]_D^{25} = -30.1°$ ($c = 0.084$, ether).

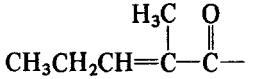
RESULTS

Pheromone Identification. Males and females were attracted to volatile chemicals collected from the males (Khorramshahi and Burkholder, 1981). Comparison of GC traces of individual collections of volatiles from males, females, and food samples showed that two components present in male

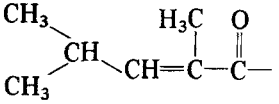
volatiles were absent from both the female and the food extracts. A sample of volatiles, collected from 2000 insects of approximately equal numbers of both sexes held in a chamber containing food for 20 days, was fractionated on the OV-101 column held at 80° for 4.5 min, then programed at 4°/min to 190°. Fractions 6 (24.5–25.5 min) and 7 (25.5–27.5 min) were found to be active. These fractions coincided with the two components found only in male volatiles. Using the same column isothermally at 130°, we collected approximately 200 μg of fraction 6 and 440 μg of fraction 7 as single peaks (present in the concentrated solution in a ratio of 1 : 1.95), with retention times of 5.3 and 7.5 min, respectively.

Fraction 6 (Figure 1) showed M^+ 184 and IR peaks at 1705 and 1648 cm^{-1}. Fraction 7 (Figure 2) showed M^+ 198 with a similar IR spectrum. The molecular formulas assigned were $C_{11}H_{20}O_2$ and $C_{12}H_{22}O_2$, respectively, with two units of unsaturation in each molecule. The IR peaks at 1705 and 1648 cm^{-1} and the NMR spectra showing typical downfield olefinic absorptions for protons for each compound suggested α,β-unsaturated esters.

In the NMR spectrum of fraction 6 (Figure 1), the olefinic proton absorption was a triplet at δ 6.73 (1H, J = 8 Hz), and since there was a pentet at 2.16 (2H, J = 8 Hz) and a triplet at δ 1.03 (3H, J = 8 Hz), the substituent on the β-carbon must be an ethyl group. A methyl substituent on the α-carbon was indicated by a 3-proton absorption at 1.82 with a small allylic coupling (J =2 Hz) to the olefinic group. Thus, the acid moiety in fraction 6 is



In the NMR spectrum of fraction 7, the downfield absorption (δ 6.64), 2H, J = 8 Hz) was a doublet with a small (J = 2 Hz) long-range coupling. This taken with the doublet at δ 1.12 (J = 8 Hz), a 1-proton multiplet at δ 2.70, and a 3-proton singlet (with allylic coupling of J = 2 Hz) at δ 1.93 defines the acid moiety as



Single-hydrogen multiplets at δ 4.98 (J = 6 Hz) in the NMR spectrum of fraction 6 and δ 5.05 (J = 6 Hz) in the NMR spectrum of fraction 7 were assigned as protons on carbons directly attached to oxygen. The doublets at δ 1.23 (3H, J = 6 Hz) in the NMR spectrum of fraction 6 and δ 1.34 (3H, J = 6 HZ) in the NMR spectrum of fraction 7 were assigned as methyls attached to the carbon attached to oxygen. The distorted 3-proton triplet in the NMR spectrum of fraction 6 at δ 0.90 (J = 7 Hz) suggests that an ethyl group must be present, which with the remaining methylene would make up

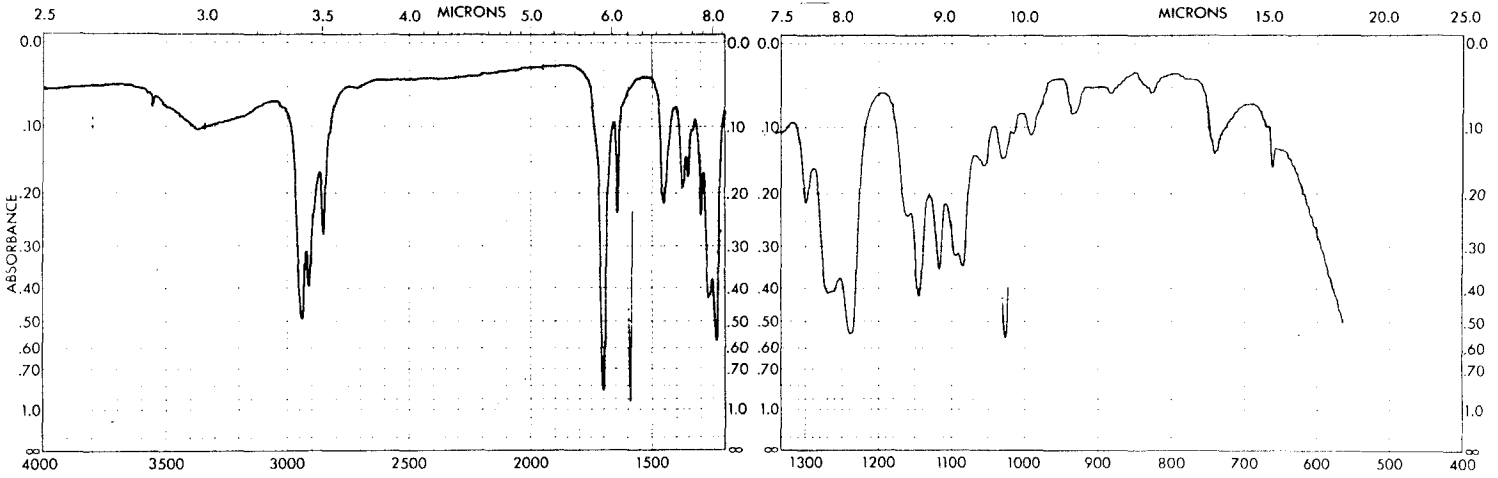


FIG. 1. Infrared (a), nuclear magnetic resonance (b) (10 ppm sweepwidth, 100 MHz), and mass spectra (c) for fraction 6.

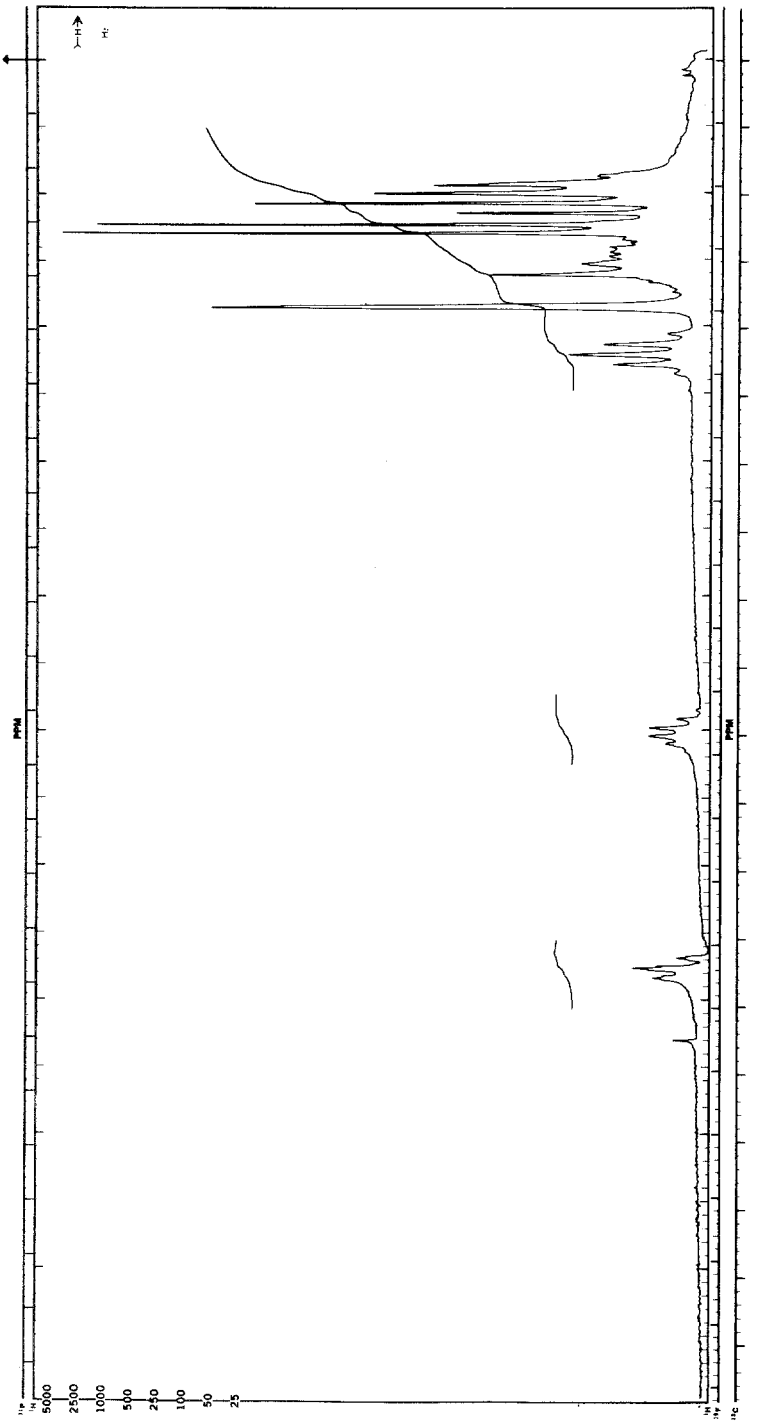


Fig. 1b.

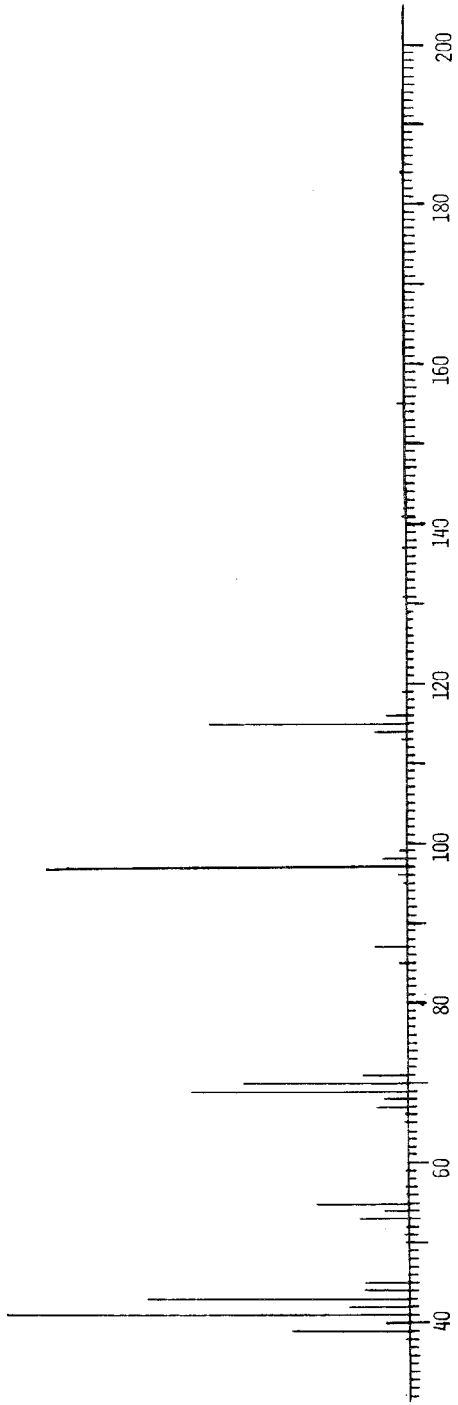


Fig. 1c.

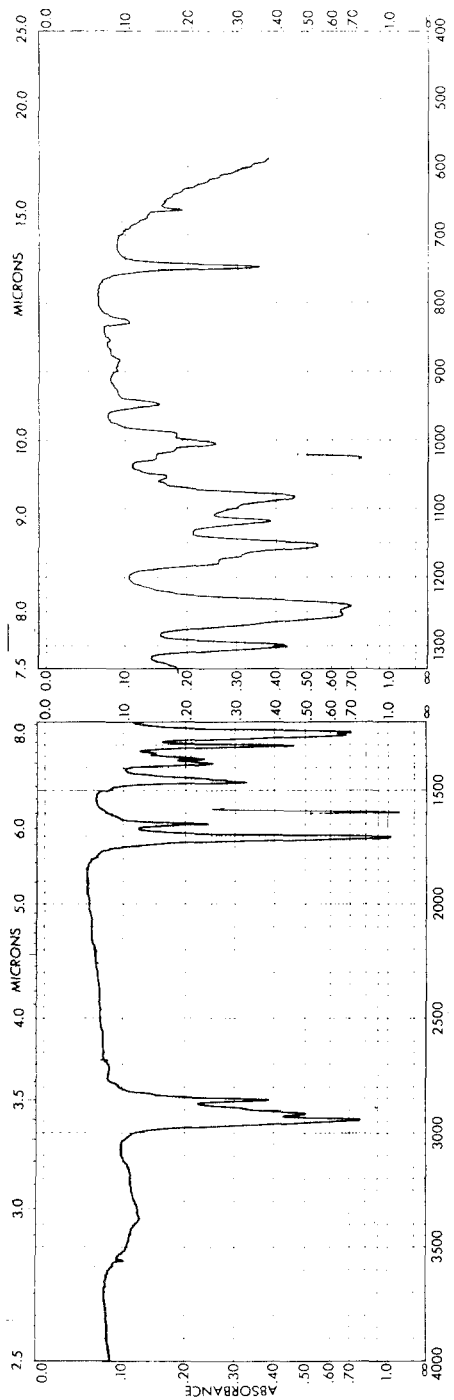


FIG. 2. Infrared (a), nuclear magnetic resonance (b) (10 ppm sweepwidth, 100 MHz), and mass spectra (c) for fraction 7.

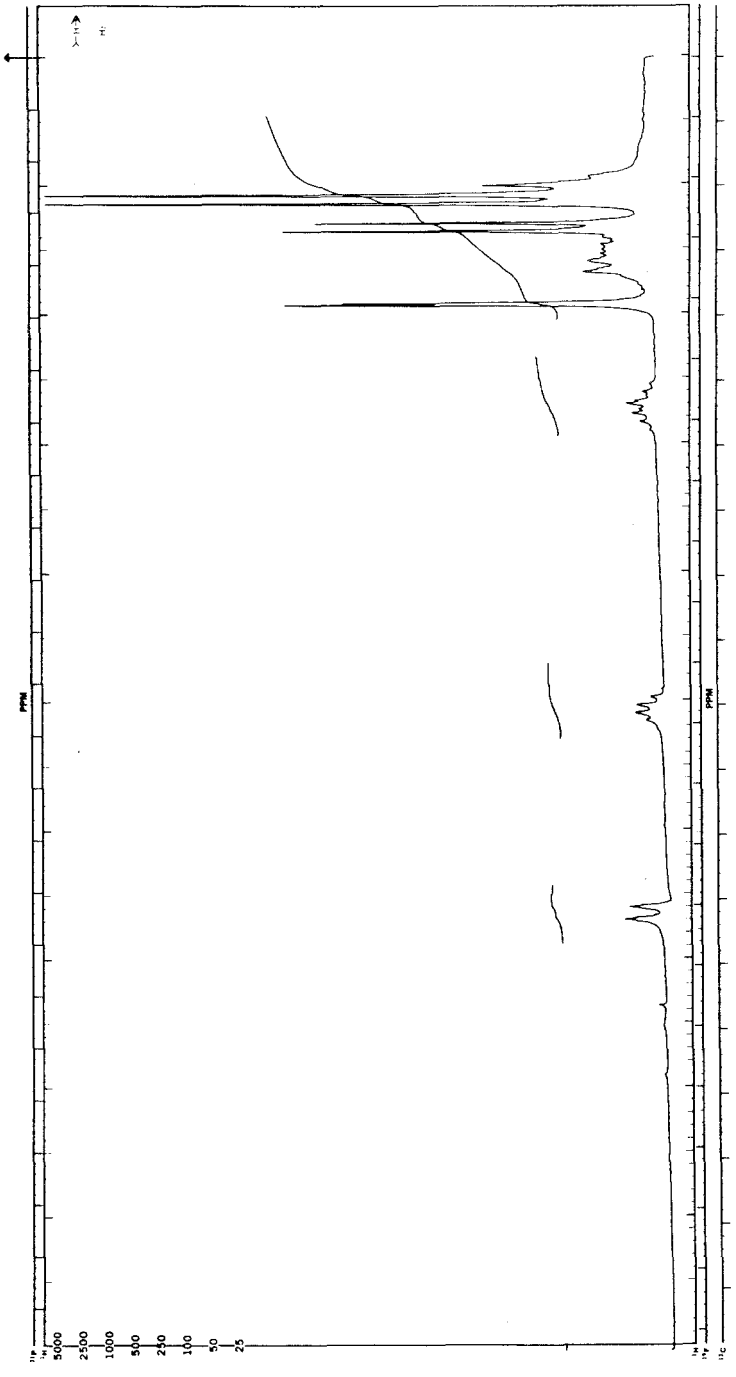


Fig. 2b.

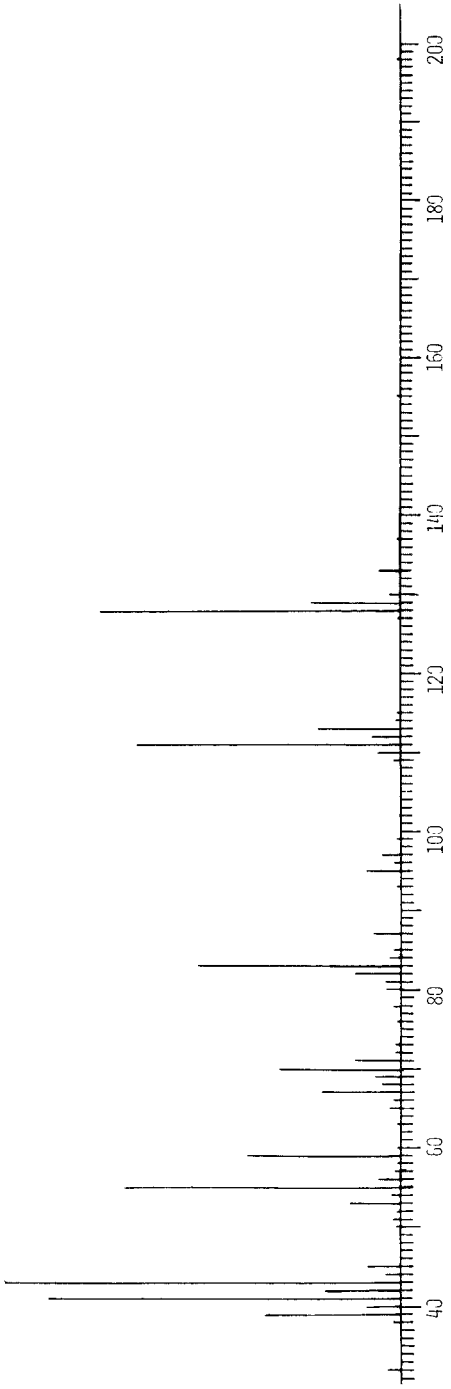
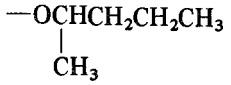
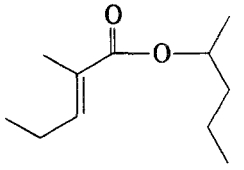


Fig. 2c.

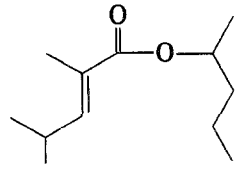
an *n*-propyl group attached to the methine carbon. The corresponding triplet in the NMR spectrum of fraction 7 is partially buried under the upfield doublets. Therefore the alcohol moiety (the same in both compounds) is:



In closely related compounds, the olefinic proton absorbed at δ 5.90 for the *Z* compound and at δ 6.64 for the *E* compound (Chan et al., 1968). We therefore assigned the *E* configuration to our compounds (δ 6.73 and 6.64 in fractions 6 and 7, respectively). The structures thus are 1-methylbutyl (*E*)-2-methyl-2-pentenoate (I) and 1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate (II), for which we propose the trivial names dominicalure 1 and 2, respectively.

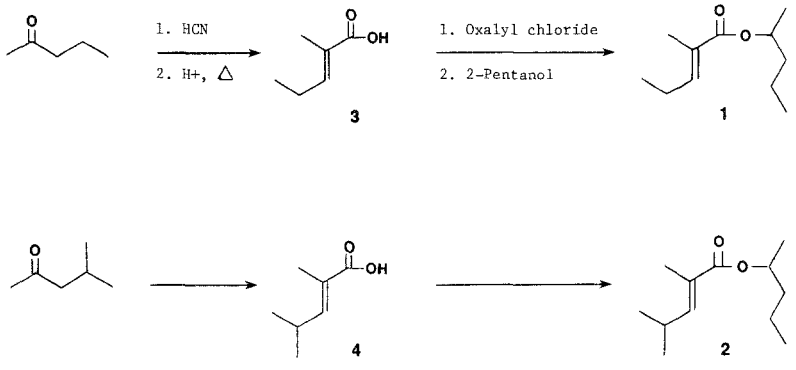


(I)



(II)

Samples of racemic dominicalure 1 and 2 were synthesized for structure confirmation and biological testing by known methods (Scheme 1) and were found to be chromatographically and spectrally identical to the natural pheromones. Samples of pure, racemic *E* isomers, prepared by preparative GC as described earlier, were tested in an arena olfactometer and found to be active. No masking effects were noted. Therefore, samples purified by simple distillation, containing small amounts of the other isomers, were used in field testing (Figure 3). In each of the field tests, a significant ($P < 0.05$) response to the pheromone occurred when compared to the control (Figure 3). In field test



SCHEME 1

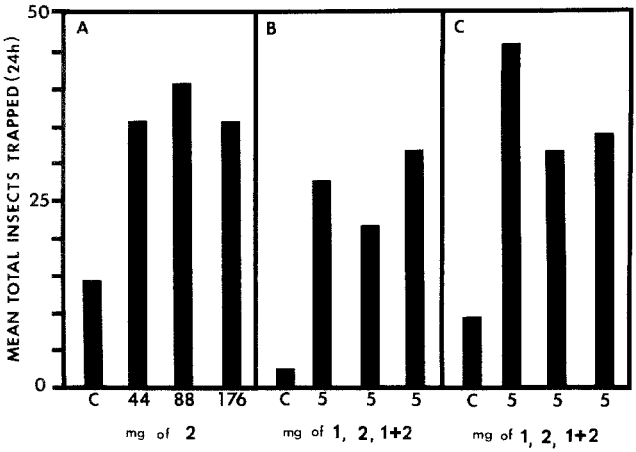


FIG. 3. Mean total of *Rhyzopertha dominica* males and females trapped after 24 hr in storage-room tests. Insects (200) were released in each room. Each experiment was replicated 4 times with 1 Sectar I trap per room in test A; 4 Sectar I traps per room in test B; and 4 corrugated paper traps per room in test C.

A, there were no significant ($P < 0.05$) differences between the several pheromone concentrations. In field tests B, with aerial traps, or in C, with floor traps, there were no significant ($P < 0.05$) differences between the several pheromone treatments.

Optical Activity. Rotations were taken of each natural ester, dominicalure 1 having $[\alpha]_D^{25} = +32.1$ ($c = 0.156$ ether) and dominicalure 2 having $[\alpha]_D^{25} = +32.3$ ($c = 0.777$ ether). Enantiomeric composition of the natural esters was determined by examination of their NMR spectra in a CCl_4–benzene-d_6 (1:1) solution to which approximately 1 equivalent of the chiral shift reagent [Eu(hfc)$_3$] had been added (Figures 4 and 5). For racemic dominicalure 1, the alcohol methyl doublet originally found at δ 1.35 (Figure 4a) is seen as a pair of doublets at δ 2.25 and 2.35 in Figure 4b. However, when a similar amount of the reagent was added to a solution of the natural ester, the peak was seen only as a doublet at δ 2.70 (Figure 4c). For racemic dominicalure 2, the alcohol methyl doublet in Figure 5a is seen as an apparent triplet (overlapping doublets at δ 2.75 and 2.85) in Figure 5b. Again the natural material plus the shift reagent (Figure 5c) shows only a doublet at δ 2.20. To the limits of NMR detection, both natural samples consist of the pure (+) enantiomer.

Enantiomers of known configuration of both pheromone components were prepared from the two isomers of glutamic acid (Scheme 2). Although there may be better methods of preparing the enantiomers of 2-pentanol, this method is general for synthesizing otherwise difficult-to-obtain enantiomers

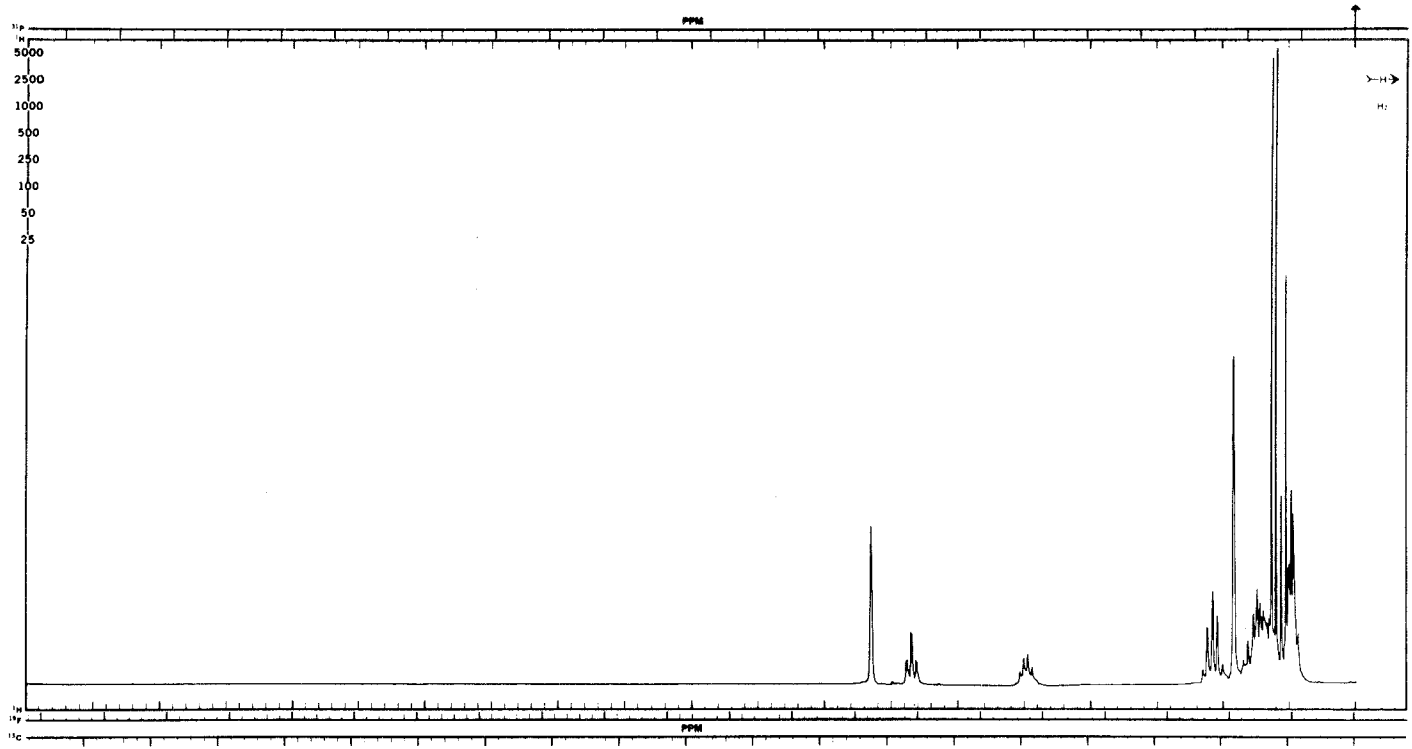


FIG. 4. Nuclear magnetic resonance spectra (20 ppm sweepwidth, 100 MHz) of (a) synthetic dominicalure 1, (b) synthetic dominicalure 1 with added shift reagent, and (c) natural dominicalure 1 with added shift reagent.

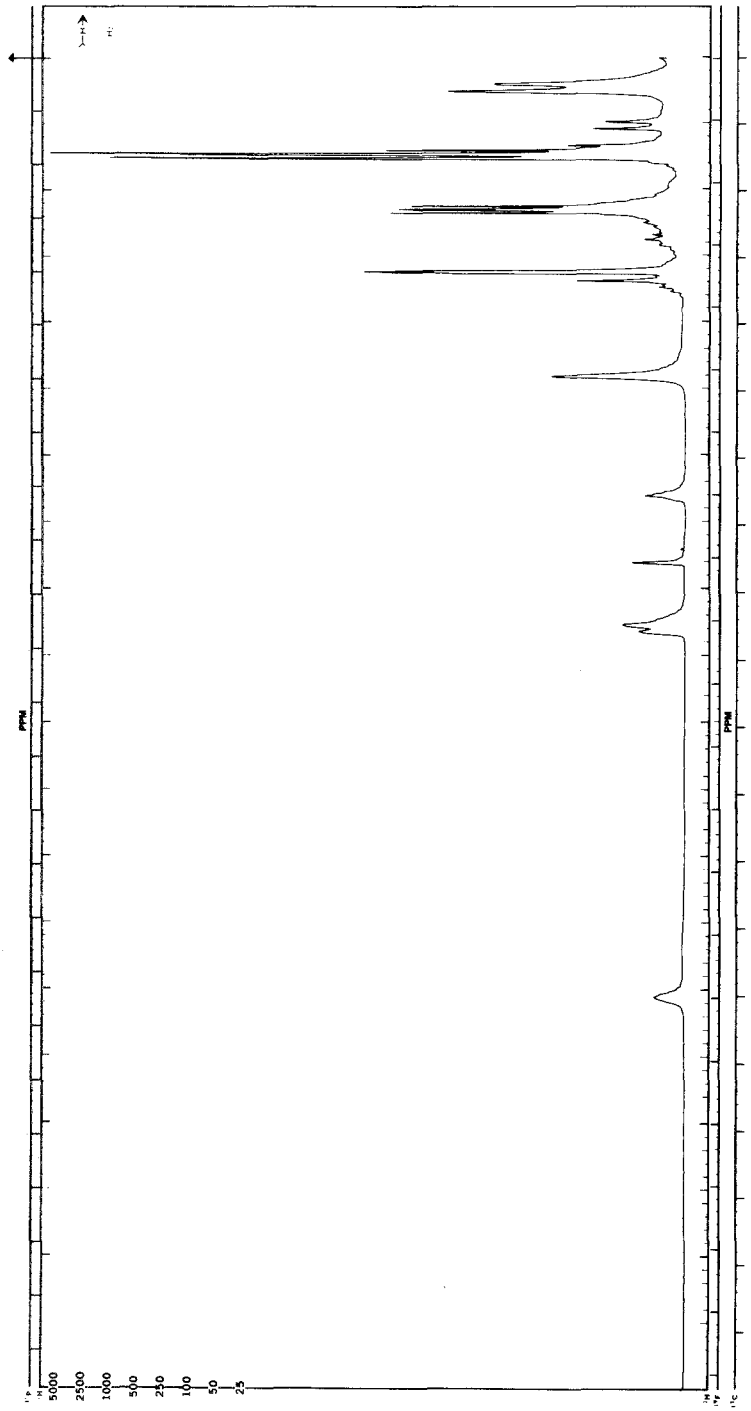


Fig. 4b.

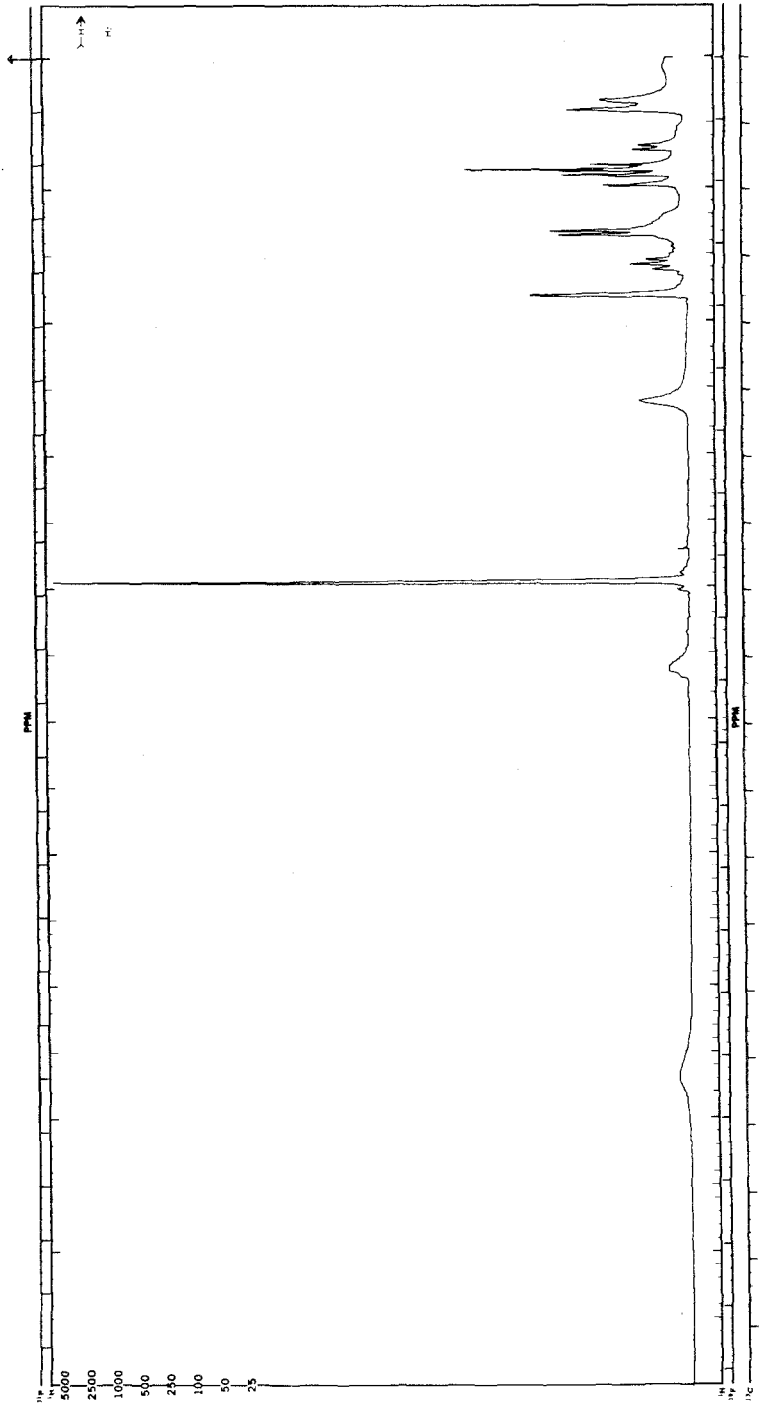


Fig. 4c.

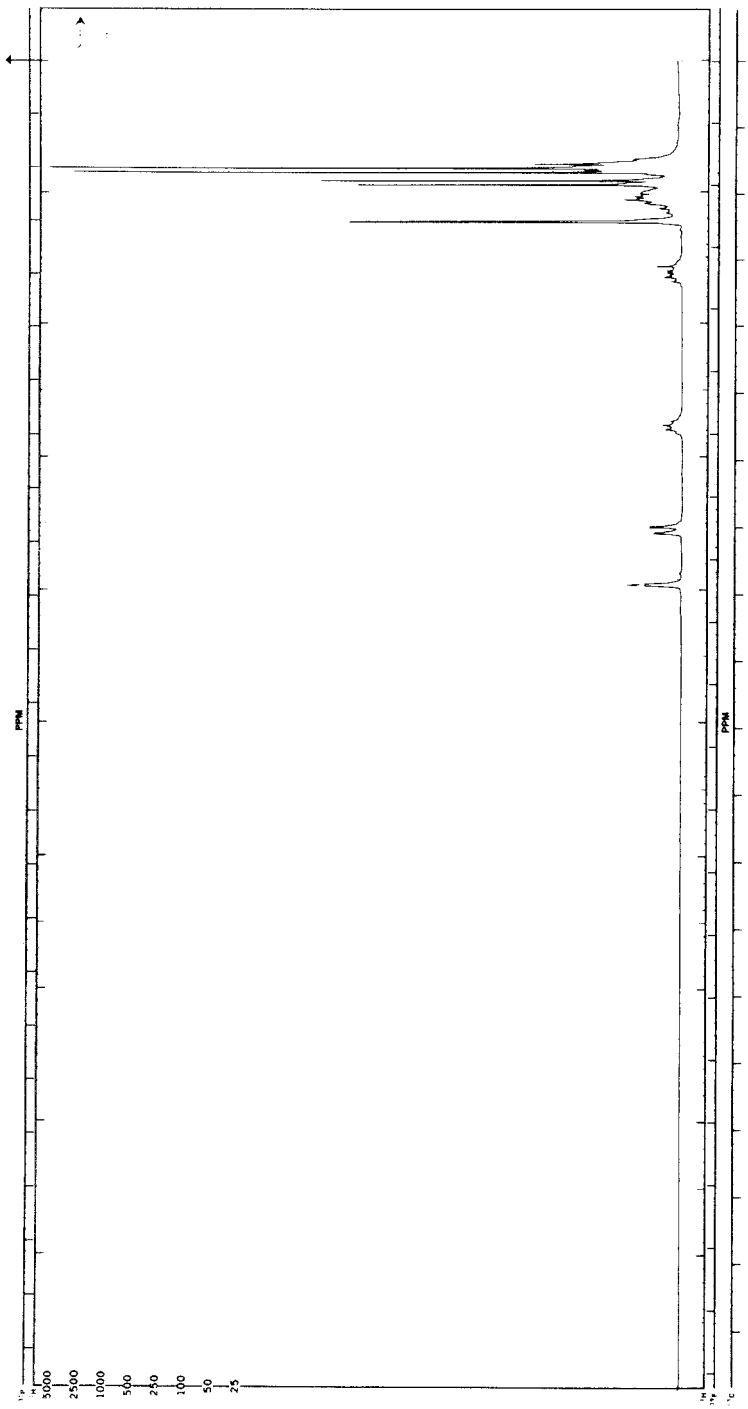


FIG. 5. Nuclear magnetic resonance spectra (200 ppm sweepwidth, 100 MHz) of (a) synthetic dominicalure 2, (b) synthetic dominicalure 2 with added shift reagent, and (c) natural dominicalure 2 with added shift reagent.

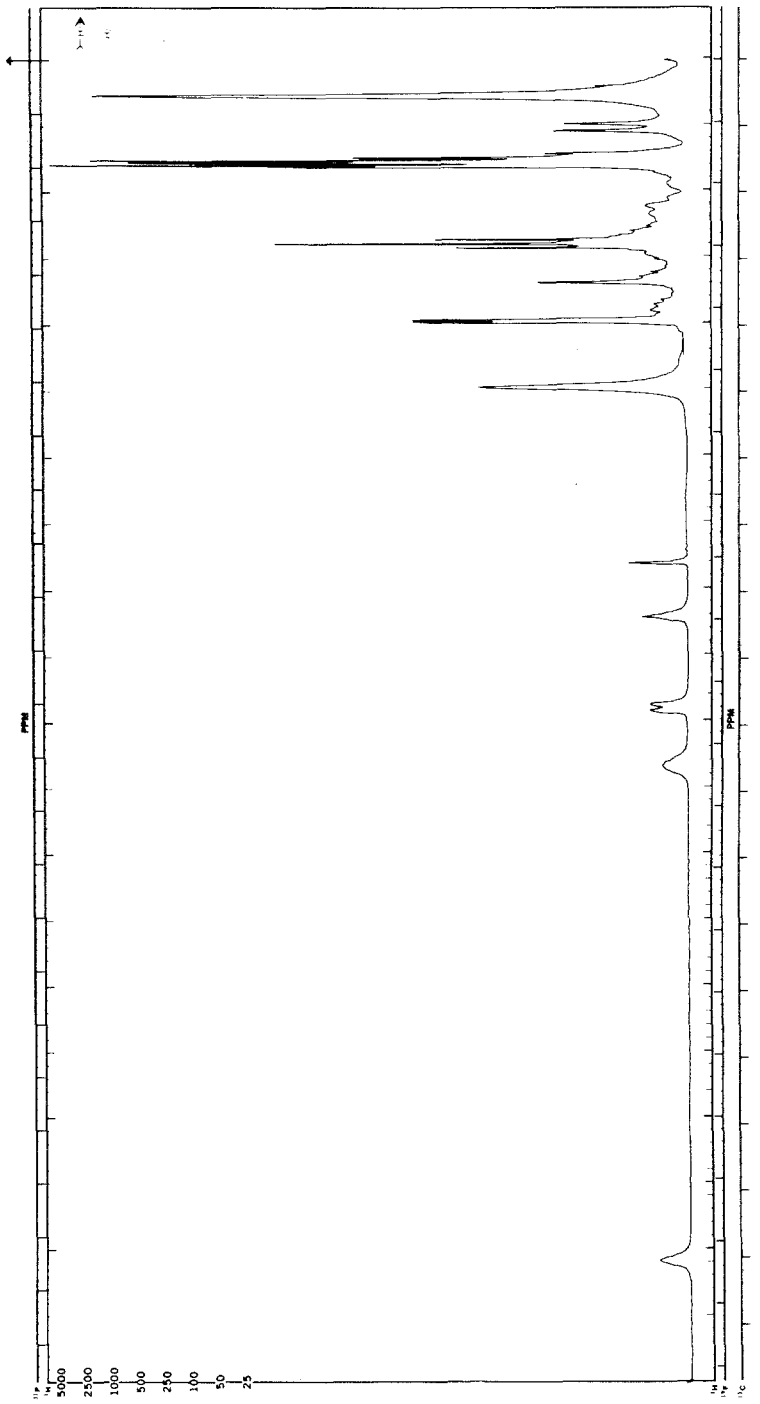


Fig. 5b.

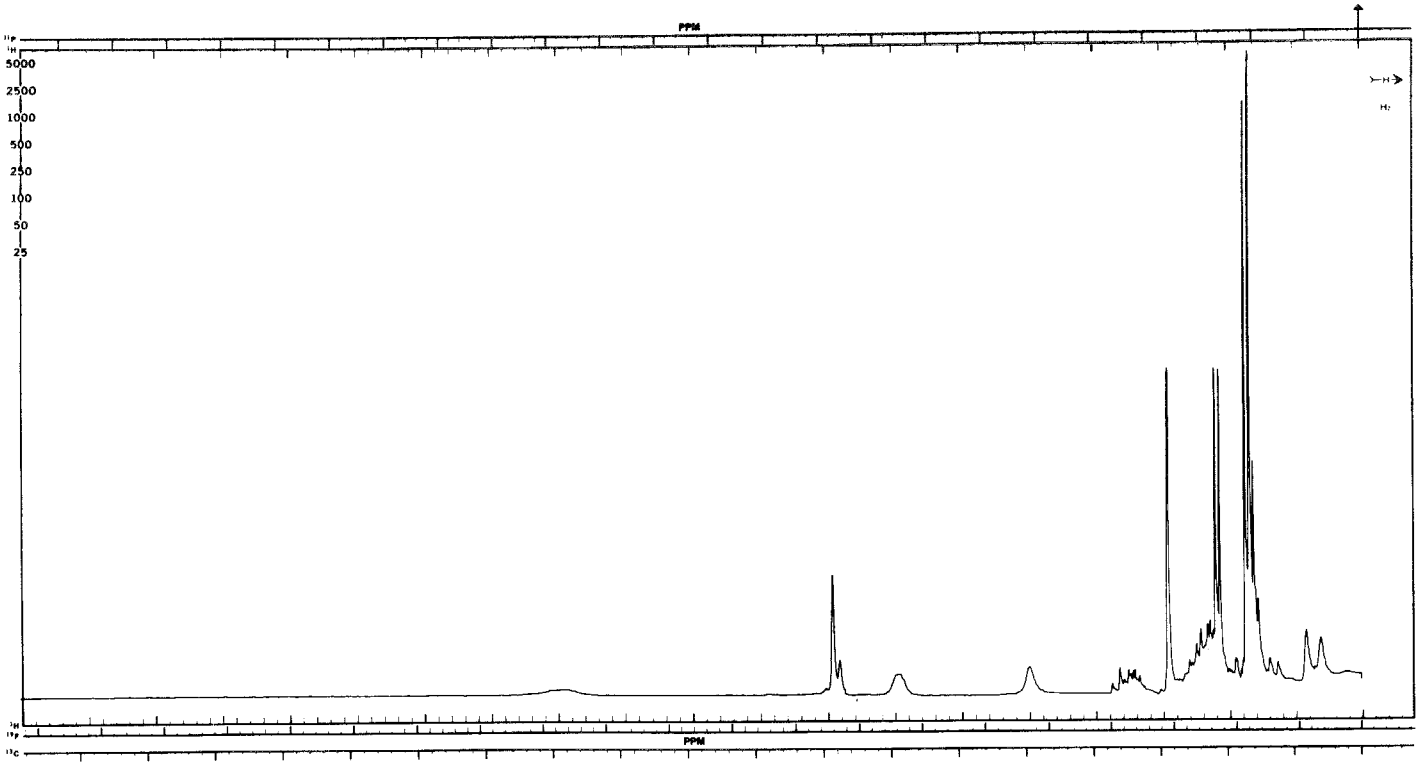
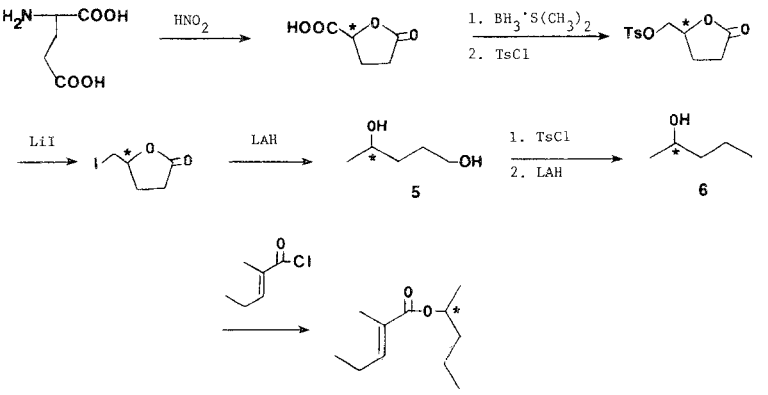


Fig. 5c.



SCHEME 2

of secondary alcohols since the carbon chain may be lengthened by addition of organocuprates to halides and tosylates (Smith and Williams, 1979). The (*S*)-(+)-alcohol produced from *D*-glutamic acid produced the natural (*S*)-(+)-dominicalure 1 and 2. On the assumption that natural dominicalure 1 and 2 are enantiomerically pure (as is indicated above), the enantiomeric excesses (optical purity) of the synthesized enantiomers are: (*S*)-(+)-dominicalure 1 = 98%; (*R*)-(−)-dominicalure 1 = 93%; (*S*)-(+)—dominicalure 2 = 99%, (*R*)-(−)-dominicalure 2 = 93%.

Preliminary testing showed the natural (+) isomers to be approximately twice as active as the unnatural isomers. No synergistic or blocking effects were noted.

DISCUSSION

The compounds identified in this paper are unlike any other pheromonal esters that we are aware of, being neither fatty acid- nor terpenoid-derived and having acid and alcohol moieties of roughly equal length. Studies of the pheromones of related insects are contemplated to determine if unsaturated esters of this type are used generally or if they are unique to this species. The unusually intense, sticky-sweet fragrance of the dominicalures may be a guide in this study since other compounds of this type could perhaps be expected to have similar odors.

Our preliminary field testing has been highly encouraging. Since the synthetic procedures developed during these studies use inexpensive starting materials and can easily be scaled up, the production of kilogram amounts of the dominicalures is possible and is at present under way. Large-scale field testing of synthetic pheromone will be performed in Texas, Australia, and elsewhere in the near future.

Acknowledgments—Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by cooperative agreement among the University of Wisconsin, the State University of New York College of Environmental Science and Forestry, and the U.S. Department of Agriculture, and by National Science Foundation grant PCM7813244. We thank Mrs. Hazel Jennison for the mass spectra and Mr. Alan Harvey for the NMR spectra.

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Journal of Chemical Ecology, Vol. 7, No. 4, 1981

Obituary

It is with profound sorrow that we communicate the untimely death as a result of acute leukemia of our colleague and friend Dr. John Brian Siddall on Sunday morning, April 26, 1981. John was born in Chesterfield, England on May 27, 1939 and obtained his education in England, completing his Ph.D. in Organic Chemistry under the direction of Professor Arthur J. Birch at Manchester. He joined Syntex Corporation in Mexico City in 1963 and was rapidly promoted to Senior Chemist (1964) and to Group Leader (1967) in this company's newly established research center in Palo Alto, California. In this latter capacity he headed Syntex's pioneering research in the synthesis of insect molting hormones and of juvenile hormones and their analogues. This work was instrumental in the establishment of Zoecon Corporation in Palo Alto, where he served as its first Director of Research, then as Vice President of Research, and lately as its first Senior Scientist. Throughout his tragically short career, John authored a prodigious number of patents and research publications in the area of the chemistry and action of insect hormones and pheromones. He was intensely interested in the biology and biochemistry of insects. In 1971 he launched a basic research project which resulted in the discovery of a new natural insect juvenile hormone in 1973, and he played a major role in the discovery and development of methoprene, the first commercial juvenoid. For his contribution to this latter invention he was corecipient of the 1979 'Inventor of the Year' award from the parent corporation, Hooker Chemical Company, a division of Occidental Petroleum Corporation. John was a member of the American Chemical Society, The Chemical Society, AAAS, and the Federation of American Scientists. He was very active in the activities of the Division of Pesticide Chemistry and recently served as a member of the executive committee. He was also on the board of editors of the *Journal of Chemical Ecology*. In addition to his scientific and professional activities, John was an avid sailor and skier, and devoted much time to the Boy Scouts of America. Our hearts go out to his wife Judie, his children, and to his parents.

We will all miss John for his brilliant scientific ideas, his eloquence, his wit, and his gentle ways.

Clive A. Henrick
Julius J. Menn

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Errata

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A. SHANI and J.T. KLUG

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TRAPPING THE WESTERN PINE BEETLE AT AND NEAR A SOURCE OF SYNTHETIC ATTRACTIVE PHEROMONE: EFFECTS OF TRAP SIZE AND POSITION

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The first sentence of the second paragraph below Table 4 on p. 528 should read, "When the surrounding traps were 0.19 m^2, the proportion of males

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caught at the source of attractant was significantly different from the proportions caught 1.5 m away, 1.5 m high and 4.5 m away, 3.0 m high (Table 2). The proportion of males caught 1.5 m away, 1.5 m high was significantly different from the proportion caught 1.5 m away, 3.0 m high." We thank J.A. Byers for bringing this error to our attention.

REFERENCE

TILDEN, P.E., BEDARD, W.D., WOOD, D.L., LINDAHL, K.Q. and RAUCH, P.A. 1979. *J. Chem. Ecol.* 5:519-531.

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DETERRENCE OF REPEATED OVIPOSITION IN SORGHUM SHOOTFLY *Atherigona soccata*

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(Received September 15, 1980; revised November 17, 1980)

Abstract—The sorghum shootfly, *Atherigona soccata*, under low population density conditions lays one egg per sorghum plant. Possible regulatory mechanisms of this oviposition behavior are reported. The presence of an egg thoroughly washed and reattached to a leaf does not deter further oviposition, indicating the absence of visual cues. When washings from eggs were sprayed on sorghum plants, or when plants from which eggs had been removed were presented to a gravid female, significant deterrence was observed. Apparently a deterrent pheromone is associated with the water-soluble glue with which the females attach their eggs to the leaves. Some deterrent effect persists for at least 7 days.

Key Words—Oviposition deterrent, pheromone, sorghum shootfly, *Atherigona soccata*, Diptera, Muscidae.

INTRODUCTION

Among phytophagous insects whose feeding sites are selected by a stage other than the one actually feeding and where the food resources are exhaustible, the species have often developed a mechanism of uniformly distributing their progeny to avoid intraspecific competition. Certain insect species are known to accomplish this through marking pheromones produced by the females and dispensed at the time of oviposition. The best known examples are from frugivorous Tephritidae (Prokopy et al., 1976). The pheromones in these cases act as markers and deter subsequent oviposition. Such pheromones have been reported from several other groups of insects by Oshima et al. (1973) in Bruchidae and Rothschild and Schoonhoven (1977) in Lepidoptera, to give a few examples.

The sorghum shootfly, *Atherigona soccata* Rondani, under low population density conditions usually lays only one egg per sorghum plant

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(Ponnaiya, 1951; Ogwaro, 1978). The eggs are glued to the underside of the leaves with a water-soluble substance. Even when more than 1 egg is laid on one plant, only 1 larva survives to maturity (Raina, 1981).

Results of the investigation of two possible regulatory mechanisms, namely visual recognition of the presence of eggs or recognition of a chemical factor controlling oviposition in the sorghum shootfly, are reported here.

METHODS AND MATERIALS

CSH-1, a hybrid variety of sorghum developed in India and very susceptible to the shootfly, was used in the study. Plants were grown in 37.5 × 45 × 10-cm metal trays at 20 plants/tray, in a greenhouse under 27 ± 5°C and 70 ± 20% relative humidity. Three-week-old plants (4- to 5-leaf stage) were used for the tests. Flies were reared on CSH-1 and maintained in an environmental chamber at 30°C, 60 ± 10% relative humidity, and a light-dark regime of 12:12 hr.

In the first experiment shootfly eggs, thoroughly washed in distilled water, were glued with a dilute solution of commercial paper glue, one per leaf, on every leaf of alternate plants in a tray. Glue without eggs was applied to control plants with a fine brush. A mated gravid female was caged among the plants in each tray for 2 days, after which the eggs were counted on all the plants. Four such trays were used each time and the test was repeated 3 times.

In the second experiment flies were allowed to lay many eggs on sorghum plants in oviposition cages. The leaves with eggs were cut, the eggs counted, and then the leaves shaken in a known quantity of distilled water for 5 min, whereby almost all the eggs became detached from the leaves. The liquid was filtered and the filtrate sprayed on two alternate rows (10 plants) of a tray, at a rate of washing from 25 eggs/plant. However, not all the spray hit the plants and no attempt was made to determine the exact amount received by the plants. A single gravid female was caged among the plants in each tray and eggs counted after 2 days. The test using 4 trays was repeated 3 times.

In the third experiment sorghum was planted in 7.5 × 2.5-cm glass specimen tubes. Half of these (20 plants) were introduced in oviposition cages and the flies allowed to lay eggs. As soon as 2–3 eggs were laid on each leaf of a plant, it was taken out and the eggs removed with a moist camel-hair brush. One test plant and a control plant (on which no eggs had been laid) were placed in a cage with a gravid female for 4–6 hr. The plants were then removed and eggs on each one of them counted.

In the last experiment, 90 plants were planted in glass specimen tubes. Forty-five of these plants were sprayed with egg washing at a rate of washing from 12 eggs/plant, whereas the control plants were sprayed with eggfree leaf wash. (Both the egg and the leaf wash were concentrated by lyophilization before use.) One treated and 1 control plant were placed in a test cage with a

gravid female. Oviposition behavior of the fly and the sequence and time of egg laying for the first 5–6 eggs was recorded. Five such pairs of plants were tested each day for 8 days to study the persistence of the pheromone. Deterrent activity was measured on a scale of 0–15 (5 for first egg laid to 1 for the 5th egg because deterrence exhibited by the treated plant decreased as more eggs were laid on the control plant) with a maximum of 15 if all the 5 eggs were laid on the control plant. All data were analyzed using analysis of variance and t test.

RESULTS

There was no significant difference in oviposition between plants on which eggs were artificially glued and those without such eggs (Table 1). The results indicate that the presence of an egg alone on a plant does not deter the fly from laying more eggs on it.

When plants on which the washing from eggs had been sprayed were presented along with control plants for oviposition, significantly fewer egg-wash-treated plants received eggs. The number of eggs per plant was also significantly lower in the former. Also when plants from which eggs had been removed were presented for oviposition along with control plants the differences were highly significant ($P > 0.01$) (Table 1). These results indicate that a chemical marker, deterring further oviposition by the shootfly, was present in the water-soluble cement used by the fly to attach the eggs. So far attempts to extract the deterrent chemical with nonpolar solvents have resulted in lack of activity.

The activity of the deterrent substances when sprayed on plants was maximum on the first day, decreased on the second day and thereafter did not change significantly during the rest of the test period (Figure 1).

TABLE 1. RESULTS OF EXPERIMENTS INVESTIGATING POSSIBILITY OF OVIPOSITION MARKING PHEROMONE IN SORGHUM SHOOTFLY

	Visual recognition		Chemical marking[a]		
			Eggs-wash spray		Eggs removed
	% plants with eggs	No. of eggs/plant	% plants with eggs	No. of eggs/plant	(average eggs/plant)
Treatment	70.0	1.76	54.4*	0.91**	3.1**
Control	77.5	1.33	72.2	1.60	13.0

[a]Significantly different at $*P = 0.05$, and at $**P = 0.01$.

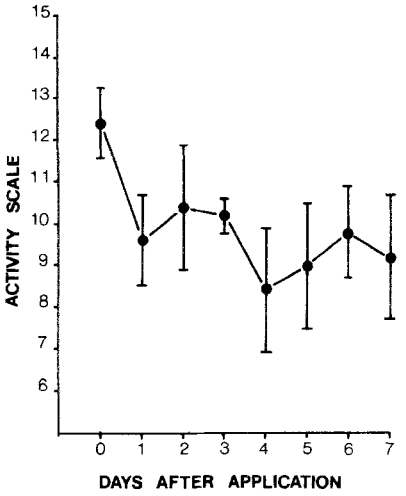

FIG. 1. Persistence of activity of the oviposition-deterring pheromone of sorghum shootfly sprayed as an aqueous solution on sorghum plants. Vertical bars indicate ±SD.

When an egg-wash treated plant and a control plant were simultaneously presented to a gravid female, the first landing of the fly was completely at random, indicating the nonvolatile nature of the deterrent. If a female landed on a treated plant, it rapidly moved up and down the plant, exhibiting characteristic tapping with its front tarsi. Only about half the flies that landed on a treated plant laid their first eggs on it. On the other hand, of those that landed on a control plant nearly 95% laid an egg. After laying an egg on the control plant, the flies flew either to the cage walls or to the treated plant. In the latter case, however, they returned quickly to the control plant. This behavior continued until 2–3 eggs were laid on the control plant. Oviposition thereafter was random.

DISCUSSION

Since only one shootfly larva can survive and grow to maturity in a single sorghum plant, it is of significant advantage to the fly to distribute its eggs evenly on the available plants. Present observations indicate that this is accomplished by a marker pheromone which deters repeated oviposition on one plant.

In most reported cases of oviposition-deterring pheromones (e.g., Tephritidae), the chemical agent is applied by dragging the ovipositor on the oviposition substrate. In case of the shootfly the deterrent is apparently present in the glue cementing the egg to the leaf. Zimmerman (1978) reported a

similar deterrent in *Hylemya* associated with the glue cementing the egg to the sepals. He further discounted the presence of any visual cues because the eggs are hidden between the sepals and petals. However, Rothschild and Schoonhoven (1977) reported that even model plastic eggs placed on cabbage leaves inhibited oviposition by the cabbage white butterfly, *Pieris brassicae* L. This is not so in case of the sorghum shootfly, where the mere presence of a thoroughly washed egg does not deter flies from subsequent oviposition. The female fly does not hover over a plant but lands directly on it. Upon landing it explores the entire leaf surface before selecting a site for oviposition. The fly actually has to come into contact with the deterrent chemical, whereupon it exhibits characteristic tapping of the foretarsi and rapid movement up and down the plant. The deterrent is therefore either nonvolatile or very slightly so. Katsoyannos (1975) reported a water- and methanol-soluble oviposition-deterring pheromone for the cherry fruit fly, *Rhagoletis cerasi* L. which elicits chemotactile responses in which no long-range olfaction is involved.

The deterrent effect of the pheromone in the shootfly was observed to last at least for 7 days when sprayed as an aqueous solution on test plants. This is longer than needed, since in about 4–5 days after an egg is laid the plant shows symptoms of dead-heart, which by itself is a deterrent for oviposition (unpublished observations). Prokopy et al. (1977) reported that deterrent activity of the pheromone in the Caribbean fruit fly *Anastrepha suspensa* (Loew) lasted for at least 6 days. Whereas water solubility of the deterrent pheromones may seem to be a disadvantage (Prokopy et al., 1978), it is not so in the shootfly, because the eggs are laid in the curvature on the underside of a leaf, well protected from rain.

Possible use of oviposition deterrent pheromones as control agents has been suggested by Katsoyannos and Boller (1976) and Prokopy et al. (1978). The applicability of such a deterrent for shootfly control will depend on, among other factors, identification of the active compound(s) and further tests under field conditions.

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BIOLOGICAL AMPLIFICATION FOR INCREASING ELECTROANTENNOGRAM DISCRIMINATION BETWEEN TWO FEMALE SEX PHEROMONES OF *Spodoptera littoralis* (LEPIDOPTERA: NOCTUIDAE).[1]

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(Received July 19, 1980; revised November 20, 1980)

Abstract—Male *Spodoptera littoralis* stimulated by a mixture of *S. littoralis* female sex pheromone with a positional isomer and by the pheromone alone, gave greatly amplified electroantennograms (EAGs) when the antennae were connected in series. The technique is described and examples are given of its use in screening extracts for EAG activity. Dose–response curves obtained with this technique are presented and discussed. Through biological amplification, EAG discrimination between the pheromones was increased.

Key Words—*Spodoptera littoralis*, Lepidoptera, Noctuidae, electroantennogram, biological amplification, pheromones, *Boarmia selenaria*, Geometridae, *Matsucoccus josephi*, Homoptera, Coccoidea.

INTRODUCTION

Among the female sex pheromones of *S. littoralis* which have been isolated and synthesized are the primary pheromone *cis*-9,*trans*-11-tetradecadienylacetate (*Z,E*-9,11–14:OAc, component I) (Nesbitt et al., 1973; Tamaki and Yushima, 1974) and *cis*-9,*trans*-12-tetradecadienylacetate (*Z,E*-9,12–14:OAc, component II) (Tamaki and Yushima, 1974). Priesner (1979) found within the receptor system of the male *S. littoralis* two specialist cells reacting to components I and II, respectively. In their field work, Kehat et al. (1976) showed that adding one part component II to 100 parts component I

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significantly increased male catches. This was confirmed recently with the same ratio of more purified substances (Dunkelblum et al., personal communication).

With these data in mind, two questions were asked: (1) Do EAG responses to the major component alone and to a 100:1 blend I:II differ in amplitude? (2) If so, at which doses are such differences largest?

Answers to these questions were sought through conventional contact EAG (i.e., with nonimplanted electrodes) applied in a novel but simple manner. In this paper the technique is described, the results are discussed, and some pertinent applications are presented.

METHODS AND MATERIALS

The test animals were males up to 24 hr old from a laboratory colony reared continuously on an artificial diet (Navon and Keren, 1980).

The pheromone components I (95% GLC grade; product of Chemada, Be'er Sheva', Israel) and II (98% GLC grade; product of USDA Labs., Beltsville, Maryland) (see above) were both serially diluted with hexane. Filter paper strips were loaded, respectively, with 0.02, 0.2, 2.0, 20.0, and 200.0 μg of I and the same doses of I with 1% II added. After solvent evaporation, the ten strips were each put in a disposable Pasteur pipet. When not in use, the pipets were stored in a refrigerator.

EAG signals received by a WPI, model M701, microprobe system were fed into the following Tektronix units: (1) one AM505 and one 5A22N differential amplifier; (2) a 5B12 dual-time base; and (3) a D12 dual-beam oscilloscope. EAG deflections were photographed with a Nikon Kohden PC-2A recording camera.

The EAG technique of Roelofs (1977) was used except for the following. The recording electrode was of the conventional type. The stage for the antennal preparations was a paraffin plate. Sodium carboxymethylcellulose (CMC; ca. 1,500 cps; BDH) served for securing the antennae and as relays. The CMC was dissolved as a 4% w/v solution in the saline used for filling the recording electrode (9.0 g NaCl/liter; 0.2 g KCl/liter; 4.0 g glucose/liter). Drops of this solution were deposited as a quincunx in the center of the plate.

For each antennal preparation, two males were used simultaneously. Their antennae were cut as close as possible to the frons; the tips remained complete. The four antennae were transferred with a fine camel-hair brush to the surface of the five securing-relay drops in a series forming an inverse letter Z. Conductivity was ensured by wetting the antennal base with CMC. The longitudinal, nonscaly, sensitive part of the antennae faced the air stream. Antennal polarity was preserved by keeping a tip-to-base order within the series.

When comparing the pheromones at a given dose, the electrodes first spanned four antennae and eight readings were taken with the two test compounds (four/compound; see below). Thereafter, the indifferent electrode was moved to the second antenna, reducing the span to three, and again eight readings were taken. This procedure was repeated until eight readings had been taken with the last antenna. At each dose, four tetrads of antennae were used.

The stimuli were given according to a fixed sequence to equalize the chances of adaptation or saturation. Thus, with four antennae, the stimuli compared (e.g., a and b) were given in the sequence ab, ba, ab, ba; with three antennae, ba, ab, ba, ab, etc. Moist air was kept flowing over the antennae; the time elapsing between stimuli was ca. 45 sec.

Linear regressions of the recorded depolarizations on the number of antennae in series were calculated with in millivolt units. Linearity of the regressions was tested by analysis of variance for repeated measurements on the response (Walpole and Myers, 1978). Other statistical work was done with millivolt data normalized for offsetting time-dependent changes in antennal responses (Roelofs and Comeau, 1971); this was done by taking the ratio "10 × EAG response to mixture/EAG response to major component" (Roelofs, 1977).

RESULTS AND DISCUSSION

Amplification of EAG signals was based on in-series resistor and capacitor theory. Assuming the antenna functions, in part at least, as a resistor and/or capacitor, connecting antennae in series should result in a voltage between the terminals equal to the sum of the individual antennal voltages. Therefore, the depolarization of antennae in series reacting to the proper stimulus was expected to be substantially larger than that of a component antenna.

In preliminary work, a correlation was sought between depolarization and antennal impedance. When the number of antennae between the electrodes was varied within a tetrad, this correlation was good ($0.86 \leqslant r \leqslant 0.97$) and agreed with theory. However, between antennal preparations the coefficient of variation of the impedances was 57.2% (four tetrads: 64 readings), as opposed to nil when the independent variable was the number of antennae. Therefore, this simpler relationship was preferred and fair agreement to a linear model was obtained ($0.81 \leqslant r \leqslant 0.93$).

An example of the relationship between the depolarization amplitude and the number of antennae in series for doses of 0.02 and 20.0 μg is given in Figure 1. The same type of relationship was obtained with the other doses.

A striking feature of all these functions was the rapid rise in EAG magnitude with the number of antennae in series. This agrees with the

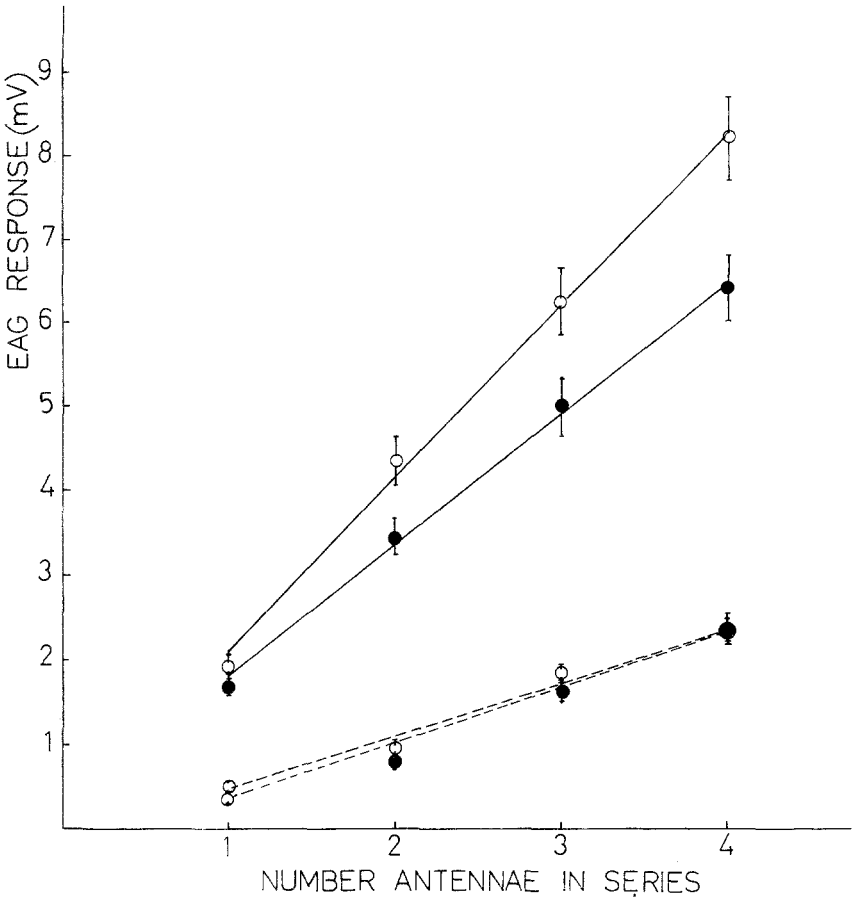

FIG. 1. EAG responses of male *Spodoptera littoralis* antennal preparations to 0.02 μg (---) and 20.0 μg (———) source concentrations of *Z,E*-9,11-14:OAc alone (●) and with 1% *Z,E*-9,12-14:OAc admixture (0). Each dot is the mean of 16 readings. Vertical bars represent standard errors.

summation theory, whereby the magnitude of the EAG reaction to a given stimulus is directly related to the number of receptors reacting (Payne, 1975). This "biological amplification" was applied in screening crude female extracts for EAG activity. In this work, the electrodes spanned four antennae connected in series. With four male *Boarmia selenaria* (Lepidoptera: Geometridae) antennae, a stimulus of as little as 4 FE crude extract of female tips gave an average EAG of 2.4 mV (I. Moore, M. Wysoki, D. Becker, and T. Kummel, unpublished results). With a very small insect, *Matsucoccus josephi* (Coccoidea: Margarodidae), EAGs of 1.3 mV were obtained with Porapak

collections of the female effluvium (I. Moore, M. Sternlicht, and E. Dunkelblum, unpublished results). It was noted also that biological amplification considerably improved the signal-to-noise ratio.

When the pheromones were given at a dose of 0.02 μg of the major component, the regression lines were very close and even converged (Figure 1). Thus, despite the presence of specialist cells (Priesner, 1979), no differences were detected in the amplitude of the responses to the major component and to the binary mixture. The same trend was observed at a dose of 200.0 μg although there was a greater distance between the regression lines. After normalization, the single antenna and tetrad data for these two doses were

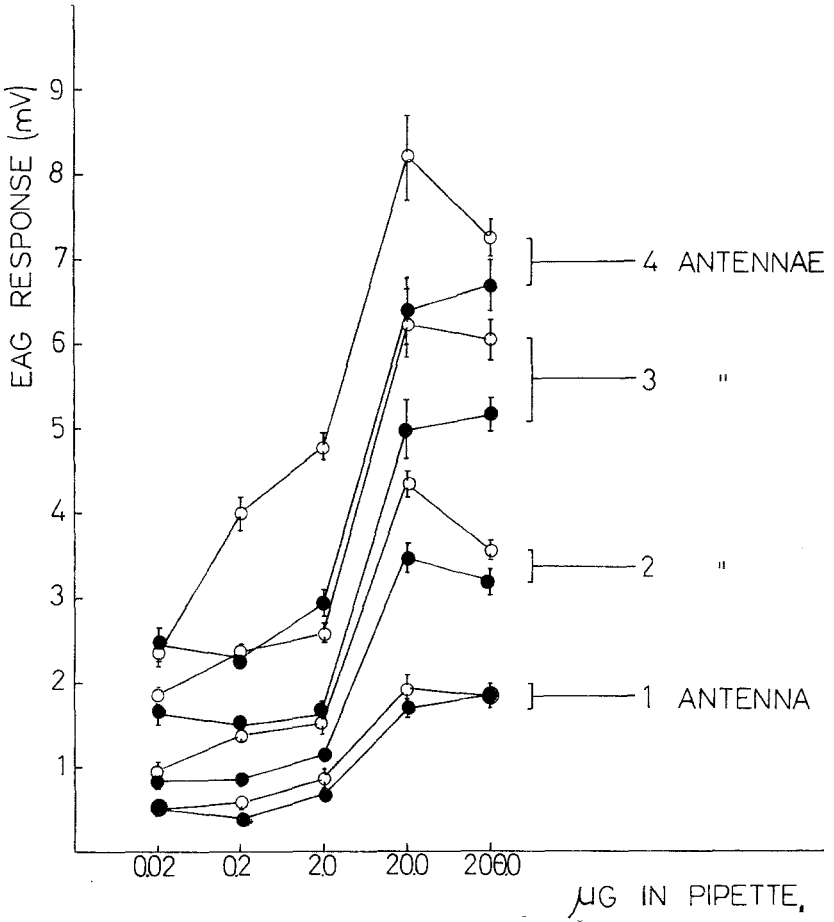

FIG. 2. Log dose-EAG response of male *Spodoptera littoralis* to various source concentrations of *Z,E*-9,11-14:OAc alone (●) and with 1% *Z,E*-9,12-14:OAc admixture (0). Antennae connected in series. Each dot is the mean of 16 readings. Vertical bars represent standard errors.

submitted to t tests for significant departures from 10 (the arbitrary value of the standard); there were none. However, at the other doses there were distinct differences in slope within and between the regression line pairs (e.g., for 20.0 μg in Figure 1). The same t tests as above confirmed that at doses of 0.2, 2.0, and 20.0 μg, four antennae in series gave highly significantly greater depolarizations ($P < 0.01$) with the binary mixture than with the major component alone; the same was true for single antennae, except at 2.0 μg, for which the probability was greater than 0.01 but less than 0.05.

The dose–response curves obtained by plotting the mean EAG response against log dose at the various antennal levels are shown in Figure 2. These curves illustrate the amplification of EAG signals with the increasing number of antennae. Also, they all exhibit a similar, typical, sigmoid trend. Thus, qualitatively, the same trend was obtained with antennae in series as with a single antenna but, quantitatively, it was greatly magnified and so were the differences in the amplitude of the responses to the pheromones. This magnification can be particularly advantageous with weak signals. Another advantage of using antennae connected in series is that for the same number of observations (but a little more work in preparing the antennae), the EAG data become based on a broader sample of antennae and receptors than those for the single antenna. This is shown by the analysis of variance of the normalized data, which provided an error mean square of only 6.88 for the tetrad data vs. 20.82 for the single antennae (75 *df* and homogeneous variance of antennal preparations in both cases). The greater precision obtained through in-series amplification was also apparent when the mean normalized values were separated by Duncan's multiple range test (Table 1).

As expected, the values indicated in Table 1 under each dose did not differ significantly, except for the 2.0-μg pair ($0.01 < P < 0.05$); since the data were normalized, this is hard to understand. Nonetheless, Table 1 shows

TABLE 1. MEAN[a] NORMALIZED[b] EAG RESPONSES OBTAINED WITH ONE ANTENNA AND FOUR ANTENNAE IN SERIES STIMULATED BY 100:1 MIXTURE OF *Z,E*-9,11-14:OAc; *Z,E*,-9,12-14:OAc (MALE *Spodoptera littoralis*)

Number of antennae	Dose in pipette (μg major component)				
	0.02	0.2	2.0	20.0	200.0
1	11.76^{b}	16.98^{a}	13.79^{ab}	11.68^{b}	10.64^{b}
4	$9.89^{c'}$	$17.76^{a'}$	$16.88^{a'}$	$12.85^{b'}$	$11.08^{b'c'}$

[a]Figures with the same superscript do not differ significantly ($P > 0.05$).
[b]10 × EAG response to mixture/EAG response to major component.

that with both techniques the largest response ratios "blend–major component" were obtained with the 0.2- and 2.0-μg doses, but the single antenna EAG did not allow any further separation.

After repeated stimulation, especially at high doses, some nonproportional (i.e., nonlinear) decreases of the responses would have been symptomatic of desensitization. This may have been a factor at the 0.2- and 2.0μg doses but not at the others, particularly 200.0 μg, for which there were no significant departures from linearity. Nevertheless, the clear separation of the curves between doses of 0.2 and 20.0 μg agrees well with the presence of the two specialist cells found by Priesner (1979). Thus, the greater amplitude recorded with the blend apparently reflects the combined responses of the two types of cells as opposed to the single cell responses to the major component alone. The asymptotic trends at the lowest and highest doses can be interpreted as threshold and saturation responses, respectively. If both specialist cells are being saturated by the blend, those reacting to the minor component have a greater affinity than the others.

The conventional EAG may not always be sensitive enough for monitoring minor pheromone components (Tamaki, 1979). When screening fractions of a multicomponent pheromone system or testing various pheromone mixtures for EAG activity, the signal amplification and improved signal-to-noise ratio obtained by connecting the antennae in series could be particularly useful.

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EFFECT OF VOLATILES COLLECTED ABOVE FECAL PELLETS ON BEHAVIOR OF THE RABBIT, *Oryctolagus cuniculus*, TESTED IN AN EXPERIMENTAL CHAMBER

I. Total Volatiles and Some Chemically Prepared Fractions

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Abstract—The fecal pellets of rabbits, *Oryctolagus cuniculus*, are coated with secretion from the anal gland, the odor from which functions as a territorial marker. The total volatiles collected from above fecal pellets and three different fractions prepared by washing the volatiles through either distilled water, 1 M sodium hydroxide, or 1 M hydrochloric acid were tested for their effect on the territorial confidence of individual animals. Tests were based on the neutral pen paradigm used in previous studies but employed a specially designed test chamber, the internal odor environment of which could be fully controlled. In the four series of bioassays a total of 140 separate tests were performed and 64 adult male rabbits were used. The results showed that the presence of the total volatiles influences the outcome of territorial competitions in pairs of male rabbits in favor of the individual from which they were derived. They were comparable with those obtained in earlier studies when fecal pellets were presented in the test pen. The effects of the different washing reagents on the total odor are illustrated by gas chromatograms, and the components most likely to be removed by the different treatments are suggested. The volatiles remaining after passing through distilled water had less effect on the rabbit's behavior than the total odor. Since the washing process not only removed water-soluble compounds but also led to a reduction in the concentrations of water-insoluble components introduced into the test chamber, either of these factors could have been responsible for the weakened effect. Fractions of the odor obtained by washing with either sodium hydroxide or hydrochloric acid did not influence the results of the tests. This suggests that the essential components of the olfactory signal include both basic and acidic compounds.

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Key Words—Rabbit, *Oryctolagus cuniculus*, anal gland secretion, feces, headspace volatiles, chemical fractions, bioassay, territorial confidence.

INTRODUCTION

The odor from the anal gland secretion of the wild rabbit, *Oryctolagus cuniculus*, functions as a territorial marker. It is distributed throughout the rabbits' living space on the surface of fecal pellets which become coated with the secretion as they pass through the anus (Mykytowycz, 1968, 1974). No practicable way has been found to collect pure anal gland secretion from live rabbits in the quantities required for bioassay and without damage to the animals. To continue our studies of the behavioral function and chemistry of this secretion we are obliged to use fecal pellets as the source for samples from live individuals. It has been shown experimentally that when a rabbit is confronted with another one in a neutral environment the presence of an individual's own fecal pellets increases the probability that it will become dominant (Mykytowycz et al., 1976). This effect has been attributed to the odor of the anal gland secretion carried on the surface of the pellets; however, the possibility that fecal pellets themselves provided essential visual or gustatory stimuli as well as odor could not be entirely excluded.

The primary aim of the present study was to establish that the olfactory signals contained in the volatiles given off by a rabbit's own fecal pellets affect its territorial confidence.

The chemical compositions of the anal gland secretion and of the total odor trapped from above fecal pellets, referred to throughout the text as headspace volatiles, are very complex (Goodrich and Mykytowycz, 1972; Goodrich et al., 1978, 1981a). It was therefore felt that the search for the components essential for forming the olfactory signals would be simplified if some groups of compounds in the total odor could be eliminated from further search.

In a previous study young rabbits were exposed to macerated anal gland materials. Their overt and heart-rate responses indicated that they reacted more strongly to the acid and neutral components than to the basic fraction (Hesterman et al., 1976). This suggested that all basic compounds might be eliminated from the search for the components of the olfactory signal. The possibility has been investigated in the present study by testing the behavioral effect of fractions of the fecal pellet volatiles from which the acidic and basic components were removed by chemical scrubbing.

Since aqueous solutions of the chemical agents were used in the scrubbing technique, it was also necessary to test the behavioral effect of the headspace volatiles after washing with water alone.

METHODS AND MATERIALS

Test Chamber. To provide the conditions necessary for these experiments, it was essential to control and manipulate the internal odor environment of

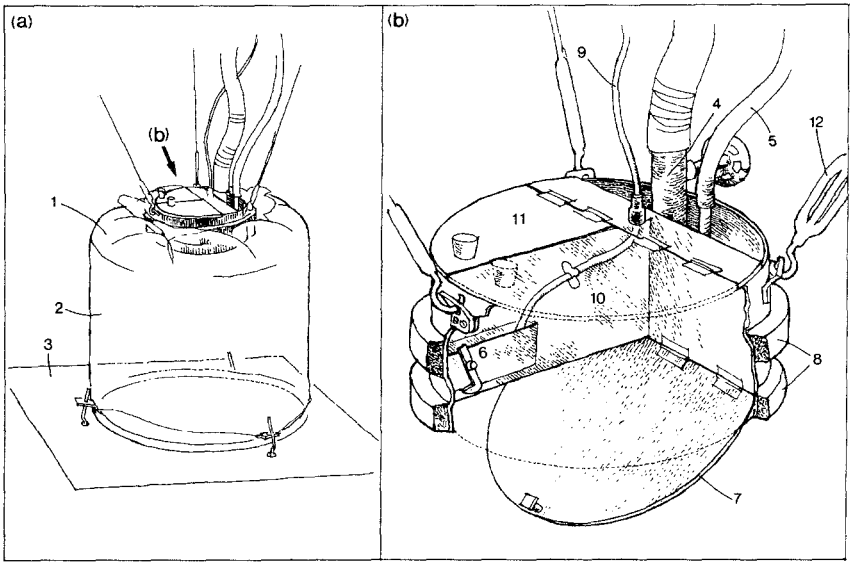

FIG. 1. Diagram of chamber used to test the behavioral reaction of rabbits to the volatiles trapped above fecal pellets. (a) Assembled chamber. (b) Sectioned view of inlet head. 1 = plastic film lining; 2 = Perspex cylinder; 3 = baseboard; 4 = air inlet pipe; 5 = Teflon tubing carrying odor-laden airstream; 6 = solenoid-operated catch mechanism; 7 = spring-loaded lower flap; 8 = sponge rubber sealing rings; 9 = electrical connection to solenoid operating switch; 10 = wall separating compartments; 11 = lid to compartment (1 of 2); 12 = suspension hook (1 of 3).

the test area. To achieve this a test chamber was designed, and its structural details are shown in Figure 1.

A cylindrical chamber 1.13 m in diameter and 0.7 m high was formed from a sheet of clear Perspex 3 mm thick. A sleeve of industrial polyethylene film (0.1 mm) of the same diameter as the cylinder was used as an internal lining. The upper end of the sleeve was clamped around a metal inlet head suspended above the center of the chamber. The lower end was turned back beneath the base of the Perspex cylinder which was clamped down against a rubber insert in the baseboard. When inflated with air at low pressure, the airtight lining molded closely to the Perspex and allowed a clear view of the inside of the chamber. The polyethylene lining and a sheet of the same material spread across the floor of the pen could be quickly renewed between tests, thus eliminating the possibility of odors being carried over from one test to another. The floor area of the chamber was 1 m^2 and its volume approximately 750 liters (see Figure 1a).

Two rabbits could be introduced simultaneously into the sealed chamber from two separate compartments in the inlet head (see Figure 1b). A single

hinged flap sealed the floor of the two compartments and was retained in the closed position by a latch mechanism which could be tripped remotely by means of an electric solenoid circuit. With the latch mechanism tripped, the weight of the rabbits forced the flap open against a return spring allowing them to fall into the chamber while the flap returned to the closed position.

Fresh air to inflate the plastic lining and the odor-carrying airstream were introduced into the chamber through separate tubes which passed through a bulkhead in the inlet head.

Animals. Sixty-four adult male wild-type rabbits were used in the experiments. They were all socially experienced and normally lived in large outdoor pens in mixed sex groups of three or four individuals. For the duration of the experiments they were kept singly in cages in an animal house. They were fed ad libitum on a standard pelletised rabbit food.

Preparation and Presentation of Odors for Testing. Collection of fecal material was carried out overnight from the test animals housed in individual cages. A wire mesh tray placed under the cage retained the pellets but allowed urine to pass through. Pellets suspected of being contaminated with urine were not used. The pellet collections were stored for no longer than one month at −20° C in sealed glass containers.

Total headspace volatiles used for the tests were obtained from 30-g samples of fecal pellets contained in a 250-ml long-necked conical flask and warmed to 37° C in a water bath (see Figure 2a). A metered flow of 300 ml/min supplied from a cylinder of medical-grade compressed air was passed downwards into the flask over the surface of the sample. In tests on total volatiles, the odor-laden vapor was fed directly into the test chamber for 30 min prior to the test. This gave a concentration of volatiles of approximately 12 liters headspace equivalent/kiloliter in the chamber.

Fractionation of headspace volatiles was carried out by bubbling the gas stream emerging from the sample flask through a series of three gas scrubbing tubes each containing 30 ml of wash solution (Figure 2b). For the removal of water-soluble components, all three scrubbing tubes contained distilled water. Acidic or basic components were removed by using 1 M NaOH or 1 M HCl, respectively, in the first two tubes and a 0.2 M phosphate buffer, pH 7, in the third tube. The acid, alkali, and buffer wash solutions were saturated with sodium chloride to reduce water solubility of the odor components.

Table 1 indicates the likely general composition of the odors after passage of the volatiles from the fecal pellets through the different scrubbing reagents.

The back-pressure generated by the gas scrubbing tubes limited the vapor flow in these experiments to 80 ml/min. The concentration of headspace volatiles in the test chamber after 30 min was therefore approximately 3.2 liters headspace equivalent/kiloliter.

Gas Chromatography of Headspace Volatiles. The total headspace volatiles and fractions (see above) obtained from a pooled sample of fecal

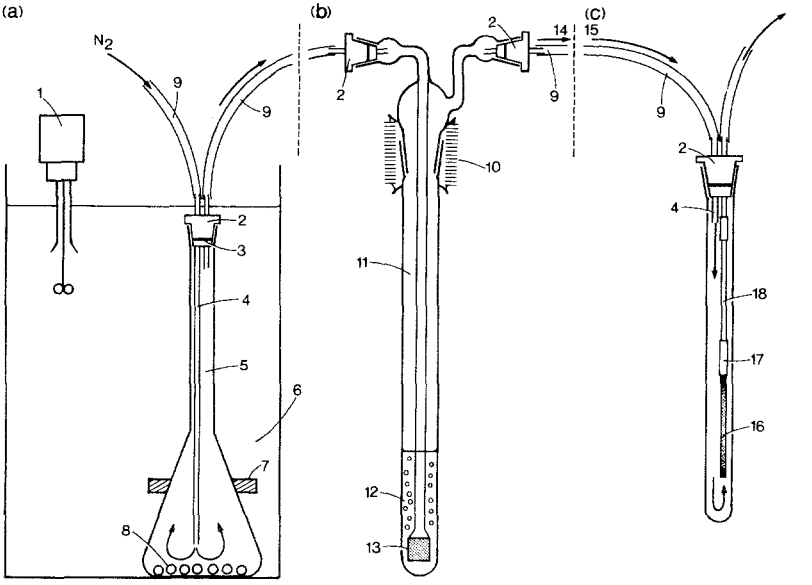

FIG. 2. Diagrams of apparatus used to prepare and fractionate the headspace volatiles from the fecal pellets of rabbits. (a) Apparatus used to prepare the total odor. (b) Gas scrubbing tube. (c) Apparatus used to collect the volatiles onto Chromosorb 105 traps for gas chromatographic analysis. 1 = thermostatic stirrer immersion heater; 2 = Teflon stopper 14/23; 3 = Viton O ring; 4 = stainless-steel tubing, 3.2 mm OD; 5 = 250-ml long-necked conical flask fitted with 14/23 joint; 6 = water bath; 7 = lead collar; 8 = fecal pellets; 9 = thin-walled Teflon tubing, 3.2 mm ID; 10 = retaining spring; 11 = gas scrubbing tube; 12 = wash solution (30 ml); 13 = glass sinter; 14 = connection to additional gas scrubbing tubes; 15 = scrubbed headspace volatiles; 16 = Chromosorb 105 trap; 17 = Teflon union; 18 = empty stainless-steel spacer tube.

pellets excreted by one adult male rabbit were collected on Chromosorb 105 traps (see Figure 2c) by the passage of oxygen-free nitrogen (30 ml/min) over 20 g of pellets at 40° C for 4 hr (9.6 liters) as previously described (Murray, 1977; Goodrich et al., 1978).

The traps were introduced into a modified Hewlett-Packard 7600A gas chromatograph (Goodrich et al., 1981a) over a period of 15 min at an inlet temperature of 170° C. The column was a glass SCOT column 60 m, 0.5 mm ID, coated with FFAP, and was temperature programed at 60° C for 20 min followed by 1° C/min rise to 80° C then 2° C/min rise to 190° C.

Experimental Design. As in previous studies in which a neutral environment was used to test the effects of different odor stimuli on the territorial confidence of rabbits (Mykytowycz, 1975; Mykytowycz et al., 1976), the present experiments were designed to allow comparisons to be

TABLE 1. COMPONENTS OF HEADSPACE VOLATILES OF RABBIT FECAL PELLETS LIKELY TO BE REMOVED BY SCRUBBING THROUGH WATER OR 1 M NaOH OR 1 M HCl SOLUTIONS

Scrubbing solution	Components likely to be removed from headspace vapors	Components likely to be retained in headspace vapors
Distilled water	Water-soluble compounds, e.g., lower members of the homologous series of acids, alcohols, aldehydes, amines, ketones, esters, etc.	Water-insoluble compounds, e.g., hydrocarbons and higher members of the homologous series of acids, alcohols, aldehydes, amines, ketones, esters, etc.
1M sodium hydroxide saturated with sodium chloride	Acidic components, e.g., carboxylic acids, phenols, etc., together with decreased amounts of water-soluble components.	Basic components, e.g., amines. Neutral components, e.g., hydrocarbons, aldehydes, ketones, esters, etc.
1 M hydrochloric acid saturated with sodium chloride	Basic components, e.g. amines, together with decreased amounts of water-soluble components	Acidic components, e.g., carboxylic acids, phenols, etc. Neutral components, e.g., hydrocarbons, aldehydes, ketones, esters, etc.

made between the performances of the same animal in the presence of its own odor—home situation—and in the presence of its opponent's odor—away situation. In this report the terms donor and nondonor are used instead of home and away.

The test pairings between rabbits were made randomly except that pairings between animals which normally lived together or were housed adjacent to one another during the test period were avoided. Each pair was tested twice, once in the presence of the odor derived from the fecal pellets of one individual and again in the presence of the odor from the other. Tests involving the same pair of animals were separated by a period of at least 4 days.

The numbers of animals, test pairings, and tests involved in the separate experimental series are listed in Table 2.

Testing Procedure. For each test the pen was set up with a new plastic lining and floor covering and the required internal odor environment was established as described above. The rabbits to be tested were placed in the compartments in the inlet head and within 2 min released into the test chamber.

Each test lasted 10 min starting from the moment when either actual physical contact was established between the animals or a directed approach was made by one to within approximately 10 cm of the other. Two observers recorded the following behavioral parameters: the animal initiating the first contact, movement around the pen (exploration), the number of directed approaches made by each rabbit towards the other, olfactory investigations made by each rabbit of the other (sniffing), aggressive interactions (chasing, biting, fighting), and sexual mounting. To record the incidences and durations of the different activities a keyboard-operated ten-place counter/timer was used. At the conclusion of each test both observers also recorded their own

TABLE 2. NUMBERS OF RABBITS, TEST PAIRINGS, AND TESTS INVOLVED IN SEPARATE EXPERIMENTAL SERIES MEASURING EFFECTS ON TERRITORIAL CONFIDENCE OF DIFFERENT FRACTIONS OF HEADSPACE VOLATILES FROM FECAL PELLETS

Treatment of headspace volatiles	Number of rabbits	Number of test pairings	Number of tests
Untreated	26	13	26
H_2O scrubbed	30	50	100
NaOH scrubbed	18	9	18
HCl scrubbed	16	8[a]	16

[a]The same pairs of animals were used as in the NaOH series except for one pair which was unavailable because of sickness.

subjective assessments of the dominance or degree of confidence of each rabbit.

Statistical Procedures. Before carrying out bioassays on the fractionated headspace volatiles, data from the tests on the total volatiles were analyzed to establish that the territorial confidence of the rabbits had been affected. Student's t tests or binomial probabilities were used as appropriate. In subsequent analyses the results from all series were considered collectively.

A preliminary examination of the correlation matrix of all behavioral variables revealed that the separate variables were not independent and univariate analysis was inappropriate. A multivariate analysis of variance (MANOVA) was therefore used complemented by canonical variate analysis (Morrison, 1976).

Analyses of the data for incidences and durations of the various behavioral components were carried out on the transformed variables:

$$\text{Log}_e[(\text{donor score} + 1)/(\text{nondonor score} + 1)]$$

where donor and nondonor scores were for the same animal when matched against the same adversary.

The frequency data from the subjective assessments and first-contact observations were examined separately for each series by means of binomial probabilities in which the probabilities of occurrence of the two main categories under the null hypothesis were considered to be equal ($P = 0.5$), and the third categories of equal confidence and simultaneous contact were disregarded. Chi-square tests of independence were calculated from 4×3 contingency tables using all three categories of results.

Since the experimental hypothesis is concerned only with effects favoring the donor situation, significance levels for binomial probabilities and t tests have been calculated from one-tailed distributions. Tests have not been applied to comparisons where the nondonor values were the greater.

RESULTS

Effect of Different Scrubbing Agents on Headspace Components. The degree of fractionation of the fecal pellet volatiles actually achieved by the scrubbing procedures is shown by the gas chromatograms in Figure 3. It should be noted that the ordinate scale recorder response in Figures 3b, c, and d is ten times greater than in Figure 3a.

A comparison of the chromatograms in Figure 3a and 3b clearly shows that washing with water has removed a number of very large peaks. This is particularly evident on the right side of the figures; it can be seen that peaks due to the high concentrations of water-soluble compounds in the total odor effectively mask the presence of other volatiles. The compositions of the major

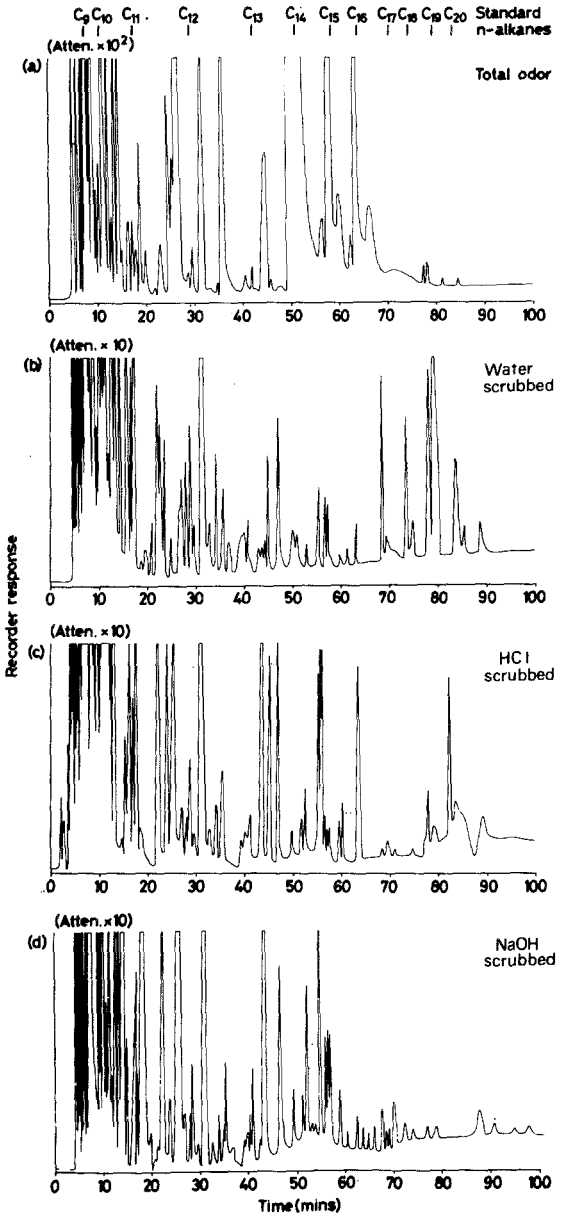

FIG. 3. Chromatograms of the headspace volatiles from the fecal pellets of a male rabbit showing the effects of different scrubbing reagents: (a) total volatiles; (b) distilled water; (c) 1 M hydrochloric acid; (d) 1 M sodium hydroxide. Note that the ordinate scale in (a) is reduced by one tenth compared to the scales for the other chromatograms.

peaks in the total odor have been established in an earlier study (Goodrich et al., 1981a).

The removal of additional compounds by washing with hydrochloric acid or sodium hydroxide is evident in chromatograms 3c and 3d. In both cases this is most apparent on the right side of the figures, although the complexity of the other parts of the chromatograms makes the absence of individual peaks difficult to detect.

Behavioral Tests. Table 3 summarises the results obtained from the first series of tests in which the total volatiles from above fecal pellets were introduced into the test chamber. The standard deviations of the mean differences are shown in the table to give an indication of the variation, but it should be noted that the transformed variables were used in the calculation of t tests.

The observers' assessments were significantly ($P < 0.01$) in favor of the donor rabbits, which were judged to have behaved more confidently in 18 of the tests as against 4 for the nondonor animals. Donor rabbits also made initial contact in a significantly ($P < 0.01$) larger number of tests (19) than the nondonors (6 tests).

Mean scores for all the behavioral parameters were higher in the donor situation. The differences between donor and nondonor situations were highly significant in incidence ($P < 0.01$) and duration ($P < 0.001$) of sniffing and in approaching adversaries ($P < 0.001$). Aggressive behavior and mounting did not occur frequently enough to allow useful statistical inferences to be made.

The results of this series of tests show convincingly that the presence of the total headspace volatiles from their own fecal pellets strongly influenced the outcome of territorial contests between pairs of male rabbits in favor of the donor animal.

The results of the tests involving the fractions produced by scrubbing the fecal pellet volatiles through either water, 1 M NaOH solution, or 1 M HCl solution are shown in Table 4.

In the H_2O scrub tests all measures were higher for the donor than for the nondonor situations. However, in the NaOH and HCl tests there was little difference between the rabbits' performances in the donor and nondonor situations. For several behavioral parameters in both these series, the mean values are actually lower in the donor situation. Since a multivariate analysis of variance was carried out on the results for the behavioral parameters (see following section) the less powerful t test technique was not applied.

Comparison of the effects of the total and fractionated headspace volatiles (multivariate analysis of variance) was carried out on the behavioral data from all four series of tests using all measures except mounting, which did not occur in two of the series. This analysis shows that there was a significant effect due to the type of headspace volatiles used in the different series (Wilk's

TABLE 3. MEAN VALUES OF VARIOUS BEHAVIORAL PARAMETERS FOR RABBITS PLACED IN PAIRS INTO A TEST CHAMBER CONTAINING TOTAL VOLATILES FROM FECAL PELLETS OF ONE OF THEM (DONOR)[a] (26 TESTS)

Behavior	No. of tests in which behavior occurred	Mean score		Mean difference ± SD (donor − nondonor)	Significance[b]
		donor situation	nondonor situation		
Exploration	26				
Incidence		21.7	17.0	4.7 ± 22.9	NS
Duration (sec)		97.8	67.8	30.0 ± 111.7	NS
Approaches	26				
Incidence		8.3	3.4	4.9 ± 8.2	***
Sniffing					
other rabbit	26				
Incidence		13.6	6.3	7.3 ± 10.7	**
Duration (sec)		39.2	12.2	27.0 ± 29.1	***
Aggression	6				
Incidence		2.5	0.6	1.9	+
Duration (sec)		3.5	1.2	2.3	
Mounting	6				
Incidence		3.3	2.0	1.3	+
Duration (sec)		38.5	9.1	29.4	
Number of tests in which donor or nondonor rabbits made initial contact					
Donor				19**	
Nondonor				6	
Simultaneous				1	
Number of tests in which donor or nondonor rabbits were judged to be most confident					
Donor				18**	
Nondonor				4	
Equal				4	

[a] NS = not significant ($P > 0.05$); ** $P < 0.01$; *** $P < 0.001$; + insufficient data.
[b] t tests were carried out on the transformed variables (see text).

TABLE 4. MEAN VALUES OF VARIOUS BEHAVIORAL PARAMETERS FOR RABBITS PLACED IN PAIRS INTO A TEST CHAMBER CONTAINING FRACTIONS OF VOLATILES FROM FECAL PELLETS OF ONE OF THEM (DONOR)[a]

	H_2O-scrubbed volatiles (100 tests)				NaOH-scrubbed volatiles (18 tests)				HCl-scrubbed volatiles (16 tests)			
Behavior	Tests in which behavior occurred	Mean score		Mean difference ± SD (donor − nondonor)	Tests in which behavior occurred	Mean score		Mean difference ± SD (donor − nondonor)	Tests in which behavior occurred	Mean score		Mean difference ± SD (donor − nondonor)
		Donor situation	Nondonor situation			Donor situation	Nondonor situation			Donor situation	Nondonor situation	
Exploration	100				18				16			
Incidence		20.6	18.5	2.1 ± 15.9		18.5	16.9	1.6 ± 14.8		14.8	18.5	−3.7 ± 15.3
Duration (sec)		46.0	36.0	10.0 ± 65.5		95.9	62.3	33.6 ± 100.8		93.4	107.8	−14.4 ± 124.1
Approach	100				18				16			
Incidence		4.1	1.9	2.2 ± 4.5		7.3	5.2	2.1 ± 9.6		3.9	4.8	−0.9 ± 6.9
Sniffing other rabbit	100				18				16			
Incidence		15.3	6.2	9.1 ± 12.5		7.2	6.4	0.8 ± 8.8		4.1	5.9	−1.8 ± 7.6
Duration (sec)		22.3	8.7	13.6 ± 26.5		14.1	21.8	−7.7 ± 35.8		8.5	16.3	−7.8 ± 28.5
Aggressive behavior	64				15				10			
Incidence		15.1	10.1	5.0 ± 29.0		10.8	19.6	−8.8 ± 30.3		2.4	2.2	0.3 ± 6.1
Duration (sec)		10.0	5.7	5.2 ± 16.1		16.4	20.3	−3.9 ± 30.7		1.9	4.3	−2.4 ± 13.5
Mounting	4				0				0			
Incidence		0.5	0.1	0.4		—	—	—		—	—	—
Duration (sec)		3.6	0.8	2.8								

Numbers of tests in which donor or nondonor rabbits made initial contact			
Donor	49**	7+	10NS
Nondonor	27	9	4
Simultaneous	17	2	2
Numbers of tests in which donor or nondonor rabbits were judged to be most confident			
Donor	62***	6+	7+
Nondonor	18	6	8
Equal	20	6	1

[a] NS = not significant ($P > 0.05$); ** $P < 0.01$; *** $P < 0.001$; + tests inappropriate.

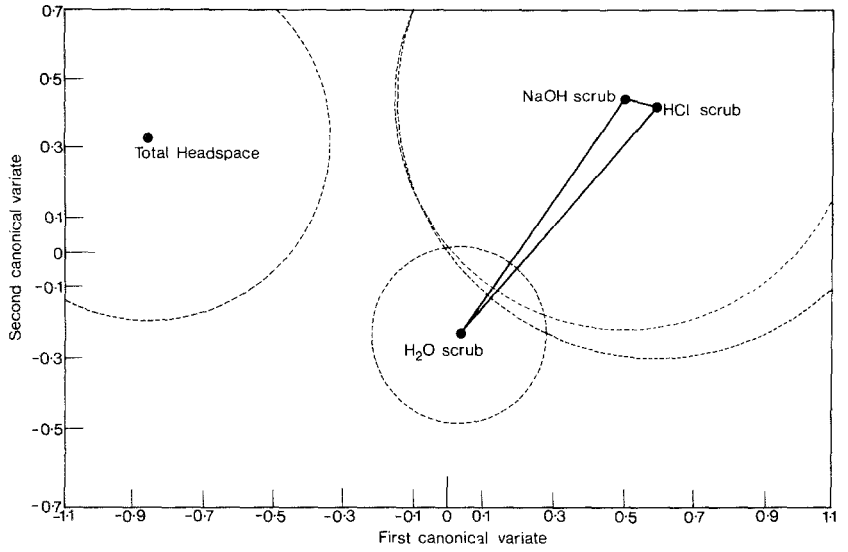

FIG. 4. The relationships among the data for the four series of tests as indicated by canonical variate analysis. Means joined by lines are not significantly different ($P > 0.05$). Broken circles indicate the 95% confidence limits.

lambda test, $P < 0.01$). Further analysis was performed to indicate which of the compound means differed, and these results are shown in Figure 4 together with the result of a canonical variate analysis of the data. In Figure 4 the positions of the canonical means are shown in relation to the first and second canonical variates which account for 89.5% of the variation. It can be seen that the position of the canonical variate mean for the total headspace series differs significantly from that of the other means. Although the H_2O scrub mean does not differ significantly from the means for NaOH and HCl, there is a distinct suggestion of a separation between them with the H_2O mean lying closer to the position of the total headspace. The large extents of the 95% confidence limits for the NaOH and HCl means are a reflection of the small numbers of tests used in these two series.

A closer look at the results for the separate forms of behavior showed that variations in the incidences and durations of sniffing accounted for most of the differences between series. Multiple comparisons of the series means for these two variables are presented in Table 5 and show a grouping of total headspace with H_2O scrub and NaOH with HCl scrub.

The results for the first contacts and subjective assessments (see Table 4) also indicate that, in contrast to the NaOH- and HCl-treated volatiles, there was some effect from the H_2O scrubbed headspace. Tests on the individual series give highly significant results for both the subjective assessments

TABLE 5. MULTIPLE COMPARISONS OF MEANS FOR SNIFFING INCIDENCE AND DURATION BETWEEN FOUR SERIES OF TESTS ON DIFFERENT FRACTIONS OF VOLATILES FROM FECAL PELLETS OF RABBITS[a]

Treatment	Sniffing incidence	Sniffing duration (sec)
Total volatiles	7.3^a	27.0^a
H_2O scrubbed	9.1^a	13.6^a
NaOH scrubbed	0.8^b	-7.7^b
HCl scrubbed	-1.8^b	-7.8^b

[a]Means for the same variable with the same superscripts are not significantly different at the 0.05 level. Statistical analyses were carried out on the transformed variables (see text).

($P < 0.01$) and first contacts ($P < 0.001$) in the case of the H_2O-scrubbed headspace but are not significant for NaOH- or HCl-treated headspaces. The relatively small number of tests involved in the latter two series does, however, give reason to view the comparisons with caution. Chi-square tests for independence on 4×3 contingency tables, using the data from all four series of experiments and the three categories of results, were significant ($\chi^2_6 = 14.5$, $P < 0.05$) for the subjective assessment scores but not significant for the first-contact data.

DISCUSSION

The results presented in this paper show conclusively that odors emanating from fecal pellets influence the outcome of territorial competitions between pairs of male rabbits in an experimental test area in the same way as the actual presence of feces.

Large numbers of tests are needed in order to demonstrate which of the numerous possible combinations of compounds present in the odor of fecal pellets is behaviorally active. To make such a program of testing practicable, the time required for the preparation and performance of each test must be short. Also, since the odor mixtures are characteristic for each individual, a clean environment uncontaminated with odors from previous tests must be provided. The design of the test chamber described in this report overcomes these difficulties to a large extent. The type of apparatus described here could find useful applications in experimental investigations of the behavioral importance of odors in many other small- to moderate-sized animals.

The results of the tests using water-scrubbed headspace volatiles are not clear-cut. Taken on their own, they indicate a bias towards better performances by the donor rabbits, but comparisons with the results for the other three treatments differ depending upon the variable considered. It seems reasonable

to interpret the overall results as indicating that the volatiles remaining after water scrubbing did have some influence on the outcome of the tests but were less effective than the total fecal pellet odor.

It is possible that the reduced activity of the water scrubbed volatiles was due to the lower concentration, in terms of headspace equivalents, used in this series of tests as compared with that for the total volatiles.

Information on the concentrations of volatiles from skin gland secretions present in the rabbits' environment under natural conditions is totally lacking, but it seems likely that they would retain their effectiveness as a means of communication over a wide range of concentrations. In deciding upon the amount of volatiles to be used in these experiments consideration was given to the fact that in previous studies (Mykytowycz et al., 1976) only 20 pellets (approximately 4–5 g) present in an open test arena produced a highly significant effect on the territorial behavior of the rabbits. The provision of 12 liters headspace equivalent/kiloliter of volatiles from 30 g of pellets seemed to allow a large margin of safety to compensate for the inevitable losses of volatiles by adsorption onto the surfaces of the apparatus, delivery tubing, and test chamber. Even when the concentration was reduced to 3.2 liters headspace equivalent/kiloliter by the restriction of gas flow through the scrubbing units, it was felt that this was still more than comparable to the concentration of volatiles probably present in the original open-pen experiments. However, the results suggest that the concentration of volatiles present in the test chamber for the water-scrubbed series of tests was approaching the lower threshold for behavioral effectiveness. The question of volatile concentrations clearly needs further investigation.

Removal of either the basic or acidic components from the headspace volatiles eliminated their effect on the rabbits' territorial confidence and shows that essential components of the olfactory signal are included in both these classes of compounds (see Table 1). The idea that the basic components might be behaviorally inactive must therefore be discarded.

It has been suggested that differences in the type of food ingested may contribute to individually characteristic odors in some species of mammals (Skeen and Thiessen, 1977). Since all the rabbits used in this study were fed on an identical diet, differences in the compounds available from varying food sources could not have been essential for the formation of the individually characteristic odor signals affecting their territorial confidence in the present experiments.

The technique of chemical scrubbing used in this first attempt to fractionate the headspace volatiles from fecal pellets for bioassay was not selective enough to give information on the exact identity of the essential components of the olfactory signal. It has, however, provided information on the types of compounds probably involved, which could be useful in further

investigations. A more precise method of separating out the numerous components of the total odor by means of gas chromatographic techniques described elsewhere in this journal (Goodrich, et al., 1981b) has provided more encouraging results.

The fact that mammalian odors are highly complex mixtures of large numbers of separate compounds makes the task of isolating the active components of olfactory signals very difficult. The techniques described in this paper show that the complex mixtures can be manipulated and selected parts bioassayed.

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IDENTIFICATION OF SOME VOLATILE COMPOUNDS IN THE ODOR OF FECAL PELLETS OF THE RABBIT, *Oryctolagus cuniculus*

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Abstract—A study has been made of the volatile constituents in the vapors collected from fecal pellets of wild rabbits, *Oryctolagus cuniculus*. Measurements of changes in the heart rates of adult male rabbits exposed to the effluent of a gas chromatographic capillary column were used to indicate the presence of compounds which may be of behavioral significance. Combined with the use of high-resolution columns in gas chromatography and gas chromatography–mass spectrometry, this approach has implicated several classes of compounds, including volatile fatty acids and phenols, which in certain absolute or relative concentrations may be involved in the formation of signals of territorial importance.

Key Words—Rabbit, *Oryctolagus cuniculus*, anal gland secretion, feces, headspace vapors, volatile constituents, gas chromatography, GC-MS, heart-rate responses.

INTRODUCTION

The anal gland plays an important role in the olfactory communication of the rabbit, *Oryctolagus cuniculus*. The secretion from the gland coats the fecal pellets as they pass through the anus. Dung-hills—formed by the frequent deposition of feces at particular places within a rabbit's home range—act as communication posts which contain information about the territorial order (Mykytowycz, 1968).

The general chemical composition of the anal gland secretion has been described (Goodrich and Mykytowycz, 1972), and changes in heart rate and in

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overt behavior in response to separate chemical fractions of the secretion have been reported (Hesterman et al., 1976). Gas chromatography–mass spectrometry (GC-MS) has been used to examine the volatiles trapped from above macerated anal glands. A number of compounds which elicited heart-rate responses in monitored rabbits were identified as saturated and monounsaturated normal and methyl-branched aldehydes in the C_9–C_{12} range (Goodrich et al., 1978).

Experimental evidence that the volatiles collected from above fecal pellets influence the territorial dominance relationships between male rabbits (Hesterman et al., 1981) has stimulated further work on the composition of this odor. As in an earlier study (Goodrich et al., 1978), heart-rate responses of adult male rabbits located to sniff the outlet of a capillary column in a gas chromatograph have been used in the work reported here to indicate the presence, in the odor from fecal pellets, of compounds of presumed behavioral importance. Some of these compounds were then selected for further examination by GC-MS.

METHODS AND MATERIALS

General Approach. The volatiles from the headspace above feces were collected by means of a technique used in the study of food aroma (Murray, 1977).

Gas chromatographic examination of the headspace vapors was made on a glass SCOT column coated with a polar phase (FFAP). The heart-rate responses of rabbits positioned to sniff the column effluent were used to aid the selection from the complex chromatogram of a number of zones of possible behavioral significance. Altogether 15 adult male rabbits were used.

The selected zones were collected in repeated runs of the chromatograph and rechromatographed on a glass SCOT column of lower polarity (Silicone SF-96), again with rabbits monitoring the column effluent to pinpoint active fractions for further study. These were subsequently collected and rechromatographed on a glass SCOT column of low polarity (Silicone OV-101), coupled to a mass spectrometer. Major volatile constituents of the "active" fractions were identified.

To identify constituents not detected with the FFAP column, the same procedure was carried out using a glass SCOT column coated with a different polar phase (Carbowax 20M).

Collection of Fecal Pellets for Analysis. Fecal pellets from individual adult male rabbits were collected overnight on wire mesh trays placed under the cages. Those obviously contaminated with urine were not used. Pellets not used immediately were stored for not longer than one month in sealed glass containers at −20° C.

Collection of Volatiles. The technique used to collect volatile compounds on Chromosorb 105 (Johns-Manville) for gas chromatographic examination was similar to that previously described by Murray (1977) and Goodrich et al. (1978). In the present work oxygen-free nitrogen (40 ml/min) was passed over 30 g of fecal pellets at 40° C for 16 hr and the effluent collected in a single preconditioned trap containing a bed of Chromosorb 105 (50/60 mesh, 100 mg) packed into a stainless-steel tube (90 mm, 3.2 mm OD). The tube was then purged with dry nitrogen to remove residual water, sealed with Teflon end caps, and stored over solid carbon dioxide until required. Headspace collections for examination by GC-MS were similarly carried out on larger quantities of pellets (60 g) over a period of 40 hr.

Gas Chromatography. A Hewlett-Packard series 7600A gas chromatograph with modifications similar to those previously described (Murray, 1977) was used. The headspace volatiles were introduced at 170° C over a period of 15 min. The glass SCOT columns used were coated with FFAP (60 m, 0.5 mm ID), or Carbowax 20M (70 m, 0.5 mm ID). The columns were temperature programed at 20 min at 60° C followed by 1° C/min rise to 80° C, then 2° C/min rise to 190° C.

Monitoring the Column Effluent. The technique of monitoring the heart rate of a rabbit positioned at the outlet of a gas chromatographic column has been described earlier (Goodrich et al., 1978). In the present study the effluent from the gas chromatographic capillary column was split and presented simultaneously to two rabbits restrained in separate soundproofed boxes (Figure 1). Heart beats of the rabbits were detected by using miniature electrocardiogram radio transmitters operating on separate frequencies and the signals were recorded on separate channels of an FM instrumentation recorder. The output from the gas chromatographic detector was also recorded on a third channel. Subsequently the heart-beat recordings were processed through a biotachometer which produced an output voltage proportional to the beat-to-beat heart rates, and these were displayed synchronously with the gas chromatogram on a multipen strip-chart recorder.

Changes in heart rate of 15% or more above or below the mean rate were considered as responses, and their positions in relation to the gas chromatograms were noted on the strip-chart records.

Because of the complexity of the total headspace gas chromatograms, it was not always possible to associate responses precisely with particular peaks. The total numbers of responses which occurred in separate 2-min periods of the gas chromatograph runs were therefore considered.

Animals. Fifteen adult male wild-type rabbits were used to monitor the column effluents. Each animal was employed on a number of occasions but only once on any one day. The rabbits normally lived in mixed-sex groups of 3 or 4 individuals in outdoor pens but for the duration of the experiments were housed singly in cages.

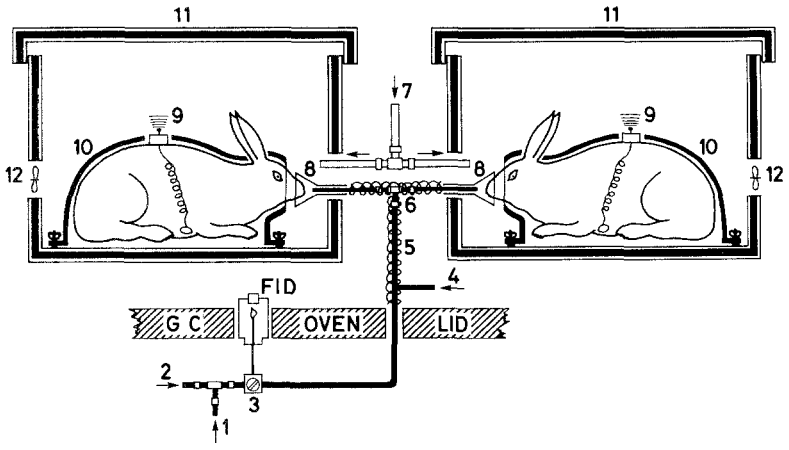

FIG. 1. Diagram showing two rabbits, *Oryctolagus cuniculus*, located to sniff the gas chromatographic column effluent with simultaneous recording of heart rate. FID = flame ionization detector; 1 = capillary column outlet; 2 = make-up N_2, 18 ml/min; 3 = variable outlet splitter; 4 = make-up air for dilution of effluent (100 ml/min); 5 = heated Teflon delivery tube (100° C) for presentation of volatile components to rabbits; 6 = T-piece union for splitting effluent between rabbits; 7 = precooled air (18° C; 5 liters/min) to each rabbit; 8 = glass funnels; 9 = miniature radio transmitters; 10 = fiberglass casts for restraining rabbits; 11 = soundproofed boxes housing rabbits; 12 = exhaust fan used to remove odorous material from boxes housing rabbits.

FFAP Column Fractions. Eleven monitoring runs were made on the FFAP column on identical fecal pellet vapor collections using a different pair of rabbits for each run. Not all animals accepted the experimental situation, but 16 satisfactory heart-rate traces were obtained.

To select the areas of the chromatograms for further fractionation, two criteria were used: first, the number of responses which occurred within separate 2-min periods of the gas chromatograph run, and second, the quantities of the volatiles contained in the "active" zones had to be sufficient for GC-MS examination without carrying out time-consuming concentration procedures. Using these criteria ten fractions of the headspace volatiles were selected for trapping (Figure 2).

The ten fractions were obtained in five repeated runs by substituting packed tubular traps cooled in dry ice for the effluent delivery line. These traps were of the same design as the Chromosorb 105 trap used for the initial collection of volatiles except that the porous polymer was replaced by a bed of 10% Silicone SF-96 on 30–40 mesh Chromosorb A (Johns-Manville). The technique of trapping and the unloading of the traps has been described

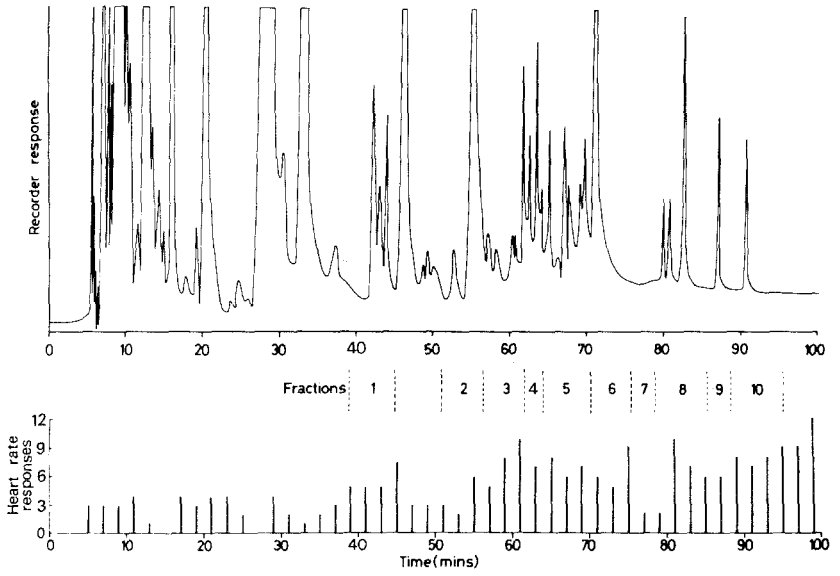

FIG. 2. Gas chromatogram on FFAP column of headspace volatiles from fecal pellets of an adult male rabbit, *Oryctolagus cuniculus*, with indication of the zones (fractions 1–10) selected on account of heart-rate responses for further studies.

previously (Murray, 1977). The five sets of ten fractions were further fractionated by GC on a glass SCOT column (43 m, 0.5 mm ID) coated with Silicone SF-96 and temperature programed from 70° C to 160° C at 2° C/min (fractions 1 and 2) and 90° C to 160° C at 2° C/min (fractions 3 to 10).

The capillary column effluents from each separated fraction were presented to pairs of rabbits. Some zones containing volatiles which most consistently elicited changes in heart rate were retrapped in subsequent runs and then examined by a gas chromatograph coupled to an Atlas CH4 mass spectrometer (Murray et al., 1972). The gas chromatograph unit was equipped with a glass SCOT column (80 m, 0.6 mm ID) coated with OV-101 and isothermally heated at a temperature most suitable for maximum column resolution for each fraction under examination. The carrier gas used for the GC-MS was helium at a flow rate of 3 ml/min. Baseline resolution was obtained for most of the peaks in the areas of interest, and high-quality mass spectra were recorded for the resolved major components.

Carbowax 20M Column. Further examination of the fecal pellet headspace was carried out on a Carbowax 20M SCOT column in three separate runs using a total of six different rabbits to monitor the gas chromatographic effluent. The same procedures were followed as for the

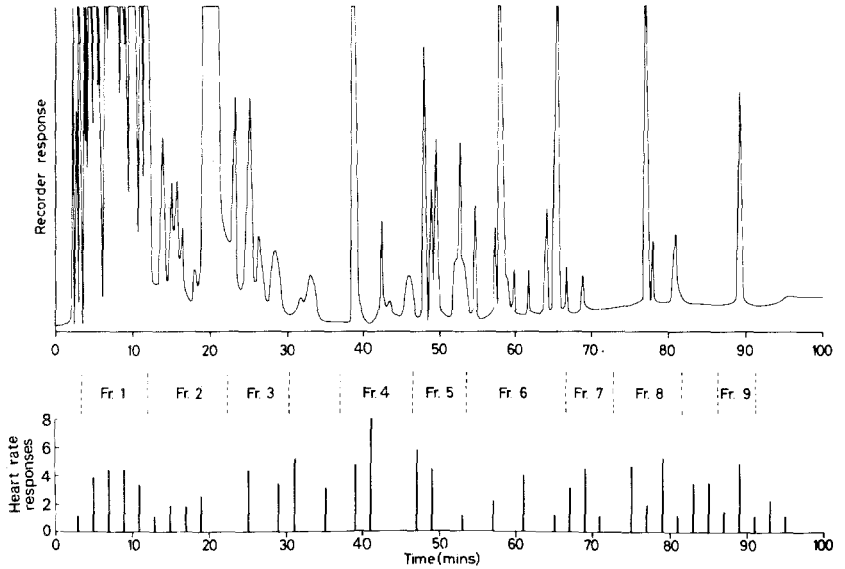

FIG. 3. Gas chromatogram on Carbowax 20M column of headspace volatiles from fecal pellets of an adult male rabbit, *Oryctolagus cuniculus*, with indication of the zones (fractions 1–9) selected on account of heart-rate responses for further studies.

FFAP column examinations. Nine fractions were selected in this series (Figure 3) and were subsequently trapped out and analyzed by GC-MS.

RESULTS AND DISCUSSION

Table 1 shows the major components of the selected fractions from both the FFAP and Carbowax 20M separations identified in the GC-MS runs. However, it is not known to what degree these compounds contribute to the overall activity of the fractions as determined by the number of heart rate responses elicited in the monitoring animals (Figures 2 and 3). In the present study, minor components which were present in concentrations too small for mass spectral identification were ignored, although it is realized that they could significantly contribute to the overall quality of the odor and consequently could be of behavioral importance.

Volatile fatty acids, which have been shown elsewhere to influence the physiological behavior of rabbits (Hesterman et al., unpublished data) were identified in a number of the selected active fractions. Short-chain acids occur also in the secretions of skin glands of a number of mammals, for example, in the anal gland of the red fox, *Vulpes vulpes* (Albone and Fox, 1971), in the vagina of the rhesus monkey, *Macaca mulatta* (Michael et al., 1971), and in

TABLE 1. VOLATILE CONSTITUENTS OF GAS CHROMATOGRAPHIC FRACTIONS OF FECAL PELLET ODOR WHICH INDUCED LARGEST NUMBER OF HEART-RATE RESPONSES IN MALE RABBITS, *Oryctolagus cuniculus*

Compound	Fraction on FFAP column in Figure 2	Fraction on Carbowax 20M in Figure 3	Molecular weight	Evidence of identification[a]
Hydrocarbons				
Two monoterpenes		4, 7	136	ms
n-Decane		1	142	MS GC
n-Dodecane		3	170	MS GC
n-Tridecane	1		184	MS GC
n-Tetradecane	2		198	MS GC
β-Gurjenene	5		204	ms
C_{15} H_{30}	4		210	ms
n-Pentadecane	2, 3		212	MS GC
C_{16} H_{32}	5		224	ms
C_{16} H_{34}	3		226	ms
n-Hexadecane	5		226	MS GC
Alcohols				
2-Methylpropan-1-ol		2	74	MS GC
n-Butanol		2	74	MS GC
3-Methylbut-2-en-1-ol		4	86	ms
3-Methylbutan-1-ol	1		88	MS gc
n-Pentanol		3	88	MS GC
n-Hexanol		4	102	MS GC
n-Heptanol	3	5	116	MS GC
2-Phenylethanol	8	8	122	MS GC
n-Octanol	4	6	130	MS GC
n-Nonanol		6	144	MS GC
Ketones				
Pentan-2,3-dione	1		100	MS gc
Heptan-2-one		2	114	MS gc
6-Methylhept-5-en-2-one	1	4	126	MS gc
6-Methylheptan-2-one		3	128	MS gc
Octan-3-one		3	128	MS GC
2,2,6-Trimethylcyclohexanone	1		140	ms
Nonan-2-one		4	142	MS gc
Carboxylic acids				
Ethanoic	2	5	60	ms
Propanoic	3		74	ms
n-Butanoic	5	6	88	ms
Esters				
Ethyl propanoate		1	102	MS gc
Methyl butanoate		1	102	MS gc
Ethyl butanoate		1	116	MS gc
Ethyl pentanoate		2	130	MS gc
3-Methylbutyl ethanoate		2	130	MS gc

TABLE 1. (cont'd.)

Compound	Fraction on FFAP column in Figure 2	Fraction on Carbowax 20M in Figure 3	Molecular weight	Evidence of identification[a]
n-Propyl butanoate		2	130	MS gc
Ethyl hexanoate		3	144	MS gc
n-Butyl pentanoate	1		158	MS gc
n-Pentyl butanoate	1		158	MS gc
n-Butyl hexanoate	2		172	MS gc
n-Hexyl butanoate	2		172	MS gc
Ethyl 2-phenylpropanoate	7		178	ms
Aryl compounds				
Phenol	9		94	ms
Styrene		3	104	MS gc
o-Cresol	7		108	MS
p-Cresol	10	9	108	MS GC
Naphthalene	6		128	MS
2,6-Di-*tert*-butyl-*p*-cresol	8		220	MS
Heterocyclic compounds				
Pyridine		2, 3	79	ms
Trimethylpyrazine	2		122	ms
Tetramethylpyrazine	3		136	MS gc
2-*n*-Pentylfuran		3	138	MS GC
2-Ethyl-3,5,6-trimethylpyrazine	4		150	ms
Others				
Dimethyl disulfide		1	94	ms
Trichloroethylene		1	131	MS gc

[a]MS = mass spectrum identical with that of pure material. GC = linear retention index agrees with that of the pure material on two columns (either FFAP or Carbowax 20M, and Silicone OV-101). gc = linear retention index agrees with that of the pure material on one column (Silicone OV-101). ms = mass spectrum identified from literature search only (Stenhagen et al., 1974; Eight Peak Index of Mass Spectra, 1974).

the anal sac of the coyote, *Canis latrans* (Preti et al., 1976). The origin of these acids in probably microbial, and their presence in the odor above rabbit feces may be due to the breakdown of dietary constituents by gastrointestinal microorganisms. A number of bacterial strains and a series of volatile fatty acids have been isolated from the inguinal pouches of rabbits (unpublished data).

Aliphatic aldehydes previously reported as active constituents in the anal gland secretion (Goodrich et al., 1978) were not detected in the headspace vapors collected above fecal pellets, although their presence was expected since the anal gland secretion is normally deposited on the feces. The concentration of these compounds in the anal gland secretion is very low and,

since they are also probably subject to degradation by the fecal microbial flora, it could be extremely difficult to detect them in the headspace vapors. It is also known that the FFAP stationary phase removes aldehydes (Allen, 1966). Apart from the use of a Carbowax 20M capillary column for the gas chromatographic fractionation of headspace vapors, no further effort was made to confirm the presence of aldehydes.

Many compounds which are present in the odor of rabbit fecal pellets have also been reported to occur in the gland secretions of other mammals. Behavioral importance has been ascribed to some of them, as for example in the case of dimethyl disulfide in the vaginal secretion of the hamster, *Mesocricetus auratus* (Singer et al., 1976), nonan-2-one in the pedal glands of the bontebok, *Damaliscus dorcas* (Burger et al., 1976), 6-methylhept-5-en-2-one in the urine of the red fox, *Vulpes vulpes* (Jorgenson et al., 1978), and phenol and *p*-cresol in the perineal scent glands of the wild guinea pigs, *Cavia porcellus* and *C. aperea*, respectively (Wellington et al., 1979).

It has to be emphasized that in the present study, as well as in earlier ones, the heart-rate responses of rabbits have been used merely to indicate the presence of volatiles to which the rabbits' olfactory mechanism is sensitive and to which the animals pay more attention. The assumption is made that such compounds are more likely to be components of the complex odors involved in the rabbits' olfactory communication system than those to which rabbits are inattentive or olfactorily insensitive. This assumption has been made to narrow down the field of search in the highly complex mixture.

The results of this and previous studies show clearly the complexity of the odor signals by which rabbits and other species communicate information of behavioral importance. In view of this complexity it is unreasonable to expect a rabbit to show any specific or characteristic response to a single isolated component. The heart-rate data therefore should not be interpreted as having any greater significance than that suggested above. For the same reasons a cautious view must be taken of many reports on the behavioral importance of some compounds in various species of mammals which were based merely on choice tests.

Elsewhere reports are presented on a series of experiments in which a more specific behavioral response in free-acting rabbits is used to measure the effectiveness of different fractions of the total fecal pellet headspace in conveying information of territorial significance (Hesterman et al., 1981).

As previously reported (Goodrich et al., 1978) responses of rabbits to large gas chromatographic peaks do not necessarily coincide with the maximum of the peaks. A possible reason for this is that during the passage of a gas chromatographic peak, rapid and wide changes in concentration take place which can cause pronounced variations in the odor quality of the eluting volatiles. Thus presentation of odor components in concentrations which differ markedly from those encountered by the animals under natural

conditions could release false physiological as well as behavioral reactions in the rabbits.

In the earlier studies (Hesterman et al., 1976; Goodrich et al., 1978) accelerative changes in heart rate were weak and were observed only infrequently. Hence only decelerative changes were considered in the selection of behaviorally active compounds. In the present study, however, decelerative and accelerative heart-rate responses occurred with equal frequency and with similar patterns of distribution. The increased occurrence of accelerative heart-rate responses during the present study may be due to the temperamental characteristics of the individual rabbits used—their cognitive style (Lacey and Lacey, 1970)—to differences in the concentrations of the volatiles present in the column effluent or to the different types of compounds present. Accelerative heart-rate changes are often associated with stimulation which is aversive, either because of the intrinsic unpleasantness of the stimulus or because of its excessive intensity (Graham and Clifton, 1966).

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USE OF SEX PHEROMONE, 2,6-DICHLOROPHENOL, TO DISRUPT MATING BY AMERICAN DOG TICK *Dermacentor variabilis* (SAY)[1]

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Abstract—Treatment of *D. variabilis*-infested dogs with formulations of the sex pheromone, 2,6-dichlorophenol, significantly reduced mating by the attached ticks. Aqueous emulsions of a gelatin microcapsule–xylene slurry and a pheromone-loaded, molecular-sieve powder were used. Concentration was an important variable influencing product efficacy. Such formulations combined with an acaricide may prove effective in reducing tick populations on livestock and pets.

Key Words—American dog tick, *Dermacentor variabilis*, Acarina, Ixodidae, sex pheromone, mating disruption, 2,6-dichlorophenol, control.

INTRODUCTION

Ticks are among the most important pests and disease vectors affecting livestock, wildlife, pets, and humans. Hard ticks (Ixodidae) transmit an impressive variety of protozoan, rickettsial, and viral agents. To date, control of ticks is accomplished almost entirely by the use of pesticides applied in dips,

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dusts, or sprays, or released from plastic collars and tags. Experience, however, suggests that this extreme dependence on pesticides constitutes a mixed blessing. Pesticides usually do not eradicate a pest species, and their continued use or misuse leads to accumulation of poisonous residues, destructive environmental effects, decreased effectiveness, and increasing costs. The emergence of tick strains resistant to a variety of acaricides demands the development and testing of an ever-increasing array of new products. Government regulation, especially in the more developed countries, reflects public awareness of the dangers inherent in the massive burden of toxic materials dispersed annually in an effort to control arthropod pests.

Despite the dangers and difficulties that attend the use of pesticides, few practical alternatives are available. Chemosterilants, sterile male release, biological control methods, and pheromones show promise. The use of pheromones in particular may serve in the selective control of important pests without intolerable side effects. In the case of ticks, an aggregation-attachment pheromone extracted from male Gulf Coast ticks (*Amblyomma maculatum Koch*) was used to attract ticks to an insecticide-treated area on cattle; ticks thus attracted to the treated site attached and died (Gladney et al., 1974). Similar use of a pheromone-baited insecticide was reported by Rechav and Whitehead (1978) for *A. hebraeum* (Koch). Unfortunately, these pheromones have not yet been identified.

We considered the use of 2,6-dichlorophenol, the sex pheromone common to several hard ticks, as an aid in tick control. Treating the host body surface with this compound may be expected to disrupt mate-seeking by males, rendering them more susceptible to insecticides and host grooming, as well as reducing mating success. Preliminary studies (Sonenshine et al., 1979) with pheromone-treated dusts led to slight delays in mating and occasional reductions in mating success, but substantial disruption of mating and reproduction was not demonstrated. Controlled-release formulations were then developed to enhance the spread and persistence of 2,6-dichlorophenol on tick-infested hosts. We report the disruption of mating of the American dog tick, *Dermacentor variabilis* (Say), in the presence of microencapsulated sex pheromone dispersed in sprays and describe tests using molecular sieves as slow release mechanisms for dispensing pheromone on tick-infested hosts. We also discuss the feasibility of tick control on animals by these methods.

METHODS AND MATERIALS

Dermacentor variabilis ticks were laboratory reared from collections obtained near Montpelier and Ashland, in central Virginia. All ticks were adults, approximately 2–4 weeks old, held in an Aminco-Aire Climate Lab® (American Instrument Co., Silver Spring, Maryland) at 27° ± 1°C and 90 ± 2% relative humidity prior to use.

Formulations. Controlled release formulation of the sex pheromone was accomplished by microencapsulation or loading on molecular sieves. Gelatin microencapsulation of the pheromone, 2,6-dichlorophenol (DCP, Aldrich Chemical Co., Milwaukee, Wisconsin, 99% pure) was done by Appleton Papers, Inc., Dayton, Ohio (originally with National Cash Register Co.). The gelatin microcapsules ranged in size from 50 to 200 μm, with a retention factor of 25% (i.e., 25% of the encapsulated material would not escape during the period of study). Two gelatin microcapsular formulation were supplied as xylene slurries containing 30% capsular solids. The xylene slurries were allowed to stand until the microcapsules rose to the surface forming a viscous layer, which was skimmed off. Ten grams of the top layer of each slurry was mixed with 2.5 ml of the emulsifier Triton X-100 (Mallinckrodt, Inc., St. Louis, Missouri) and diluted with distilled water to 250 ml to give final preparations containing 5.6 μg/ml or 1.1 mg/ml DCP. Approximately 150 ml of each of these preparations was sprayed on each tick-infested dog, and rubbed into the fur by hand to ensure thorough wetting. Thus, the dogs were treated with a total of about 0.84 mg or 162 mg DCP, respectively. To some preparations, 5% polyethylene glycol, mol wt 200 (Sigma Chemical Co., St. Louis, Missouri) or 1% low-viscosity sodium carboxymethylcellulose (Sigma Chemical Co.) was added to retard desiccation of the capsules and enhance their adhesion to the host fur or skin.

The controlled-released dust formulation was prepared by holding 2.5 g Linde molecular sieve type 13X (MS) powder (Union Carbide Corp., S. Plainfield, New Jersey) and 250 mg DCP under vacuum in a sealed container for 48 hr. The MS powder was reweighed, and the percent loading was calculated to be approximately 9.2%. The DCP-loaded MS powder was diluted 1:20 with unodorized talc (Mallinckrodt, Inc., St. Louis, Missouri) to which 3% magnesium stearate (Fisher-Scientific Co., Fair Lawn, New Jersey) was added as a "sticker." The final preparation was estimated at approximately 5 mg DCP/g dust. Ten grams of the mixture was rubbed into the fur of each treated dog by hand, each dog thus receiving 50 mg DCP.

Test Procedures. Mixed-breed adult dogs in good health and found to be capable of sustaining tick populations were supplied by Environmental Consultants, Inc., at their Suffolk, Virginia, kennel. The dogs were infested with 50 female and 75 male, laboratory-reared, unfed *D. variabilis.* More males than females were used to increase opportunities for mating. The ticks were confined under stockinet sleeves affixed loosely to the host to minimize loss of ticks during attachment. A wide plastic collar was installed around each dog's neck to minimize tick loss due to grooming. The tick-infested dogs were held in individual cages with solid metal trays for recovery of ticks that dropped off. Kennel temperatures averaged 18° ± 3° C; humidity was not controlled.

Four days after infestation, the stockinet was removed, and the dogs were

inspected. The number, sex, and location of the ticks were recorded. All unattached ticks were counted and removed. Immediately afterwards, the test animals were treated with the various pheromone treatments either in spray (150 ml/dog) or dust (10 g/dog) form, with the wet or dry deposit being rubbed by hand into the hair. Control animals were treated with pheromone-free, xylene–water emulsions or MS talc dusts, depending on the experiment. After treatment, fresh stockinet was placed on the dogs.

Four days after treatment (i.e., 8 days after the original infestation), the tick populations on the dogs were examined again. Counts were made of all ticks; sex, location, and the number of males and females in copula were recorded. Fully engorged females found loose in the stockinet sleeves or in the trays below the cages were collected. The number of detached males, if any, was also recorded. Finally on the fifth day after treatment, all ticks were removed from the dogs, returned to the laboratory, and weighed.

Mating in female ticks was determined by weight classification as well as visual observation. Balashov (1968) reported that unmated, feeding ixodid females engorged slowly or not at all, even though attached for many weeks. In *D. variabilis*, feeding of mated females is completed in 7–9 days; the average engorged weight of mated females was reported to be 625 mg (data from Sonenshine and Tignor, 1969). Unmated female *D. variabilis* attached 9 days to rabbits were found to weigh only 88.78 ± 33.40 mg ($N = 20$). Consequently, a value of 190 mg, i.e., the mean $\pm$ 3 standard deviations (to include 99% of the sample population) was used to separate the lighter unmated females from the much heavier, mated females. The weight distribution (Figure 1) of female ticks feeding on control dogs was bimodal: the lighter, unmated ticks with a peak at 75 mg, and the heavier, mated ticks with a broad peak at 375–425 mg.

Statistical tests of the differences between the percentages of mated and unmated ticks were done by the t test with arcsin transformation as described by Sokal and Rohl, (1969, p. 607).

To determine persistence of pheromone on the host after treatment, samples of hair and rubbings (with cotton applicators) of exposed skin surfaces of pheromone-treated dogs were taken at the time of examination, 4 days after treatment. Weighed samples of hair and unmeasured material rubbed from the skin were extracted in hexane. DCP in the hexane was removed by extracting with 3% NaOH, and the DCP was recovered by acidifying the basic solution with 4% HCl and extracting with ether. The amounts of 2,6-dichlorophenol in the different samples were determined by injecting the ether extracts into a Varian model 2700 gas chromatograph fitted with an electron capture detector (tritium–titanium foil). GC columns, packing materials, temperatures, and other conditions were as described by Sonenshine et al. (1976).

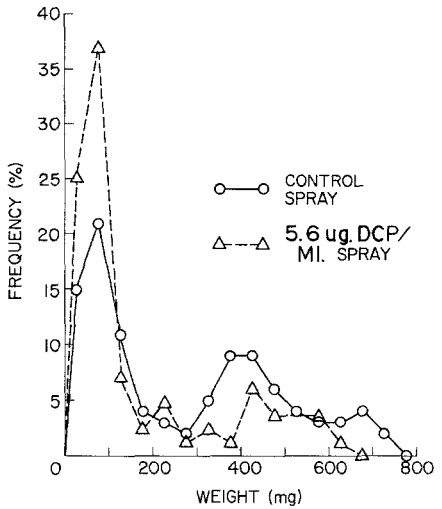

FIG 1. Comparison of weight distribution of female ticks, *D. variabilis*, on pheromone-treated and control dogs 8 days postinfestation (4 days posttreatment). Data points represent the number of female ticks in each 50-mg class. Triangles represent ticks from the dogs treated with 5.6 μg/ml formulation; circles, from the control dogs (washed with an aqueous placebo).

RESULTS

Microencapsulated Pheromone. Table 1 summarizes the visual observations of tick-mating frequency on dogs following treatment with the two different concentrations of microencapsulated pheromone. Matings were reduced when the dog body surface was treated with the 5.6 μg/ml formulation to a frequency ranging from 25.9% to 41.7% in the five replicates; overall frequency was only 34.2%. In contrast, mating frequency on the untreated controls ranged from 54.5% to 75.0%, with an overall average of 69.9%. The 5.6 μg/ml formulation thus depressed mating frequency to approximately half that encountered on the control animals. This difference is highly significant ($T = 5.58$, $P < 0.001$, 232 *df*). Treatment with the 1.1 mg/ml formulation did not significantly reduce mating in the two replications in which it was tested ($T = 0.86$, NS, 161 *df*).

The addition of "stickers," did not appear to improve the performance of the 5.6 μg/ml formulation. In the two replications in which 5% PEG was the sticker additive, observed mating frequency rose to 61.3%, which is not significantly different from the controls ($T = 0.90$, NS, 152 *df*). In the one test with carboxymethylcellulose as the sticker additive, 57.7% of the females were observed in copula, again not significantly different from the controls ($T = 1.18$, NS, 147 *df*).

TABLE 1. EFFECT OF PHEROMONE TREATMENT ON MATING IN *Dermacentor variabilis* ON DOGS[a]

Replicate No.	Treatment											
	5.6 μg/ml pheromone				1.1 mg/ml pheromone				Control			
	Mating		Not mating		Mating		Not mating		Mating		Not mating	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1	7	25.9	20	74.1	8	42.1	11	57.9	17	65.4	9	34.6
2	8	30.8	18	69.2	17	81.0	4	19.0	6	54.5	5	45.5
3	6	37.5	10	62.5					12	75.0	4	25.0
4	7	38.9	11	61.1					13	72.2	5	27.8
5	10	41.7	14	58.3					17	70.8	7	29.2
6									21	75.0	7	25.0
Summary	38	34.2[b]	73	65.8	25	62.5[c]	15	37.5	86	69.9	37	30.1

[a] Microencapsulated pheromone spray based on visual determinations of mating.
[b] Highly significantly different from the control, $T = 5.583$, $P < 0.005$, 232 *df*.
[c] Difference from the control not significant $T = 0.861$, NS 161 *df*.

TABLE 2. COMPARISON OF DISTRIBUTION OF ENGORGED AND UNENGORGED FEMALE TICKS CLASSIFIED BY WEIGHT IN RELATION TO DIFFERENT MICROENCAPSULATED PHEROMONE SPRAY TREATMENTS

Spray treatment	No. in Sample	Not mating		Mating	
		% Females < 190 mg	Weight (mg) ± SD	% Females > 190 mg	Weight (mg) ± SD
Control	101	50.5	78.7 ± 43.6	49.5	455.9 ± 130.3
5.6 μg/ml microcapsules	84	71.4[a]	66.2 ± 38.5	28.6	420.7 ± 119.6
5.6 μg/ml microcapsules + PEG	32	75.0[b]	71.1 ± 34.9	25.0	424.5 ± 115.8
1.1 mg/ml microcapsules	34	35.3[c]	90.7 ± 36.8	64.7	544.5 ± 132.4
1.1 mg/ml microcapsules + CMC	25	48.0[d]	91.3 ± 42.9	52.0	473.8 ± 98.1
1:10 "dilution" 1.1 mg/ml microcapsules	22	49.9[e]	83.1 ± 41.1	59.1	492.2 ± 90.2

[a] Highly significantly different from the control, $T = 2.927$, $P < 0.005$, 183 *df*.
[b] Highly significantly different from the control, $T = 2.531$, $P < 0.01$, 131 *df*.
[c] Difference from the control not significant, $T = 1.556$, NS, 133 *df*.
[d] Difference from the control not significant, $T = 0.225$, NS, 124 *df*.
[e] Difference from the control not significant, $T = 0.82$, NS, 121 *df*.

Table 2 summarizes the results of the same pheromone treatments described above, but with weight distribution as the determinant of mating. Analysis of these data confirms and reinforces the findings described above. The 5.6 μg/ml formulation was the most effective of the various treatments in restricting female tick engorgement, with only 28.6% of these ticks in the high weight category characteristic of mated females (420.7 $\pm$ 119.6 mg). In contrast, 50.5% of the females from the control dogs weighed more than 190 mg (455.9 $\pm$ 130.3 mg). This difference is highly significant ($T = 2.93$, $P < 0.005$, 183 *df*). Figure 1 illustrates the contrast between the weight distribution of female ticks feeding on the control and the dogs treated with the 5.6 μg/ml formulation. On the treated dogs, the lighter weight (unmated) class is much greater, with 37% of the ticks at the 75-mg peak, and only 21% of the same weight class on the control dogs.

The addition of 5% PEG to the 5.6 μg/ml pheromone microcapsule formulation did not improve the performance of this preparation, although the percentage of high weight (i.e., mated) females (25%) was still significantly less than the control (49.5%) ($T = 2.53$, $P < 0.01$, 131 *df*). The 1.1 mg/ml formulation and the various dilutions or additions to it did not significantly restrict female tick engorgement. In each case, the percentage of females in the heavy weight class was not significantly different from the control.

Pheromone-Impregnated Dust. Table 3 summarizes visual observations of tick mating frequency on dogs following treatment with the 5 mg/g pheromone-loaded molecular sieves dispersed as a dust. Mating frequency was reduced substantially, particularly when compared with some of the spray treatments described previously. However, mating frequency was also reduced in the controls, and the difference between mating frequency on the treated dogs (41.5%) and the control dogs (45.8%) was not significant ($T = 0.34$, NS, 63 *df*).

Table 4 summarizes the weight distribution of the fed or feeding female ticks on the dogs receiving the pheromone dust treatment and the controls. The same weight criteria described previously were used to characterize the ticks as mated or unmated. Most of the female ticks on the pheromone dust-treated dogs were light and, presumably, unmated (73.2%). In contrast, slightly more than 50% of the females on the control dogs (dust only) were heavy, presumably mated ticks. This difference was significant at the 5% level ($T = 1.98$, $P < 0.01$, 60 *df*). The ticks in the mated female class on both the experimental and control dogs were notably lighter than the ticks of the same class on the spray treated dogs.

Persistence of Pheromone on the Host. Under a stereoscopic microscope, the gelatin microcapsules in the emulsified formulation appeared as spherical objects of various diameters. This indicates that the microcapsules do not disintegrate when the gelatin microcapsule–xylene slurry is emulsified with Triton X-100 water. When this dilute aqueous preparation was allowed to dry,

TABLE 3. EFFECT OF PHEROMONE TREATMENT ON MATING IN *Dermacentor variabilis* ON DOGS (PHEROMONE IN MOLUCULAR SIEVE DUST)

	5 mg/g Dust DCP MS[a]				Control dust MS[b]			
	Mating		Not mating		Mating		Not mating	
Replicate	No.	%	No.	%	No.	%	No.	%
1	4	66.7	2	33.3	5	50.0	5	50.0
2	6	28.6	15	71.4	6	42.9	8	57.1
3	5	50.0	5	50.0				
4	2	50.0	2	50.0				
	17	41.5[c]	24	58.5	11	45.8	13	54.2

[a]Linde molecular sieve loaded with 2,6-dichlorophenol and mixed with talc.
[b]Linde molecular sieve not loaded, mixed with talc.
[c]Difference from control not significant, $T = 0.337$, NS, 63 *df*.

the microcapsules shriveled and coagulated into irregular clusters. The addition of 5% PEG to the preparation did not prevent desiccation of the microcapsules.

Microcapsules were found adhering to the hair shafts of animals 24 hr post treatment with the emulsified 5.6 μg/ml formation. The microcapsules appeared shriveled, but were attached to the hair, either singly or in clusters. Extracts of hair and skin surface samples analyzed by GC revealed 2,6-dichlorophenol in both types of host material. Two samples of hair removed from the back of each of two dogs 4 days posttreatment had 4.8 and 3.4 ng

TABLE 4. COMPARISON OF DISTRIBUTION OF PRESUMPTIVE MATED AND UNMATED FEMALE TICKS CLASSIFIED BY WEIGHT IN RELATION TO PHEROMONE DUST AND DUST CONTROL TREATMENTS

		Not mating		Mating	
Dust treatment	No. in Sample	% Females < 190 mg	Weight (mg) ± SD	% Females > 190 mg	Weight (mg) ± SD
5 mg/g pheromone MS[a] dust	41	73.2[c]	66.5 ± 29.2	26.8	380.7 ± 85.8
MS[b] dust control	21	47.6	54.7 ± 65.5	52.4	326.8 ± 91.4

[a]Linde molecular sieve loaded with 2,6-dichlorophenol and mixed with talc dust.
[b]Linde molecular sieve not loaded, mixed with talc dust.
[c]Significantly different from the control, $T = 1.987$, $P \simeq 0.005$, 60 *df*.

pheromone/100 mg hair, respectively. Two samples from the abdominal area of the same two dogs had 0.8 and 1.2 ng pheromone/100 mg hair, respectively. Skin surface extracts from a closely shaved area of the back of the same two dogs each had 5 and 3 ng, respectively, in an unknown amount of skin debris.

DISCUSSION

Treatment of tick-infested dogs with the 5.6 μg/ml microencapsulated pheromone formulation effectively reduced mating in *D. variabilis* during the 8-day observation period. This effect was probably related to the ubiquitous presence of pheromone on the host, since microscopic examination revealed gelatin microcapsules adhering to the hair, while GLC analysis demonstrated nanogram amounts of 2,6-dichlorophenol in hair and skin. The 5.0 mg/g pheromone dust formulation also appeared to significantly reduce mating, although the results were not as conclusive as those obtained with the 5.6 μg/ml formulation. Significant reductions in mating were observed for the dust formulation only when weight classification was used, perhaps because mating was not delayed as long with the dust formulation as it was with the microcapsular formulation. Sonenshine et al. (1979) also observed slight delays of 1 or 2 days in mating with their 5% and 20% microencapsulated formulations dispersed as a dust. Greater differences might have been revealed had they removed and weighed the female ticks.

The tick sex pheromone, 2,6-dichlorophenol, guides the mate-seeking male ticks to the female and identifies the female as a sex partner. In addition, vapors of this same compound excite feeding male ticks, stimulating them to detach and seek mates (Sonenshine et al., 1979). Consequently, it is possible that the 5.6 μg/ml formulation, and to a lesser extent the 5 mg/g dust formulations were effective because we treated the environment on the host with sufficient 2,6-dichlorophenol to confuse mate-seeking males, but not enough to excite them to a vigorous search. Sexually active *D. variabilis* males are extremely sensitive to 2,6-dichlorophenol, detecting as little as 0.5 ng on host animals (Sonenshine et al., 1976), or 0.00005 ng in closed Petri dishes (Leahy and Booth, 1978). The concentration of pheromone appears to be critical. Above a certain effective threshold, behavior may be modified in an unwanted direction. This phenomenon, which has been observed in insects, is reviewed by Shorey (1977). Thus, the presence of up to 5 ng of pheromone persisting in small samples of skin or hair 4 days after treatment with the 5.6 μg/ml formulation was well above the threshold of perception of male ticks. Increasing the concentrations of pheromone by treatment with the 1.1 mg/ml microencapsulated formulation may have excited the male ticks without effecting additional confusion. As a result, mating was not decreased and, according to one type of analysis (Table 2), may even have increased. The 1:10

dilution (Table 2) of the 1.1 mg/ml formulation did not depress mating significantly, perhaps because the gelatin microcapsules were dispersed too widely, and many sites on the host body surface were free of microcapsules. The addition of "stickers," i.e., compounds to coat the gelatin microcapsules and bind them to the hair and skin, did not significantly alter the activity of the formulations. Several of the test results with PEG and CMC even suggest that these additives had a negative effect on the efficacy of the formulation, thereby allowing more mating to occur. The reason for this effect is unknown.

The disruption of tick mating by certain of the slow-release pheromone formulations represents the first reported evidence that 2,6-dichlorophenol may have practical value as an aid in the control of hard ticks. Research with different concentrations of pheromone and pheromone delivery systems may lead to formulations that will reduce tick mating even further. Total inhibition of mating by this method may be more difficult or even impossible to achieve in view of the simplicity of the mate-finding process in metastriate ticks. Moreover, some males might remain to inseminate females later than the 8 days allowed in this study, i.e., some matings may only be delayed. Nevertheless, when combined with an acaricide, reproduction-inhibiting formulations may prove to be beneficial by increasing tick movement and thus exposure to the acaricide in addition to reducing the mating of surviving ticks.

Although not immediately apparent to the pet or livestock owner, reduced mating will lead to reduced infestation pressure in subsequent parasite generations breeding in homes, kennels, barns, and other restricted environments. Consequently, it may be possible to reduce pest populations to acceptable levels or even eradicate ticks in these situations without excessive pesticide treatment.

The authors are not unaware of the risks involved in releasing yet another potentially hazardous compound into the environment. However, 2,6-dichlorophenol exhibits relatively low toxicity; its oral LD_{50} rate of 2940 mg/kg (Anon., 1978) is much lower than that of some insecticides in common use. When used in the manner and amounts found most effective in this study (approximately 0.84 mg/animal), 2,6-dichlorophenol does not appear to present a serious hazard to human or animal health.

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SEX PHEROMONE COMPONENTS ISOLATED FROM CHINA CORN BORER, *Ostrinia furnacalis* GUENÉE (LEPIDOPTERA: PYRALIDAE), (*E*)- AND (*Z*)-12-TETRADECENYL ACETATES

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Abstract—The sex pheromone components from the corn borer spreading widely in China, *Ostrinia furnacalis* Guenée, have been identified as (*E*)-and (*Z*)-12-tetradecenyl acetates (*E* and *Z* 12–14: Ac). The ratio of *E* isomer to *Z* isomer was 53:47. Traps containing 1×10^{-7}–1×10^{-5} g of these compounds captured more males than did live females or their tip extract (3–6 female equivalents). Tetradecyl acetate (14: Ac) was also identified in the tip extract. Its quantity was about 1.8 times the sum of the other two isomers. However, including this compound in its natural ratio in pheromone traps resulted in a decrease in trap catches ($P < 0.05$).

Key Words—Lepidoptera, Pyralidae, *Ostrinia furnacalis*, sex pheromone, corn borer, (*E*)-12-tetradecenyl acetate, (*Z*)-12-tetradecenyl acetate, tetradecyl acetate, gas chromatography–mass spectrometry, selected ion monitoring technique.

INTRODUCTION

From external morphology, the corn borer spreading widely in China is almost indistinguishable from the European corn borer, *Ostrinia nubilalis* Hübner. Therefore, some authors (e.g., Cai, 1973) described the China corn borer as *O. nubilalis*. However, Mutuura and Munroe (1970) identified this species as *O. furnacalis* Guenée.

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It has been known for several years that two species of the genus *Ostrinia*, *O. nubilalis* and *O. obumbratalis* Lederer, use the same compounds, namely, (*Z*)- and (*E*)-11-tetradecenyl acetates (*Z* and *E*11-14:Ac) in their sex pheromone communication systems (Klun and Junk, 1977; Klun and Robinson, 1972). However, the sex pheromone of *O. furnacalis* has not yet been identified.

During the period August 1974 to March 1975, we carried out field screening tests with synthetic unsaturated and saturated straight-chain acetates in corn fields in Yangshan County and Hainan Island, Kwangtung Province, of South China. Neither pure *Z*11-14:Ac, *E*11-14:Ac, nor their mixtures in different ratios attracted male corn borer moths in our field experiments.

The preliminary experiments on female tip extracts started in the summer of 1977. According to the results of chemical reactions and GC-MS experiments, we concluded that the pheromone components of *O. furnacalis* were tetradecenyl acetates, but the position of the double bond was not known (Yang, 1978). At the end of 1978, the sex pheromone system was finally characterized qualitatively and quantitatively. Field tests on synthetic pheromonal acetates were carried out during the period 1979–1980. In the present paper the chemical composition of the sex pheromone of *O. furnacalis* and the pheromonal activity of the synthetic compounds are described.

METHODS AND MATERIALS

Collection and Extraction. All corn borers used in this study were collected in corn fields in Yangshan County. They were mainly collected in the form of pupae, but some of them were obtained in the final larval stage. The larvae collected in such a way were allowed to stay until pupation. The pupae were sexed, and the females were allowed to emerge under room conditions. The healthy female moths were transferred into a screened cage (60 cm high × 40 cm diameter), sprayed with water twice a day, and held at room temperature for 1–2 days. Then the moths were narcotized by treatment with methylene chloride or diethyl ether vapor at 3–5 AM (mating time for the corn borer). As was proved by bioassay, the pheromone gland of *O. furnacalis* was located on the surface of the intersegmental membrane between the 8th and 9th abdominal segments. These segments were normally retracted in the 7th segment. In order to obtain the gland, the females were forced to extrude the 8th and 9th abdominal segments by gentle squeezing of the base of the abdomen. The abdominal tip, consisting of the 8th and 9th segments, was snipped into methylene chloride solvent. The crude preparations were stored at 0° C in a refrigerator before use. After removal of solvent from the filtrate, a

yellow oil was obtained in amounts corresponding to about $3\text{–}5 \times 10^{-5}$ g per female tip.

TLC and Column Chromatography. The thin-layer chromatography was performed on silica gel G (about 0.25 mm thick), using petroleum ether (60–90° C) and diethyl ether (95:5, v/v) as a solvent system. This procedure was used to check the purity of fractions eluted from the silica gel column before they were injected into GC columns.

A glass column (1 cm ID) packed with 30 g of 100–140 mesh silica gel was used to clean up the crude extracts utilizing the same solvent system as above. Extract from 2000 female tips was loaded onto a column each time.

Microchemical Reactions. A detailed report on microchemical reactions for sex pheromone has been published separately (Yang, 1979). For each reaction a purified fraction or crude extract corresponding to about 70–150 FE was usually used. Generally, the reactions were carried out in a volume as small as 1 ml or less. Transfer of material from one vessel to another was avoided in order to reduce loss.

The oxidation reaction of crude or purified extract was carried out at room temperature with $KMnO_4$ in acetone, acidified by H_2SO_4.

Addition reactions of pheromone-active fractions eluted from a silica gel column were performed with bromine solution in CCl_4. Elimination of bromine from brominated products were accomplished by grinding the dibromides with zinc powder in ethanol.

Saponification of pheromone-active fractions was carried out in a 4% KOH–ethanol solution under reflux for an hour. Reacetylation was performed by addition of acetyl chloride to the alcoholic constituents, which were obtained by extraction of the saponified mixture with petroleum ether or diethyl ether.

The microozonolysis reaction procedure has been described elsewhere (Beroza and Bierl, 1967).

GC-MS Experiments. All chromatographic data in the present work were obtained on a Varian Aerograph 2740 GC-MAT 311 A MS system fitted with a two-stage Watson-Biemann separator using the selected ion monitoring (SIM) technique at 70 eV. The advantages of this technique over the conventional total ion current monitoring method are its good selectivity and high sensitivity. We have successfully detected as little as 2×10^{-11} g of (*Z*)-9-dodecenyl acetate, while researching the sex pheromone of the sugarcane borer, *Argyroploce schistaceana* Snellen, utilizing m/z 61 $(CH_3COOH_2)^+$ as a characteristic ion for straight-chain acetates (Laboratory No. 1, Kwangtung Institute of Analysis, 1978).

It is most important to choose the proper ions for the SIM technique. As shown above, the ion at m/z 61 $(CH_3COOH_2)^+$ is suitable for detection of straight-chain acetates. Its intensity is always high, and in most cases the

interference from impurities is negligible. On the other hand, in some cases the intensity of the molecular ion M^+ is too weak to be detected, but fortunately the intense ion $(M- CH_3COOH)^+$ may be used to calculate the molecular weight of the original compound.

Both Carbowax 20 M support-coated open tubular (SCOT) glass capillary columns and a 5% DEGS (on 80–100 Gas Chrom Q) stainless-steel column (3.5 m × 2 mm ID) were used in the present study.

Field Trapping Tests. All steps in purification and identification were monitored for pheromonal activity by field trapping tests. The synthetic pheromone components were also tested by the same technique. A simple water trap was used for this purpose. It was a 30-cm-diameter vessel filled with water, with detergent added to reduce the surface tension of water. A paper roll impregnated with pheromonal solution and supported 1–1.5 cm above the water surface was used as bait. The height of the trap above the ground depended on the height of the plants. Usually, it was about 80–100 cm. The lures in the traps were changed every night.

In 1979 no pure *Z* 12–14: Ac was available, so a pure *E* 12–14: Ac and a crude *Z* isomer sample (*Z* 12–14: Ac/*E* 12–14: Ac/ 14: Ac = 92: 1: 7) were used to make lures for trapping tests (Tables 2 and 3). In 1980 all three acetates were obtained in pure form; therefore, more accurate field experiments could be done (Tables 4 and 5).

Since the numbers of replicates changed from sample to sample, we could not use the statistical test especially designed for comparison of several samples of equal size. We had to compare every pair of samples using the *t* table (Anderson, 1978; Fryer, 1966), and then summarize the results (Tables 2–5).

All field tests were carried out in Yangshan County, Kwangtung.

RESULTS AND DISCUSSION

Identification of Pheromone Components. The pheromone-active components from female corn borers were completely destroyed by saponification and could be restored by reacetylation. The activity was also destroyed by bromination or oxidation by $KMnO_4$ and restored in the first case by debromination. The results of these microchemical reactions suggest that the pheromone of *O. furnacalis* consists of acetates of unsaturated alcohols.

Only a narrow band on the TLC plate was found to be pheromone-active. Its R_f value (about 0.5–0.6) coincided with that of straight-chain acetate esters and was slightly higher than that of glycerides.

Further support for esters was also obtained when a crude extract was purified by chromatography on silica gel. During the clean-up procedure one to two 6-ml (usually 13th and/or 14th) fractions showed biological activity.

The eluted volume of active fractions was also coincident with that of synthetic long straight-chain acetates.

The purity of all active fractions from the silica gel column was checked separately by TLC. Only the highly pure fractions were injected onto GC columns.

As discussed above the acetate character of the pheromone compounds was indicated by preliminary experiments, including microchemical reactions and thin-layer and column chromatography. The next step involved detection and identification of the straight-chain acetates present in female tip extracts using GC-MS and the SIM technique.

When the ion at m/z 61 was monitored, three well-separated GC peaks (retention times for peaks I, II, and III were 20.43, 26.70, and 28.85 min, respectively, Table 1) were found in the pheromone-active fractions on a Carbowax 20 M SCOT capillary column (36 m × 0.29 mm ID) under the following conditions: the carrier gas (helium) was adjusted to a flow rate of 3.6 ml/min with 30 ml/min makeup helium; the column was heated isothermally at 150° C. Only peak I at 20.43 min could be found when monitoring m/z 196; peaks II and III at 26.70 and 28.85 min could be found when monitoring m/z 194; and finally none of these peaks appeared when monitoring m/z 192 (Figure 1). These results indicate that I was a peak from tetradecyl acetate, and II and III were peaks from tetradecenyl acetates.

According to the data of synthetic samples on Carbowax 20 M SCOT capillary columns (Table 1), the retention times of GC peaks were positively correlated with increasing distance between the acetate group and the carbon–carbon double bond. In addition, the retention times of *Z* isomers were longer than those for corresponding *E* isomers. The compounds represented by GC peaks I, II, and III were identical with 14:Ac (I), *E*12-14:Ac (II), and

TABLE 1. RETENTION TIMES OF GC PEAKS OF SEX PHEROMONE COMPONENTS AND SOME STANDARD STRAIGHT-CHAIN ACETATES (OCTOBER 5–17, 1978)

	Retention time (min)						
Column number[a]	Peak I	*E*9-14:Ac	*Z*9-14:Ac	*E*11-14:Ac	*Z*11-14:Ac	Peak II	Peak III
1	10.38	12.30	12.85		13.16	13.63	14.80
2	20.43		23.90	24.52	25.63	26.70	28.85
3	35.62			41.30	43.85	45.73	49.23

[a]GC conditions: SCOT glass capillary columns, coated with Carbowax 20 M, column temperature 150° C, makeup helium 30 ml/min, two-stage Watson-Biemann type separator. Column 1: 37 m × 0.32 mm ID, helium flow 1.5 ml/min; column 2: 36 m × 0.29 mm ID, helium flow 3.6 ml/min; column 3: 50 m × 0.31 mm ID, helium flow 3.6 ml/min.

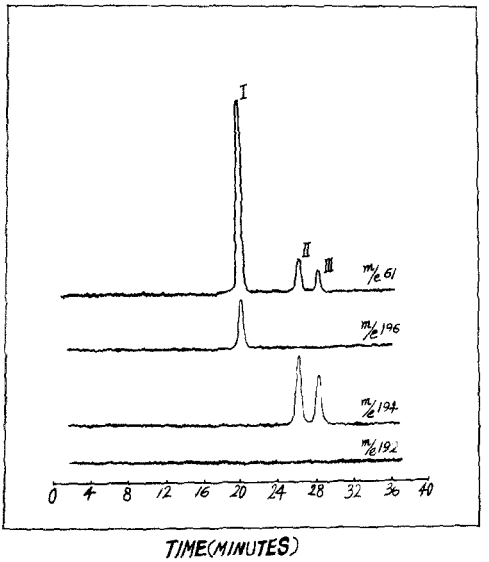

FIG. 1. Mass chromatograms of sex pheromone components of *Ostrinia furnacalis* (GC conditions: see Table 1, column 2).

Z 12–14:Ac (III), respectively, in retention times and mass fragmentation patterns. These three components were eluted in the same order from a DEGS-packed column, operating at 160° C and a flow rate (helium) of 20 ml/min. Their retention times were 6.78, 8.93, and 9.70 min, respectively. They showed the same spectral characteristics as the components eluted from capillary columns. Coinjection of active fractions with authentic compounds was also conducted. Upon coinjection, the intensities of the GC peaks increased, but their retention times and widths remained unchanged. These enhancement experiments further confirmed the above-mentioned assignments.

The unsaturated acetates were ozonized to the corresponding aldehydes by Beroza's method. A peak at 14.23 min was detected in the ozonized products on the DEGS column, operating at 180° C and a helium flow of 30 ml/min, when the ion of *m/z* 61 was monitored using the SIM technique. This compound was identical with the authentic 12-acetoxydodecanal in retention time and mass spectral characteristics (*m/z* 61 and 139); thus, the ozonolysis experiment further supported the conclusion that 12-tetradecenyl acetates were present in the pheromone system of *O. furnacalis*.

The *E*-to-*Z* ratio for 12-tetradecenyl acetates determined by gas chromatographic peak areas was 53:47. At the same time, the quantity of tetradecyl acetate was about 1.8 times the sum of the other two compounds.

Field Trapping Tests. The preliminary field tests showed that mixtures of

E and *Z* 12–14: Ac in nearly all possible ratios were attractive to males, but the most efficient lures were the mixtures with ratios like the natural one (Table 2). On the basis of data listed in Table 2, we came to the conclusion that synthetic mixtures at microgram levels were more attractive to males than three live virgin females. For example, the mean catches for the artificial mixture in natural *E*/*Z* ratio were nearly six times higher than those for live female traps (9.3 against 1.6).

The mixture with the natural *E*/*Z* ratio which showed the highest attractiveness in field tests (trap No. 6 in Table 2) was used to investigate the relationship between lure doses and mean catches (July 29–August 1, August 15–September 5, 1979). The maximum sexual attractiveness was reached within a rather broad range of doses (1×10^{-7}–1×10^{-5} g per trap). Again the efficiency for traps at this dose level was higher than that for three virgin females. At the same time, no dependence of mean catches on dose could be found within this dose range, but mean catches diminished significantly as the lure dose either decreased or increased from this range (Table 3). The males were not trapped with lure dose higher than 1×10^{-4} g per trap.

Although populations of corn borer adults were relatively low in Yangshan County in late August and early September 1979, mean catches for artificial mixtures reached two figures, and maximum catches were 44 males per trap per night.

TABLE 2. BIOASSAY RESULTS FOR SYNTHETIC MIXTURES (JULY 22–AUGUST 1, 1979)

Trap number	Mixture composition (%)[a]			Number of replicates	Mean trap catches (males/trap/night)[b]
	*E*12–14: Ac	*Z*12–14: Ac	14: Ac		
1	1	92	7	10	4.2bcd
2	11	83	6	6	3.5bcde
3	21	74	5	8	5.0abc
4	31	64	5	6	5.2abc
5	41	55	4	8	8.0ab
6	51	46	3	19	9.3a
7	60	37	3	8	5.8abc
8	70	28	2	6	3.7cde
9	80	19	1	8	4.1cd
10	90	9	1	6	3.7cde
11	100	0	0	12	2.3de
12	3 Virgin females			7	1.6e
13	Unbaited			13	0.08f

[a] Total dose of lure was 2×10^{-6}–1×10^{-5} g per trap.
[b] Means followed by the same letter in each experiment were not significantly different from each other at 5% level.

TABLE 3. RELATIONSHIP BETWEEN MEAN CATCHES AND DOSES OF LURE (JULY 29–AUGUST 1, AUGUST 15–SEPTEMBER 5, 1979)

Dose (g/trap)[a]	Number of replicates	Mean catches (males/trap/night)[b]
5×10^{-9}	15	4.3b
1×10^{-8}	17	2.2bc
2×10^{-8}	12	1.0c
1×10^{-7}	16	9.6a
2×10^{-7}	44	11.4a
5×10^{-7}	22	10.7a
1×10^{-6}	58	12.3a
2×10^{-6}	32	12.2a
5×10^{-6}	12	10.6a
1×10^{-5}	10	8.7a
2×10^{-5}	10	2.7b
1×10^{-4}	8	0d
2.6×10^{-4}	4	0.25cd
1×10^{-3}	10	0d
Unbaited	35	0.1d
1 Virgin female	2	1.0
2 Virgin females	2	4.0
3 Virgin females	4	4.7

[a]Percentage composition of synthetic mixture: *E*12–14:Ac/*Z*12–14:Ac/14:Ac = 51:46:3.
[b]Means followed by the same letter in each experiment were not significantly different from each other at 5% level. Mean catches for virgin females were not included in the statistical calculation, because only a few live females were available for tests.

As mentioned above, there were three long-chain acetates in tip extract from female *O. furnacalis*, and the saturated tetradecyl acetate was the major constituent in the mixture, so it was imperative to learn what role this compound could play in the pheromonal communication system for this species. During a period of 1977–1978, we attempted to use pure 14:Ac as a bait in field screening experiments, but these experiments failed. Moreover, after the carbon–carbon double bond in female extract had been destroyed by treatment with bromine or $KMnO_4$, the resultant mixtures, still containing unchanged 14:Ac, became unattractive to males.

As the role of 14:Ac in the reproductive behavior of *O. furnacalis* was quite unclear, a special study was designed to evaluate its synergetic or inhibitory effects on the attractiveness of *E* and *Z* 12–14:Ac mixture in the field. Side-by-side comparison of a three-component lure (*E*12–14:Ac/*Z*12–14:Ac/14:Ac = 17:15:68) with a two-component lure (*E*12–14:Ac/*Z*12–14:Ac/14:Ac = 51:46:3) showed that the mean catch for the first was 4.7 males/trap/night vs. 11.7 for the last ($P < 0.05$). The ratio of *E*/*Z* in three-component lure was 53:47, which was identical with that for the two-

component lure and natural extract from female tips. As mentioned above, the percentage composition of the natural extract was *E*12–14:Ac/*Z*12–14:Ac/14:Ac = 19:17:64 with a *E*/*Z* ratio of 53:47. The total dose of (*E*)-and (*Z*)-12-tetradecenyl acetates in the three-component lure was the same as in the two-component one ($2\text{–}2.5 \times 10^{-6}$ g per trap). The only difference for these two kinds of lures was in the amount of saturated tetradecyl acetate.

Results from additional field experiments were obtained in July 1980. Most of these data are presented here. As shown in Table 4, pure 14:Ac and *Z*12–14:Ac did not have attractivity significantly different from zero (unbaited traps). The pure *E*12–14:Ac, however, showed real activity which was significantly above zero (unbaited traps). A comparison between the attractiveness of pure and mixed acetates revealed the mixture of *E* and *Z*12–14:Ac in their natural ratio to be about 4.5 times more attractive than the pure *E* isomer. Nevertheless, it is difficult to explain the difference between pure *Z* and *E* isomers in attractive behavior. These results are similar to those obtained in the summer of 1979 (Table 2).

At present, the major purpose of field trapping tests is to research the role of 14:Ac in sex attraction. On the basis of data in Table 5, we can conclude that tetradecyl acetate, even though it is present in female tip extract, is not a sex pheromone component for *O. furnacalis*. As a matter of fact it acts as an inhibitor in the sex communication system. A small amount of 14:Ac added to the artificial sex pheromone mixture significantly diminishes the mean trap catches. The inhibitory effect of 14:Ac on sex attractiveness increases as its content in the mixture rises.

Since the role of 14:Ac in the pheromonal behavior for *O. furnacalis* is

TABLE 4. BIOASSAY RESULTS FOR SYNTHETIC ACETATES (JULY 6–11, 1980)

Composition of lures (μg)			Number of replicates	Mean catches (males/trap/night)[a]
*E*12–14:Ac	*Z*12–14:Ac	14:Ac		
1	1	0	44	2.89a
2	0	0	40	0.65b
0	2	0	38	0.08c
0	0	2	85	0.07c
	Unbaited		35	0c
	3 Virgin females		9	0.22

[a]Means followed by the same letter in each experiment are not significantly different from each other at 5% level. Mean catches for virgin females are not included in statistical calculation, because only a few live female moths were available for tests.

TABLE 5. TEST OF ROLE OF 14:AC IN SEX ATTRACTION (JULY 13–17, 1980)

Composition of lures (μg)			
*E*12–14:Ac	*Z*12–14:Ac	14:Ac	Mean catches (males/trap/night)[a]
2	2	0	8.3a
2	2	0.2	4.2b
2	2	0.4	2.9b
2	2	0.8	3.6b
2	2	1.2	2.1bc
2	2	1.6	1.7bc
2	2	2.0	1.5bc
2	2	2.4	2.0bc
2	2	2.8	0.2de
2	2	3.2	0.9bcd
2	2	3.6	0.7cd
2	2	4.0	0.9bcd
2	2	6.0	0.7cd
2	2	8.0	0.4cd
2	2	10.0	0.5cd
2	2	12.0	0.5cd
2	2	14.0	0.3cde
2	2	16.0	0.7cd
	Unbaited		0.05e

[a]Mean catches in 4 replicates (for unbaited traps and traps without 14:Ac) or 2 replicates (for all other traps) over 6 successive nights of trapping. Means followed by the same letter in each experiment are not significantly different from each other at 5% level.

not that of a sex pheromone constituent, we are planning to determine: (1) whether or not this compound is present in the other parts of the female moth body apart from the sex pheromone gland or tissues near to it; (2) if it is, whether or not it is also present in the male moth body; and (3) whether or not this compound is actually released together with the other two acetates by the female moth during the mating time.

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EDITORS' NOTE

The same compounds in a ca. 1:1 ratio have been reported as the sex pheromone components of *Ostrinia furnacalis* (Guenée) (called the Asian corn borer moth) from the

Philippines (Klun, J.A., Bierl-Leonhardt, B.A., Schwarz, M., Litsinger, J.A., Barrion, A.T., Chiang, H.C., and Jiang, Zhungxie. 1980. Sex pheromone of the Asian corn borer moth. *Life Sci.* 27:1603–1606).

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SEX PHEROMONAL ACTIVITY OF (+)-*trans*-VERBENYL ACETATE AND RELATED COMPOUNDS TO THE AMERICAN COCKROACH, *Periplaneta americana* L.[1]

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Abstract—The sex pheromone mimic of the American cockroach, (+)-*trans*-verbenyl acetate, and 67 synthesized analogs were tested for sex pheromonal activity using males of the cockroach. Only three esters in addition to the original (+)-*trans*-verbenyl acetate exhibited activity. (+)-*trans*-Verbenyl propionate and (+)-verbanyl acetate in particular showed stronger activity than the original mimic. None of the other analogs caused a response at the 1-mg level. The lower threshold levels at which even the most active analogs showed activity (0.02 mg) were many orders of magnitude higher than that of the natural pheromone, periplanone-B (10^{-8} mg). From the structure–activity relationships, important structural factors for sex pheromonal activity in the original mimic were discussed.

Key Words—Sex pheromone mimics, American cockroach, *Periplaneta americana* L., Orthoptera, Blattidae, (+)-*trans*-verbenyl acetate, (+)-*trans*-verbenyl propionate, (+)-verbanyl acetate.

INTRODUCTION

The chemical structure of a component of the female sex pheromone of the American cockroach (*Periplaneta americana* L.), periplanone-B, was suggested to be a germacrane derivative (Ritter and Persoons, 1975). Later its structure was reported to be an oxygen-containing germacrene-D (Figure 1) (Persoons et al., 1976) and was determined completely quite recently (Persoons et al., 1979; Still, 1979; Adams et al., 1979). The carbon skeleton,

[1]Studies on the sex pheromone mimic of the American cockroach, (+)-*trans*-verbenyl acetate. Part VI. For Part V, *Agric. Biol. Chem.* 44:2877 (1980).

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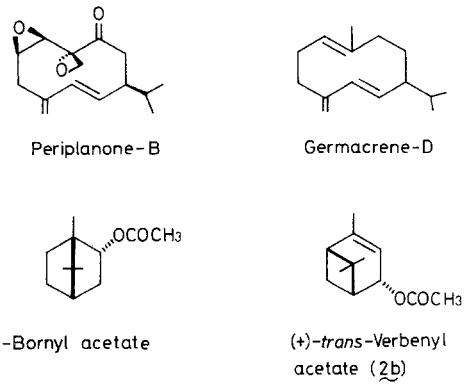


FIG. 1. Sexually active compounds toward the American cockroach. Periplanone-B: natural sex pheromone; others: sex pheromone mimics obtained from plants or plant-derived sources.

germacrene-D, itself, was found to possess sex pheromonal activity in this cockroach (Tahara et al., 1975).

Besides these active sesquiterpenoids, a bicyclic monoterpenoid, (+)-bornyl acetate (Figure 1), was also discovered as a sex pheromone mimic of the American cockroach (Bowers and Bodenstein, 1971).

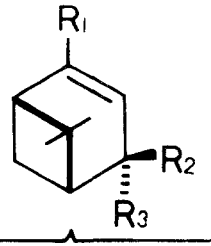
Stimulated by this discovery, we examined many bicyclic monoterpenoids for sex pheromonal activity. (+)-*trans*-Verbenyl acetate (Figures 1 and 2) was subsequently found to be a sex pheromone mimic for the cockroach (Nishino et al., 1977).

Since the discovery of the monoterpenoid mimics, we have attempted to explain why such monoterpenoids cause sex pheromonal activity, although the structures are quite different from that of the natural pheromone (see Figure 1). As the first step for solving the problem, the structure–activity relationships needed to be elucidated. Therefore, many compounds related to (+)-*trans*-verbenyl acetate were synthesized (Nishino and Takayanagi, 1979a,b, 1980; Takayanagi and Nishino, 1980). In the present work, esters and ethers of the synthesized alcohols were prepared, and sex pheromonal activity toward the American cockroach was evaluated for all of the compounds shown in Figure 2. We also wish to report here several important chemical factors necessary for the activity implicit in (+)-*trans*-verbenyl acetate on the basis of the obtained structure–activity relationships.

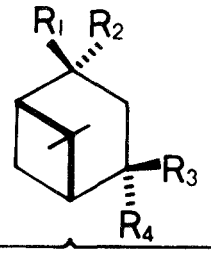
METHODS AND MATERIALS

Preparation of Analogs

Synthetic methods and physical data of all of the alcohols (**a** series in Figure 2), acids (**7** and **8**), and acid derivatives (**9–14**) have been reported in

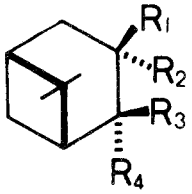


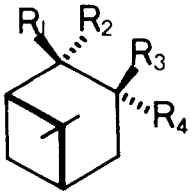
1a: R_1=CH_3, R_2=OH, R_3=H
1b: R_1=CH_3, R_2=$OCOCH_3$, R_3=H
1c: R_1=CH_3, R_2=OCOH, R_3=H
1d: R_1=CH_3, R_2=$OCOC_2H_5$, R_3=H
1e: R_1=CH_3, R_2=$OCOC_3H_7$, R_3=H
2a: R_1=CH_3, R_2=H, R_3=OH
2b: R_1=CH_3, R_2=H, R_3=$OCOCH_3$
2c: R_1=CH_3, R_2=H, R_3=OCOH
2d: R_1=CH_3, R_2=H, R_3=$OCOC_2H_5$
2e: R_1=CH_3, R_2=H, R_3=$OCOC_3H_7$
3 : R_1=CH_3, R_2=OCH_3, R_3=H
4 : R_1=CH_3, R_2=H, R_3=OCH_3
5 : R_1=CH_3, R_2=OC_2H_5, R_3=H
6 : R_1=CH_3, R_2=H, R_3=OC_2H_5
7 : R_1=CH_3, R_2=COOH, R_3=H
8 : R_1=CH_3, R_2=H, R_3=COOH
9 : R_1=CH_3, R_2=$COOCH_3$, R_3=H
10 : R_1=CH_3, R_2=H, R_3=$COOCH_3$
11a: R_1=CH_3, R_2=CH_2OH, R_3=H
11b: R_1=CH_3, R_2=CH_2OCOCH_3, R_3=H
12a: R_1=CH_3, R_2=H, R_3=CH_2OH
12b: R_1=CH_3, R_2=H, R_3=CH_2OCOCH_3
13 : R_1=CH_3, R_2=$COCH_3$, R_3=H
14 : R_1=CH_3, R_2=H, R_3=$COCH_3$
15a: R_1=R_3=H, R_2=OH
15b: R_1=R_3=H, R_2=$OCOCH_3$
16a: R_1=R_2=H, R_3=OH
16b: R_1=R_2=H, R_3=$OCOCH_3$



17a: R_1=CH_3, R_2=R_4=H, R_3=OH
17b: R_1=CH_3, R_2=R_4=H, R_3=$OCOCH_3$
18a: R_1=CH_3, R_2=R_3=H, R_4=OH
18b: R_1=CH_3, R_2=R_3=H, R_4=$OCOCH_3$
19a: R_1=R_4=H, R_2=CH_3, R_3=OH
19b: R_1=R_4=H, R_2=CH_3, R_3=$OCOCH_3$
20a: R_1=R_3=H, R_2=CH_3, R_4=OH
20b: R_1=R_3=H, R_2=CH_3, R_4=$OCOCH_3$
21a: R_1=R_2=R_4=H, R_3=OH
21b: R_1=R_2=R_4=H, R_3=$OCOCH_3$
22a: R_1=R_2=R_3=H, R_4=OH
22b: R_1=R_2=R_3=H, R_4=$OCOCH_3$

FIG. 2. (+)-*trans*-Verbenyl acetate (**2b**) and its analogs tested for sex pheromonal activity toward the American cockroach.





23a: R_1=CH_3, R_2=R_4=H, R_3=OH
23b: R_1=CH_3, R_2=R_4=H, R_3=$OCOCH_3$
24a: R_1=CH_3, R_2=R_3=H, R_4=OH
24b: R_1=CH_3, R_2=R_3=H, R_4=$OCOCH_3$
25a: R_1=R_4=H, R_2=CH_3, R_3=OH
25b: R_1=R_4=H, R_2=CH_3, R_3=$OCOCH_3$
26a: R_1=R_3=H, R_2=CH_3, R_4=OH
26b: R_1=R_3=H, R_2=CH_3, R_4=$OCOCH_3$
27a: $R_1 \cdot R_2$=$=CH_2$, R_3=OH, R_4=H
27b: $R_1 \cdot R_2$=$=CH_2$, R_3=$OCOCH_3$, R_4=H
28a: $R_1 \cdot R_2$=$=CH_2$, R_3=H, R_4=OH
28b: $R_1 \cdot R_2$=$=CH_2$, R_3=H, R_4=$OCOCH_3$

29a: R_1=CH_3, R_2=R_4=H, R_3=OH
29b: R_1=CH_3, R_2=R_4=H, R_3=$OCOCH_3$
30a: R_1=CH_3, R_2=R_3=H, R_4=OH
30b: R_1=CH_3, R_2=R_3=H, R_4=$OCOCH_3$
31a: R_1=R_4=H, R_2=CH_3, R_3=OH
31b: R_1=R_4=H, R_2=CH_3, R_3=$OCOCH_3$
32a: R_1=R_3=H, R_2=CH_3, R_4=OH
32b: R_1=R_3=H, R_2=CH_3, R_4=$OCOCH_3$
33a: $R_1 \cdot R_2$=$=CH_2$, R_3=OH, R_4=H
33b: $R_1 \cdot R_2$=$=CH_2$, R_3=$OCOCH_3$, R_4=H
34a: $R_1 \cdot R_2$=$=CH_2$, R_3=H, R_4=OH
34b: $R_1 \cdot R_2$=$=CH_2$, R_3=H, R_4=$OCOCH_3$
35a: R_1=R_2=R_4=H, R_3=OH
35b: R_1=R_2=R_4=H, R_3=$OCOCH_3$
36a: R_1=R_2=R_3=H, R_4=OH
36b: R_1=R_2=R_3=H, R_4=$OCOCH_3$

FIG. 2. Continued.

previous papers (Nishino and Takayanagi, 1979a,b, 1980; Takayanagi and Nishino, 1980). Verbenyl formates (**1c** and **2c**) were prepared from verbenols (**1a** and **2a**) in pyridine by the addition of a 1:1 mixture of formic acid and acetic anhydride. The other esters [acetates (**b** series), propionates (**d** series), and butyrates (**e** series)] were obtained from alcohols by the usual method using acid anhydride and pyridine. The ethers (**3–6**) were synthesized as follows: *n*-Butyllithium (1.6 M soln, 0.42 ml) was added dropwise to a mixture of alcohol (0.67 mmol) and triphenylmethane (2 mg) in tetrahydrofuran (1 ml) and hexamethylphosphoric triamide (1 ml) at 0° C, until the red color persisted. Alkyl iodide (0.75 mmol) was added to the reaction mixture. The mixture was diluted with water and extracted with ether. The residue of the extract was chromatographed (silica gel, *n*-hexane–ethyl acetate, 25:1) to give the ether (90% yield).

Physical Data of Analogs

Specific rotations were measured in benzene at 25°C. Infrared (IR) spectra were recorded as liquid films. Proton nuclear magnetic resonance (PMR) spectra were taken in deuteriochloroform with tetramethylsilane (TMS) as an internal standard at 60 MHz. Chemical shifts (ppm) were measured from the TMS signal. Mass spectra (MS) were taken at 70 eV. Gas chromatography was carried out using a 2-m × 3-mm glass column packed with a 3% OV-255 at 110°C with nitrogen as the carrier gas.

(+)-cis-*Verbenyl Acetate* (**1b**). $[\alpha]_D$ −89° (c = 1.1); IR: 1730, 1640, 1245 cm^{-1}; PMR: 1.01 [3H, singlet (s)], 1.36 (3H,s), 1.42 [1H, doublet (d), J = 9.0], 1.77 [3H, triplet (t), J = 1.5], 2.01 (3H,s), 5.36 [1H, multiplet (m)], 5.51 (1H,m); MS: m/e 134 (M^+—CH_3CO_2H), 119 [base peak (B^+)]; retention time (t_R, min) in GC: 5.1.

(+)-trans-*Verbenyl Acetate* (**2b**). $[\alpha]_D$ +147.0° (c = 2.0), +153.0° (c = 1.0, $CHCl_3$); IR: 1730, 1240 cm^{-1}; PMR: 0.96 (3H,s), 1.37 (3H,s), 1.43 (1H,d, J = 9.0), 1.73 (3H,s), 1.92 (3H,s), 5.28 (2H,m); MS: m/e 134 (M^+—CH_3CO_2H), 119 (B^+); t_R: 8.3.

(+)-cis-*Verbenyl Formate* (**1c**). $[\alpha]_D$ −62.3° (c = 1.0); IR: 1720, 1650, 1170 cm^{-1}; PMR: 1.02 (3H,s), 1.37 (3H,s), 1.77 (3H,t, J = 1.5), 5.36 (1H,m), 5.67 (1H,m), 8.08 (1H,d, J = 2.0); MS: m/e 134 (M^+—HCO_2H), 119 (B^+), 91: t_R: 4.3.

(+)-trans-*Verbenyl Formate* (**2c**). $[\alpha]_D$ +154.5° (c = 0.4); IR: 1720, 1650, 1180 cm^{-1}, PMR: 0.95 (3H,s), 1.45 (3H,s), 1.75 (3H,s), 5.40 (1H,m), 5.65 (1H,m), 8.10 (1H,d, J = 2.0); MS: m/e 134 (M^+—HCO_2H), 119 (B^+), 91; t_R: 4.5.

(+)-cis-*Verbenyl Propionate* (**1d**). $[\alpha]_D$ −83.4° (c = 0.9); IR: 1740, 1655, 1190 cm^{-1}; PMR: 1.02 (3H,s), 1.37 (3H,s), 1.76 (3H,t, J = 1.5), 5.37 (1H,m), 5.52 (1H,m); MS: m/e 134 (M^+—$C_2H_5CO_2H$), 119 (B^+), 91; t_R: 7.4.

(+)-trans-*Verbenyl Propionate* (**2d**). $[\alpha]_D$ +132.0 (c = 0.5); IR: 1735, 1650, 1185 cm^{-1}; PMR: 0.91 (3H,s), 1.11 (3H,t, J = 6.5), 1.32 (3H,s), 1.71 [3H, broad singlet (bs)], 5.32 (2H,m); MS: m/e 134 (M^+—$C_2H_5CO_2H$), 119 (B^+), 91; t_R: 7.6.

(+)-cis-*Verbenyl Butyrate* (**1e**). $[\alpha]_D$ −82.2° (c = 1.0); IR: 1730, 1655, 1255, 1175 cm^{-1}; PMR: 1.00 (3H,s), 1.32(3H,s), 1.72 (3H,t, J = 1.5), 5.32 (1H,m), 5.51 (1H,m); MS: m/e 134 (M^+—$C_3H_7CO_2H$), 119 (B^+), 91; t_R: 10.7.

(+)-trans-*Verbenyl Butyrate* (**2e**). $[\alpha]_D$ +134.0° (c = 0.5); IR: 1730, 1650, 1255, 1180 cm^{-1}; PMR: 0.92 (3H,t, J = 6.5), 0.92 (3H,s), 1.33 (3H,s), 1.73 (3H,bs), 5.33 (2H,bs); MS: m/e 134 (M^+—$C_3H_7CO_2H$), 119 (B^+), 91; t_R: 11.3.

Methyl Ether of (+)-cis-*Verbenol* (**3**). $[\alpha]_D$ −34.8° (c = 0.5); IR: 1645, 1085 cm^{-1}; PMR: 1.00 (3H,s), 1.34 (3H,s), 1.73 (3H,t, J = 1.5), 3.36 (3H,s),

4.01 (1H,m), 5.44 (1H,m); MS: *m/e* 151 (M^+—CH_3), 123, 119, 86, 73 (B^+); t_R: 1.7.

Methyl Ether of (+)-trans-*Verbenol* (**4**). $[\alpha]_D$ +106.8° (*c* = 0.4); IR: 1650, 1090 cm^{-1}; PMR: 0.84 (3H,s), 1.30 (3H,s), 1.68 (3H,t, *J* = 1.5), 3.32 (3H,s), 3.79 (1H,m), 5.38 (1H,m); MS: *m/e* 151 (M^+—CH_3), 123, 119, 91, 86 (B^+), 73; t_R: 2.1.

Ethyl Ether of (+)-cis-*Verbenol* (**5**). $[\alpha]_D$ −20.3° (*c* = 1.0); IR: 1650, 1085 cm^{-1}; PMR: 1.01 (3H,s), 1.18 (3H,t, *J* = 7.0), 1.32 (3H,s), 1.70 (3H,t, *J* = 1.5), 1.95 [1H, double triplet (dt), *J* = 2.0, 5.5), 3.53 [2H, quartet (q), *J* = 7.0], 4.13 (1H,m), 5.40 (1H,m); MS: *m/e* 165 (M^+—CH_3), 137, 119, 109, 100 (B^+): t_R: 1.8.

Ethyl Ether of (+)-trans-*Verbenol* (**6**). $[\alpha]_D$ +97.8° (*c* = 0.5); IR: 1650, 1080 cm^{-1}; PMR: 0.87 (3H,s), 1.18 (3H,t, *J* = 7.0), 1.32 (3H,s), 1.70 (3H,t, *J* = 1.5), 3.53 (2H,q, *J* = 7.0), 3.90 (1H,m); MS: *m/e* 165 (M^+—CH_3), 137, 119, 109, 100 (B^+); t_R: 1.8.

(−)-cis-*Apopinenyl Acetate* (**15b**). $[\alpha]_D$ −64.0° (*c* = 0.2); IR: 1730, 1620, 1245 cm^{-1}; PMR: 1.03 (3H,s), 1.33 (3H,s), 2.02 (3H,s), 5.32 (1H,m), 5.65 [1H, broad doublet (bd), *J* = 9.0], 6.42 [1H, double doublet (dd), *J* = 6.0, 9.0]; MS: *m/e* 138 (M^+—CH_2CO), 123, 120 (M^+—CH_3CO_2H), 105, 95 (B^+), 91, 89; t_R: 4.2.

(+)-trans-*Apopinenyl Acetate* (**16b**). $[\alpha]_D$ +172.8° (*c* = 1.0); IR: 1735, 1620, 1240 cm^{-1}; PMR: 0.96 (3H,s), 1.35 (3H,s), 2.03 (3H,s), 5.41 (1H,m), 5.62 (1H,bd, *J* = 10.0), 6.42 (1H,m); MS: *m/e* 138 (M^+—CH_2CO), 123, 120 (M^+—CH_3CO_2H), 105, 95 (B^+), 91, 89; t_R: 4.4.

(+)-*Neoisoverbanyl Acetate* (**17b**). $[\alpha]_D$ −9.5° (*c* = 1.2); IR: 1735, 1255 cm^{-1}; PMR: 0.78 (1H,d, *J* = 9.5), 1.06 (3H,d, *J* = 7.0), 1.12 (3H,s), 1.23 (3H,s), 2.00 (3H,s), 5.19 [1H, double quartet (dq), *J* = 2.5, 6.5, 9.5); MS: *m/e* 136 (M^+—CH_3CO_2H), 121, 93 (B^+); t_R: 6.4.

(+)-*Isoverbanyl Acetate* (**18b**). $[\alpha]_D$ +36.3° (*c* = 1.3); IR: 1730, 1250 cm^{-1}; PMR: 1.05 (3H,d, *J* = 6.0), 1.15 (3H,s), 1.25 (3H,s), 2.05 (3H,s), 5.07 (1H,m); MS: *m/e* 136 (M^+—CH_3CO_2H), 121, 93 (B^+); t_R: 6.4.

(−)-*Neoverbanyl Acetate* (**19b**). $[\alpha]_D$ −9.0° (*c* = 1.1); IR: 1730, 1255, 1020 cm^{-1}; PMR: 0.91 (3H,d, *J* = 6.5), 1.02 (3H,s), 1.22 (3H,s), 1.97 (3H,s), 5.20 (1H,m); MS: *m/e* 136 (M^+—CH_3CO_2H), 121, 93 (B^+); t_R: 6.9.

(+)-*Verbanyl Acetate* (**20b**). $[\alpha]_D$ +10.0° (*c* = 1.0); IR: 1730, 1250 cm^{-1}; PMR: 0.87 (3H,s), 0.92 (3H,d, *J* = 7.0), 1.26 (3H,s), 1.98 (3H,s), 5.12 (1H,m); MS: *m/e* 196 (M^+, $C_{12}H_{20}O_2$), 154, 136 (M^+—CH_3CO_2H), 121, 107, 93 (B^+); t_R: 6.6.

(−)-cis-*Nopinyl Acetate* (**21b**). $[\alpha]_D$ −7.1° (*c* = 1.0); IR: 1725, 1240 cm^{-1}; PMR: 0.88 (3H,s), 1.20 (3H,s), 1.98 (3H,s), 5.27 (1H,m); MS: *m/e* 182 (M^+, $C_{11}H_{18}O_2$), 167 (M^+—CH_3), 139 (M^+—CH_3CO), 122 (M^+—CH_3CO_2H), 107, 93, 91, 79 (B^+); t_R: 5.5.

(−)-trans-*Nopinyl Acetate* (**22b**). $[\alpha]_D$ −25.0° (*c* = 1.0); IR: 1725, 1245 cm^{-1}; PMR: 0.87 (3H,s), 1.23 (3H,s), 1.98 (3H,s), 5.20 (1H,m); MS: *m/e* 182

(M^+, $C_{11}H_{18}O_2$), 167 (M^+—CH_3), 140, 122 (M^+—CH_3CO_2H), 107, 93, 85, 79 (B^+); t_R: 5.5.

(−)-*3β-Methyl*-cis-*nopinyl Acetate* (**23b**). IR: 1735, 1250 cm^{-1}; PMR: 0.95 (3H,d, J = 7.0), 0.96 (3H,s), 1.18 (3H,s), 2.06 (3H,s), 5.39 (1H,dd, J = 4.0, 8.0); MS: m/e 196 (M^+, $C_{12}H_{20}O_2$), 181 (M^+—CH_3), 136 (M^+—CH_3CO_2H), 121, 93, 85 (B^+); t_R: 4.2.

(−)-*3β-Methyl*-trans-*nopinyl Acetate* (**24b**). $[\alpha]_D$ −98.7° (c = 1.0); IR: 1735, 1250 cm^{-1}; PMR: 0.83 (3H,s), 1.05 (3H,d, J = 6.0), 1.22 (3H,s), 1.98 (3H,s), 4.88 (1H,bd, J = 6.5); MS: m/e 196 (M^+, $C_{12}H_{20}O_2$), 181 (M^+—CH_3), 136 (M^+—CH_3CO_2H), 121, 93, 85 (B^+); t_R: 4.6.

(+)-*3α-Methyl*-cis-*nopinyl Acetate* (**25b**). $[\alpha]_D$ +55.2° (c = 1.0); IR: 1730, 1250 cm^{-1}; PMR: 0.88 (1H,d, J = 9.5), 1.01 (3H,s), 1.15 (3H,d, J = 7.0), 1.19 (3H,s), 1.98 (3H,s), 4.87 [1H, broad triplet (bt), J = 3.0]; MS: m/e 196 (M^+, $C_{12}H_{20}O_2$), 181 (M^+—CH_3), 153 (M^+—CH_3CO), 136 (M^+—CH_3CO_2H), 121, 107, 93 (B^+); t_R: 4.6.

(−)-*3α-Methyl*-trans-*nopinyl Acetate* (**26b**). $[\alpha]_D$ −8.7° (c = 0.7); IR: 1735, 1250 cm^{-1}; PMR: 0.95 (3H,s), 0.97 (3H,d, J = 7.0), 1.23 (3H,s), 2.02 (3H,s), 5.33 (1H,bd, J = 8.5); MS: m/e 136 (M^+—CH_3CO_2H), 121, 107, 93 (B^+); t_R: 6.7.

(+)-*3-Methylene*-cis-*nopinyl Acetate* (**27b**). $[\alpha]_D$ +25.5° (c = 0.6); IR: 3080, 1735, 1640, 1250, 900 cm^{-1}; PMR: 0.95 (1H,d, J = 9.0), 0.98 (3H,s), 1.21 (3H,s), 2.03 (3H,s), 5.16 (2H,bs), 5.65 (1H,m); MS: m/e 194 (M^+, $C_{12}H_{18}O_2$), 152, 134 (M^+—CH_3CO_2H), 119, 109, 91 (B^+); t_R: 5.8.

(−)-*3-Methylene*-trans-*nopinyl Acetate* (**28b**). $[\alpha]_D$ −45.0° (c = 1.0); IR: 3075, 1735, 1635, 1250, 895 cm^{-1}; PMR: 0.90 (3H,s), 1.26 (3H,s), 2.05 (3H,s), 5.19 (2H,bs), 5.75 (1H,bs); MS: m/e 194 (M^+, $C_{12}H_{18}O_2$), 152, 134 (M^+—CH_3CO_2H), 119, 109, 91 (B^+); t_R: 6.0.

(+)-*Neoisopinocampheyl Acetate* (**29b**). $[\alpha]_D$ +8.0° (c = 1.0); IR: 1740, 1250 cm^{-1}; PMR: 0.98 (3H,d, J = 7.0), 1.03 (3H,s), 1.06 (1H,d, J = 9.0), 2.05 (3H,s), 5.38 (1H,dt, J = 5.5, 9.5); MS: m/e 136 (M^+—CH_3CO_2H), 121, 93 (B^+); t_R: 6.9.

(−)-*Isopinocampheyl Acetate* (**30b**). $[\alpha]_D$ −38.7° (c = 1.0); IR: 1740, 1250 cm^{-1}; PMR: 0.97 (3H,s), 1.10 (3H,d, J = 7.0), 1.22 (3H,s), 2.02 (3H,s), 5.08 (1H,dt, J = 5.0, 9.0); MS: m/e 136 (M^+—CH_3CO_2H), 121, 93 (B^+); t_R: 5.6.

(+)-*Pinocampheyl Acetate* (**31b**). $[\alpha]_D$ +63.9° (c = 1.0); IR: 1740, 1250 cm^{-1}; PMR: 0.90 (3H,s), 0.95 (3H,d, J = 6.0), 1.03 (3H,s), 2.03 (3H,s), 4.73 (1H,dt, J = 7.0 9.5); MS: m/e 136 (M^+—CH_3CO_2H), 121, 93 (B^+); t_R: 5.2.

(−)-*Neopinocampheyl Acetate* (**32b**). $[\alpha]_D$ −12.8° (c = 0.7); IR: 1730, 1245, 1010 cm^{-1}; PMR: 0.82 (3H,s), 0.88 (3H,d, J = 7.0), 1.22 (3H,s), 2.00 (3H,s), 5.23 (1H,dt, J = 7.0, 1.0); MS: m/e 136 (M^+—CH_3CO_2H), 121, 107, 93 (B^+), 83; t_R: 6.9.

(+)-cis-*Pinocarveyl Acetate* (**33b**). $[\alpha]_D$ +50.0° (c = 1.0); IR: 1735, 1645, 1250, 905 cm^{-1}, PMR: 0.78 (3H,s), 1.25 (3H,s), 2.11 (3H,s), 4.78 (2H,bs), 5.67

(1H,m); MS: m/e 152 (M^+—CH_2CO), 134 (M^+—CH_3CO_2H), 119, 91 (B^+); t_R: 7.1.

(−)-trans-*Pinocarveyl Acetate* (**34b**). $[\alpha]_D$ +32.0° (c = 1.0); IR: 1735, 1645, 1245, 1010, 900 cm^{-1}; PMR: 0.70 (3H,s), 1.28 (3H,s), 2.03 (3H,s), 4.86 (1H,bs), 5.04 (1H,bs), 5.55 (1H,bd, J = 7.5); MS: m/e 152 (M^+—CH_2CO), 134 (M^+—CH_3CO_2H), 119, 91 (B^+); t_R: 6.4.

cis-*Isonopinyl Acetate* (**35b**). IR: 1725, 1250 cm^{-1}; PMR: 0.87 (3H,s), 1.23 (3H,s), 1.98 (3H,s), 5.17 (1H,m); MS: m/e 182 (M^+, $C_{11}H_{18}O_2$), 167 (M^+—CH_3), 140, 122 (M^+—CH_3CO_3H), 107, 93, 91, 85, 79 (B^+); t_R: 5.2.

trans-*Isonopinyl Acetate* (**36b**). IR: 1730, 1240 cm^{-1}; PMR: 0.80 (3H,s), 1.21 (3H,s), 1.43 (1H,d, J = 10.0), 2.01 (3H,s), 5.12 (1H,m); MS: m/e 182 (M^+, $C_{11}H_{18}O_2$) 167 (M^+—CH_3), 140, 122 (M^+—CH_3CO_2H), 107, 93, 85, 79 (B^+); t_R: 5.2.

Bioassay

Sample Preparation. For a solid compound (i.e., several of the alcohols), 1 mg of the compound was dissolved in 50 μl of *n*-hexane, and the total volume was absorbed onto filter paper (0.5 × 2.5 cm). The solvent was allowed to evaporate at room temperature. In the case of a liquid compound, 1 mg of the compound was directly impregnated onto the filter paper. The bioassay at the 1-mg dose was repeated five times for each compound. For a compound which exhibited sex pheromonal activity at the dosage level of 1 mg, 10 mg of the compound was dissolved in 1 ml of refined *n*-hexane. Serial dilutions of 50, 10, 5, and 2 μl which correspond to 0.5, 0.1, 0.05 and 0.02 mg of the compound, respectively, were dispensed onto different filter papers and subjected to our behavioral assay. This final procedure was performed just before the behavioral assay in a room separated from the bioassay room.

Assay Test. The details of our behavioral assay test were reported in a preceding paper (Nishino et al., 1980).

Males used for the assay were isolated from females for more than 1 month. A group of 25 males was housed in a plastic container (24 × 30 × 9 cm), and reared in a separate controlled-environment room which was maintained at 26° C and 40% relative humidity. The room was lighted for 7 hr (9:00 AM–4:00 PM), and darkened for 17 hr. The assay was initiated at 9:30 PM on Monday and Thursday under dimly lighted conditions in the rearing (assaying) room of the males.

Filter paper containing a quantity of a compound was placed in the container. The number of males displaying typical sexual behavior (Roth and Willis, 1952) near the sample source was counted within 3 min. None of the groups of males which showed typical sexual responses to a test compound were reused in the same evening.

RESULTS

In the bioassay of the analogs, the compounds listed in Table 1 induced the typical sexual response of the males at 1 mg and finally at the amounts in the table. All other analogs did not cause a response at the dosage level of 1 mg.

Cockroach Behavior. Behavior caused by the active analogs showed basically no difference from that induced by the natural pheromone except for an induction period and the duration of the behavior.

A general description of the behavior elicited by the pheromone mimics is as follows: 10–30 sec after exposure to a sample, most of the males in the shelters showed interest in vapor from the sample by waving their antennae. Movement gradually became rapid in their shelters, and then a few individuals rushed out from the shelters to the sample source. Upon arrival on the source, they extended their abdomens and raised and vibrated the wings. In some cases, these sexual displays were observed before they reached the sample. This excitement of a few was immediately propagated to the other cockroaches. Near the sample, they ran about, vigorously raising and vibrating the wings, and moved backward to the sample or to the other males with the abdomen extended in precopulatory display. Quite often, homosexual copulations were attempted (Figure 3). Such sexual behavior persisted for 5–10 min even after the sample was eliminated from the testing cage.

Since formate **2c** was much weaker than the other mimics, **2b, 2d** and **20b,** in pheromonal activity (Table 1), the behavior elicited by **2c** was different from that elicited by the other compounds. The formate **2c** required 1.5–2 min after the sample exposure to induce the initial rushing-out response, whereas the other esters required 1 min at the most. The males excited by **2b, 2d,** and **20b** were always on or quite near the sample source, while the males exposed to **2c** were excited at a distance from the source rather than attracted to it. In

TABLE 1. SEX PHEROMONAL ACTIVITY OF ACTIVE ANALOGS TOWARD AMERICAN COCKROACH

Compound	Amount (mg)	Number of repetitions	Activity[a]
(+)-*trans*-Verbenyl acetate (**2b**)	0.02	20	9
(+)-*trans*-Verbenyl formate (**2c**)	0.5	8	5
(+)-*trans*-Verbenyl propionate (**2d**)	0.02	20	15
(+)-Verbanyl acetate (**20b**)	0.02	20	15

[a]Average number of cockroaches showing typical sexual display (Roth and Willis, 1952) within 3 min in a group containing 25 males.

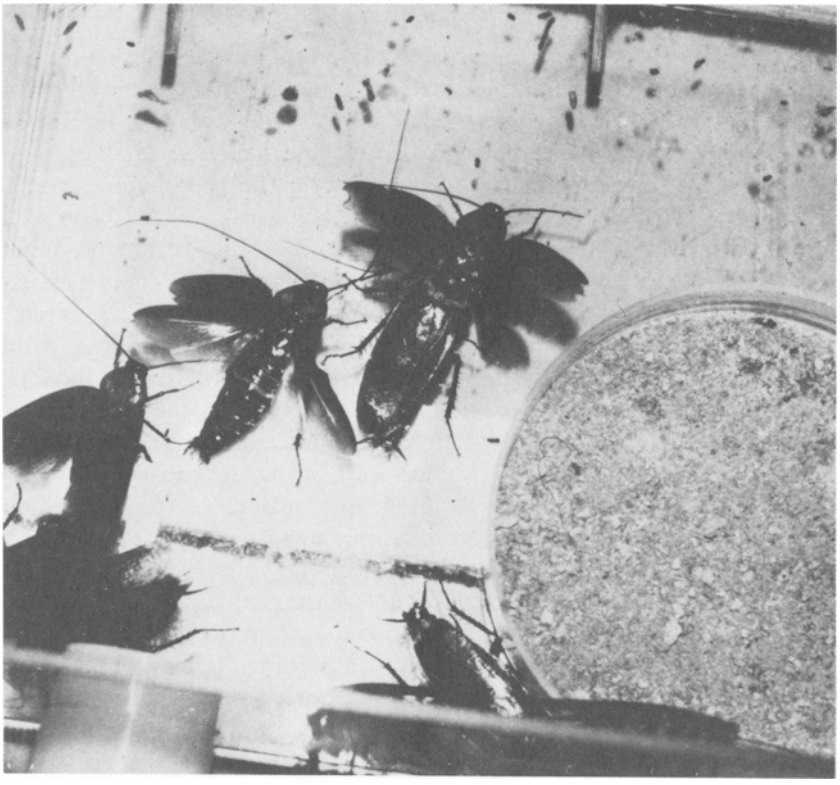


FIG. 3. Typical sexual display of males of the American cockroach induced by 0.02 mg of (+)-verbanyl acetate (**20b**).

other words, the attractivity of **2c** was less than that of the other mimics. Duration of pheromonal activity of **2b, 2d,** and **20b** after elimination of the sample was observed for 5–10 min. However, such aftereffects remained for only 1–2 min in **2c**.

DISCUSSION

(+)-Bornyl acetate, found by Bowers and Bodenstein (1971), was called a "sex pheromone mimic" because of its sex pheromonal activity toward the male of the American cockroach. The activity of this mimic (7×10^{-2} mg) (Bowers and Bodenstein, 1971) was, however, many orders of magnitude lower than that of the natural sex pheromone, periplanone-B (10^{-8} mg) (Persoons, 1977; Still, 1979; Adams et al., 1979), as were the active compounds in the present work (2×10^{-2} mg).

To account for the great discrepancy of the activities among the mimics and the pheromone, two interpretations may be possible: (1) The male cockroaches possess a special receptor responsible for the mimics, which is different from the sex pheromone receptor. (2) Both the mimics and the pheromone are received by the sex pheromone receptor, but only few of active sites of the receptor are responsible for the mimics, whereas all of the sites are sensitive to the pheromone.

If key structural factors for sex pheromonal activity implicit in the mimics could be correlated with those in the pheromone, key points for the above interpretations might be revealed. Hence, we attempt here to elucidate the factors in (+)-*trans*-verbenyl acetate **2**.

Our interest was first directed to the ethyl ethers, **5** and **6**, since the arrangement of atoms in one of the side chains of these ethers, —O—C—C, is the same as that of **2b**. The ethers were, however, inactive as were the methyl ethers, **3** and **4**, and all of the alcohols. On the other hand, esterification of several *trans* alcohols produced remarkable activities (Table 1), but all *cis* esters were inactive. Thus, an ester group possessing *trans* configuration has a critical effect on pheromonal activity.

Kikuchi and Ogura (1976, and references cited therein) suggested, in their "functional units theory" of pheromones, the presence of proton acceptors (OH, CO and π bond) and donors (OH) in pheromones. From the above results, the carbonyl oxygen atom in the acetoxyl group of **2b** was expected to be the most reactive site for accepting a proton in the receptor, while the alcoholic oxygen in **2b** has no function as the acceptor; however, the inactivity of the *trans*-methyl ketone **14** which corresponds to **2b** without the alcoholic oxygen atom implies the importance of a complete ester linkage.

When the sequence of atoms in the ester group exchanged, for example, an acetoxyl (—O—CO—CH_3, **2b**) for a methyl ester (—CO—O—CH_3, **10**), the activity was completely eliminated. The addition of a methylene group to the acetoxyl group of **2b** (—CH_2—O—CO—CH_3, **12b**) also caused loss of the activity. From these facts, it seems important that the alcoholic oxygen atom of an ester group be directly linked to the pinane skeleton.

The size of the alkyl group of the ester moiety influences the pheromonal activity. The activity increased according to size from H (**2c**) to CH_2CH_3 (**2d**). However, butyrate **2e**, possessing excess bulkiness ($CH_2CH_2CH_3$) for the receptor space, caused complete loss of the activity.

Elimination of the olefinic methyl group from **2b** (compound **16b**) brought about loss of activity. This methyl was assumed to serve as a prop for settling the molecule of **2b** in the receptor space. On the other hand, since the saturated compound **20b** showed a good activity, the double bond of **2b** is presumably not necessary for the activity.

The precise interrelation among the important chemical functions revealed here must be significant, as suggested in the case of the bark beetle

aggregation pheromones (Kikuchi and Ogura, 1976). Only *trans* esters possessing a 1 (C-2):3 (C-4) relationship between the single methyl and the ester group were active. Nevertheless **18b**, which is a configurational isomer of the active **20b** for the C-2 methyl group, was inactive, although this compound maintains the desired 1:3 relationship. Thus, delicate changes of configuration and conformation (Nishino and Takayanagi, 1979c) affect the pheromonal activity critically and also suggests the importance of precise relationships among the key functions for the pheromonal activity.

The significance of the length of the C-2 alkyl group will be a next target of our investigation.

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COMPARATIVE DISTRIBUTION AND PERSISTENCE OF DISPARLURE IN WOODLAND AIR AFTER AERIAL APPLICATION OF THREE CONTROLLED-RELEASE FORMULATIONS

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Abstract—Disparlure, the gypsy moth sex pheromone, was aerially applied to three plots, using a different controlled-release formulation—gelatin microcapsules, flakes of plastic laminates, or hollow fibers—in each plot. Disparlure concentrations in air were measured intermittently over a 34-day period after application. Measurements were made at four heights above ground: 0.3, 2, 5, and 10 m. Wind speed and air temperature were measured concurrently. Disparlure was emitted from the microcapsules and fibers at very high levels for the first day or two; thereafter, all three formulations produced gradually decreasing aerial concentrations. After 32–34 days, concentrations in all plots ranged from 1.5% to 15.5% of those on the first day, although most of the pheromone remained in the formulations. Results suggest that the effective life of all three formulations for mating disruption by atmospheric permeation would be near 30 days under the test conditions, with fibers likely to have the shortest life. Concentrations varied diurnally throughout the experiment, with highest levels generally between 1400 and 2200 hr each day. Concentrations were highest at the 10-m height with microcapsules and at the 0.3- and 10-m heights with flakes, but were similar at all heights with fibers. Biological implications of the results are presented.

Key Words—Disparlure, gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, controlled release, laminated dispensers, hollow fibers, microcapsules, atmospheric permeation, mating disruption, air concentration.

INTRODUCTION

Controlled-release formulations are used to dispense synthetic sex pheromones in field experiments directed to the control of lepidopteran insects by

the atmospheric permeation technique. It is widely recognized that, to optimize the technique, the performance of these formulations should be evaluated in the field or forest rather than in the laboratory. Only by field examination of the emission rates of the formulations, their comparative longevity, the effects of weather, and the spatial distribution of the particles can a rational choice of formulation be made (Granett, 1976). For aerial applications over forests, it is essential to evaluate the evenness of application throughout the depth under the canopy (Cardé, 1976).

In 1976, we conducted an experiment with a single microencapsulated formulation to measure the release and dissipation in the forest of synthetic disparlure, the racemic form of the sex attractant of the gypsy moth, *Lymantria dispar* (L.) (Plimmer et al., 1978). We report here the results of a similar experiment, in which the performances of three test formulations of disparlure were compared.

METHODS AND MATERIALS

Microcapsules. This formulation was manufactured by Capsular Products, Appleton Paper Co., Dayton, Ohio, and, except for particle size, was no different from that used in our 1976 experiment (Plimmer et al., 1978). It consisted of an aqueous suspension of 20% by weight of gelatin-walled microcapsules, 20–60 μm in diameter, and contained hydroxyethylcellulose as thickener. The capsules contained a 2.2% solution of disparlure in 3:1 xylene–amyl acetate. Because the gelatin walls comprised 10% of the weight of the capsules, the disparlure concentration in the total capsules was 2.0%. A sticker (RA-1645, Monsanto Company, Indian Orchard, Massachusetts) was added (1%) to aid adhesion of the formulation to foliage.

With microcapsules, pheromone emission rates depend on controllable factors such as wall composition, wall thickness, pheromone concentration, solvent, particle size, and other ingredients of the formulation (including the adhesive coating), and on uncontrollable variables such as temperature, wind, and humidity (Plimmer and Inscoe, 1979). Theoretically, release rates from microcapsules should decrease exponentially as the residual pheromone concentration decreases, but in practice, a "burst" of pheromone is often observed in the first hours after application in the field, followed by decrease in the slope of release rate vs. time (Plimmer et al., 1977, 1978). The burst occurs even though only a small proportion of the pheromone is actually released, and has been attributed to loss of pheromone from capsule walls that had become saturated during storage of the formulation. After some time in the field, microcapsules frequently stop emitting pheromones even though a substantial amount of the chemical remains in the formulation (Plimmer et al., 1977; Caro et al., 1977). Despite these shortcomings, atmospheric permeation experiments with microcapsules have given generally encouraging results.

Laminate Flakes. Three-layer plastic laminates for controlled release of volatile chemicals have been produced by the Herculite Corporation, New York, for some years, but size reduction of the laminates to sprayable flakes is a relatively recent innovation of the manufacturer. The flakes used in our experiment consisted of two layers of vinyl, each 0.08 mm (3 mils) thick, sandwiching a central porous layer containing the disparlure. Nominal concentration of disparlure in the flakes was 9.1%. Flakes were flat and irregular, ranging in surface area (one side) roughly from 7 to 35 mm^2. The formulation for spraying was a 1 : 1 : 1 mixture, by volume, of a sticker (RA-1645, as in the microcapsule formulation), a 1% aqueous solution of hydroxyethylcellulose thickening agent, and the flakes.

The emission rate from plastic laminates is readily controlled by regulating the thickness of the layers, the concentration of chemical in the middle-layer reservoir, and the stiffness of the plastic membranes (Kydonieus, 1977). Emission rate is also affected by temperature, wind speed, and additives in the formulation. The pheromone is emitted not only by diffusion through the membranes but also from the perimeter edges of the individual pieces (Bierl-Leonhardt et al., 1979), a particularly important consideration with small particles such as flakes. Theoretically, the laminates should exhibit zero-order release, and in fact the emission of disparlure under controlled conditions has been found to be relatively constant over long periods (Bierl et al., 1976). Ease of handling and controllable release characteristics have generated considerable interest in these dispensers, and they have been used experimentally not only against the gypsy moth but also against the pink bollworm, Oriental fruit moth, and boll weevil, among others (Kydonieus, 1977).

Hollow Fibers. Controlled-release hollow-fiber formulations are manufactured by FRL, an Albany International Company, at Dedham, Massachusetts, under the Conrel trademark. The fibers consist of short lengths of thermoplastic tubing, sealed at one end and filled with liquid pheromone. The plastic material is impermeable and nonreactive with the pheromone. The length of open column in the fibers used in our experiment ranged from 8 to 10 mm and the diameter was 0.15 mm (6 mils). Active ingredient was nominally 11.5% by weight of the fiber. The formulation as dispensed was made up of 2 parts by volume of a sticker ("Biotac") and 1 part of fibers. When mixed, it had the consistency of a highly viscous syrup.

Pheromone release from hollow fibers involves three basic processes: evaporation at the liquid–air interface, diffusion through the air column to the open end of the fiber, and convection away from the end (Ashare et al., 1976). If the liquid level is at least a few millimeters from the open end, diffusion controls the emission rate. Theoretically, emission rate is not constant, but will steadily decrease from an initial high value as the tube empties of pheromone. Release rate also depends on temperature, about doubling for

each 18° C rise. The biologically effective life of the formulation can be controlled by adjusting the length of fiber filled. The promise of hollow fibers as pheromone dispensers is evidenced by the fact that experiments have been conducted on over 20 insect species (Ashare et al., 1976).

Plots. The three treatment plots were located in woodland in the northeastern corner of the Beltsville Agricultural Research Center. Trees were 12–15 m high, in a dense stand of primarily deciduous varieties, with some evergreens intermixed. Each plot was a 4-hectare square (200 m × 200 m). Plot A (microcapsule treatment) had more abundant underbrush than the other two plots. Plot A was 300 m from plot B (flake treatment) at the point of least separation, and plot C (fiber treatment) was 1200 m beyond plot B in the opposite direction.

Pheromone Application. All formulations were applied by fixed-wing aircraft, with multiple passes over the treatment plots to produce as uniform a distribution as possible. Each formulation required a specially designed delivery system mounted on the aircraft. Spraying of the microcapsules on plot A required 1.5 hr of flying time: from 1530 to 1630 hr EDT on September 11, 1979, and from 0730 to 0800 hr on September 12. The flakes were applied to plot B in 4 hr: from 1500 to 1900 hr on September 12. The application of the fibers on plot C, using a second plane, was difficult and slow; spraying was discontinued after 9 hr of flying (1400–1600 hr on September 11 and 1400–2100 hr on September 13), at which time only about 2/3 of the fibers had been applied. Weather during spraying remained clear and warm until September 13, which was cooler and with a light intermittent drizzle during the afternoon.

Rate of disparlure application on plots A and B was 500 g ai/hectare and that of the fiber application on plot C was 330 g ai/hectare, as determined by analysis of the unsprayed formulation. These rates, intentionally high to permit disparlure analysis in air for an extended period, were about 25 and 17 times that used normally for insect mating disruption, ca. 20 g/hectare (Beroza, 1976).

Air Sampling. Portable generators on each plot supplied power for the air-sampling pumps and meteorological equipment, the latter on plot B only. Air samplers were 10-cm × 1.6-cm glass tubes, drawn down at one end for connection by flexible tubing to a pump. Each tube contained a 4-cm-deep bed (ca. 6 g) of pelletized type 4A molecular sieves (14–30 mesh) to adsorb the disparlure. The bed of sieves was held in place by wads of glass wool at both ends. For sampling, air was drawn at a measured rate (ca. 1–2 m^3/hr) through the samplers, which were fixed in a vertical, inverted position for protection from rain, for a predetermined 4- or 8-hr period. At the end of a given sampling period, the samplers were replaced with fresh tubes and capped for later analysis of the adsorbent.

A tree near the center of each plot was equipped with a system of pulleys to raise and lower a set of four samplers; these were kept at 0.3-, 2-, 5-, and 10-m heights for sampling. On each plot, sampling was begun as soon as possible after completion of the spraying (September 12–14) and continued for at least 32 hr. Sampling was also conducted on all three plots for 24-hr periods on September 17–18, September 24–25, October 4–5, and October 15–16, in each case beginning at 1000 hr and ending at 1000 hr the next morning. Throughout the experiment, sampling periods were ended at 0600, 1000, 1400, 1800, and 2200 hr, providing four 4-hr periods and one 8-hr period (overnight) for each 24 hr of sampling. Several samples were lost during the experiment because of generator failure or tubing disconnection.

Formulation Sampling and Analysis. After application, samples of the flakes and fibers were collected manually at random from leaves about 1 m above ground throughout wide areas of the plots for residual disparlure analysis. Collections were made within a day of application and on four subsequent days during the following 34-day period. Microcapsules could not be collected because they were not readily visible on the leaves.

For analysis, the disparlure in the flakes and fibers was extracted by shaking the formulations in hexane, and extract concentrations were determined by flame-ionization gas chromatography.

Chemical Analysis of Air Samples. All samples of molecular sieves were analyzed for disparlure content within 3½ months after collection. Disparlure was determined by the method of Caro et al. (1978), slightly modified to improve cleanup and recovery. The sieves were extracted with 9:1 hexane–acetone, and the *vic*-dibromide derivative of disparlure was formed in the extracts by reaction with triphenylphosphine dibromide. The solution containing the derivative was cleaned up by passage through a bed of Florisil® with hexane as eluent, then was analyzed by gas chromatography with a ^{63}Ni electron-capture detector. Results were corrected for recovery, using a curve of recovery vs. sample storage time that had been determined from analysis of 12 sets of duplicate fortified samples. This curve showed that recovery ranged from over 90% for 1-day storage down to 70% for storage exceeding 2 months.

Meteorological Measurements and Weather. To measure wind speeds under the woodland canopy, two rotating-cup anemometers were mounted in plot B at the 1.5-m and 5.5-m heights on a mast about 10 m from the air samplers. A recording thermograph placed on the ground nearby continuously recorded air temperature throughout the experiment.

After disparlure application, weather was generally cool (temperature range 2–27° C, but mostly 15–20° C), with intermittent periods of rain. These occurred on September 14 (heavy shower, 1600–1630 hr), September 21–22 (36-hr steady rain), September 30 (light rain all day), October 1 (steady rain all day), October 2 (heavy rain in evening), October 5 (rain in afternoon),

October 9 (rain all day), and October 10–12 (periods of rain and some snow throughout).

RESULTS AND DISCUSSION

A total of 332 air samples was generated in this experiment and subjected to disparlure analysis. Results are summarized in the tables and figures, which have been selected to illustrate specific conclusions.

Temporal Changes in Disparlure Concentrations. Aerial concentrations as a function of time are shown for the three test plots in Figure 1. Each point on the curves represents an average of all time periods and all heights for a particular sampling day. The figure clearly illustrates an early "burst" of airborne disparlure from the microcapsules and fibers, paralleling the behavior of the formulations known from direct measurement of emission rates. The laminated flakes produced an entirely different pattern; concentrations were initially low and did not peak until the second day after application. After the first 5 or 6 days, concentrations gradually decreased on all three plots, with the rate of decrease from the hollow fibers being slightly greater than from the others.

The curves show that the air in the hollow-fiber plot had higher disparlure concentrations than that in the other two plots for about 8 days after application, even though the rate of application of the fibers was only

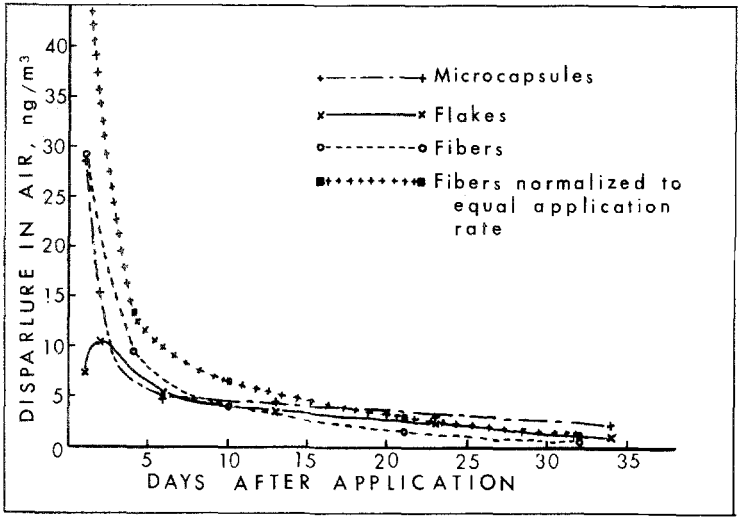

FIG. 1. Disparlure concentrations in air during the first month after application.

about 2/3 that of the others. If the concentrations from fibers are normalized to a disparlure application rate of 500 g/hectare, the curve illustrated by the crosshatched line in Figure 1 results. This shows that, for equal applications of the three formulations, hollow fibers would supply the highest levels of disparlure to the air for about 18 days after application.

Persistence of Emission from Formulations. A comparison of disparlure concentrations at each of the sampling heights on the first day after application and on the last sampling day about a month later is shown in Table 1. The primary point of interest here is the extent to which the concentrations declined within the month. The later concentrations averaged 7.3, 15.5, and 1.5% of those on the first day for the microcapsules, flakes, and fibers, respectively. These values stand in contrast to the analytical results for flakes and fibers collected by hand on the plots: the flakes contained 7.1% disparlure shortly after spraying and 6.0% after 30 days in the field, and the fibers contained 5.0% disparlure initially and 3.8% 30 days later. In both cases, therefore, most of the disparlure remained in the formulation, yet emission rates (as reflected by aerial concentrations) had decreased to very low levels. The microcapsules exhibited the same characteristic when we used them in an earlier field experiment (Caro et al., 1977).

TABLE 1. AVERAGE[a] AERIAL DISPARLURE CONCENTRATIONS ON FIRST AND LAST DAY OF SAMPLING[b]

Plot and formulation	Height above ground (m)	Disparlure concentration in air (ng/m^3)	
		Sept. 12–13[c,d]	Oct. 15–16
A, microcapsules	10	36	2.5
	5	29	2.0
	2	27	2.2
	0.3	22	1.6
B, flakes	10	7.3	1.2
	5	6.9	1.2
	2	6.5	1.2
	0.3	8.2	0.8
C, fibers	10	28	0.5
	5	26	0.4
	2	28	0.4
	0.3	34	0.4

[a] Average of five sampling periods, 1000 EDT to 1000 EDT.
[b] Approximate average air temperature, 19° C on first sampling days and 11° C on last day.
[c] 1900 EDT to 1800 EDT, 23-hr total, on plot B.
[d] September 14–15 on plot C.

The reasons for the early slowdown in emission from these formulations—earlier than that which would be predicted on theoretical grounds even with due allowance for temperature differences—are obscure. One might speculate that superficial weathering of formulation surfaces decreased permeability or that rapid loss of solvent left too little solvent to carry the pheromone through the walls of the formulation, but these would not apply to the open-end fibers. The latter would, however, be subject to blockage by dust particles. It is evident that detailed study of the field weathering of these formulations is required before their performance can be improved significantly.

The measured concentrations one month after application (Table 1) also yield information to gauge how long the formulations would be effective for insect mating disruption by the atmospheric permeation technique. The disparlure application rate in the experiment was intentionally very high, 25-fold that of the normal rate used in mating disruption of the gypsy moth (500 g/hectare vs. 20 g/hectare). With the hollow fibers, the 330-g/hectare rate was about 17 times higher than normal. Applications at the normal rate would be identical to those used in this experiment, except that fewer particles of the formulations would be distributed. Consequently, measured aerial concentrations can be feasibly scaled down to normal rates, and show that, 30 days after disparlure application at 20 g/hectare, aerial concentrations would average 0.08, 0.04, and 0.03 ng/m^3 for microcapsules, flakes, and fibers, respectively. These are of the same order of magnitude as the reported threshold concentrations for response of many species of insects to pheromones (Caro, 1980). It is likely, then, that 30 days is about the length of time that these formulations could be expected to produce adequate mating disruption under the conditions of these tests. In warmer midsummer weather, effective lifespans might be somewhat different. The present finding, however, agrees with our earlier projection for effective life of plastic laminate formulations containing (*Z*)-9-tetradecen-1-ol formate, a mating disruptant of *Heliothis* spp. (Caro et al., 1980).

First-Day Concentrations. Aerial disparlure concentrations during the first day after application, averages of which are plotted at the left in Figure 1, are shown in detail in Table 2. The periods during which concentrations were exceptionally high in both the microcapsules and fibers plots are apparent in the table, as is the quite different behavior of the laminated flakes. There is a strong dependence of these early values on the time of day, reflecting the diurnal fluctuations in meteorological parameters, rather than on the number of hours after application. In the fibers plot, for example, spraying ended at dark and air sampling was not begun until the following morning, yet an afternoon surge in aerial concentrations was observed just as in the microcapsule plot, where spraying ended in the morning and air was sampled the same afternoon. Statistical analysis of the data (Table 2) identifies the significant diurnal differences.

TABLE 2. AERIAL DISPARLURE CONCENTRATIONS DURING FIRST 24 HR OF SAMPLING[a]

Plot and formulation	Date 1979	Time Period (EDT)	Disparlure concentration in air (ng/m³) Height above ground (m) 0.3	2	5	10	Mean[b]
A, microcapsules	Sept. 12–13	10–1400	17	21	25	33	24.0 b
		14–1800	38	37	43	57	43.8 a
		18–2200	32	45	51	55	45.8 a
		22–0600	11	18	15	22	16.5 c
		06–1000	11	14	10	14	12.3 c
B, flakes	Sept. 12–13	19–2200	10.	5.6	8.9	11.	8.9 a
		22–0600	6.1	5.4	8.5	6.4	6.6 ab
		06–1000	4.6	4.7	3.8	5.7	4.7 b
		10–1400	10.	6.7	6.4	7.2	7.6 a
		14–1800	10.	10.	6.8	6.3	8.3 a
C, fibers	Sept. 14–15	10–1400	25	26	34	41	31.5 b
		14–1800	105	78	62	60	76.3 a
		18–2200	23	21	19	24	21.8 bc
		22–0600	11	7.5	8.8	10	9.3 c
		06–1000	4.7	5.8	5.9	6.2	5.7 c

[a] Air sampling began at conclusion of spraying on plots A and B and 13 hr after spraying on plot C.
[b] Within individual plots, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple range test.

The heavy shower that occurred during the afternoon of September 14 apparently did not stifle the pheromone emission from the hollow fibers for very long. Aerial disparlure concentrations during that period were, in fact, higher than at any other time in the experiment. However, concentrations did decrease dramatically in succeeding time periods as the air temperature dropped from almost 27°C at 1500 hr to 12°C at 0700 hr the following morning.

General Diurnal Variations. Full 24-hr sampling was conducted, with no missing data, on September 17 and October 15, 1979, beginning at 1000 hr each day. This permitted definitive statistical analysis of diurnal variations in aerial disparlure concentrations, with results shown in Table 3.

The diurnal variations on the first day after spraying of the formulations (Table 2) essentially continued throughout the experiment. In general, concentrations were highest in the afternoon and early evening, a pattern that does not parallel those of the two dominant meteorological factors, air temperature and wind speed. Averages of these are shown in Table 4 and Figure 2 for those sampling days during which full sets of the respective data were collected. The measurements indicate that both variables were consis-

TABLE 3. DIURNAL VARIATION IN AERIAL DISPARLURE CONCENTRATIONS ON TWO SAMPLING DAYS

Time period (hr EDT)	Average[a] disparlure concentrations (ng/m^3)							
	September 17–18				October 15–16			
	Microcaps	Flakes	Fibers	Mean[b]	Microcaps	Flakes	Fibers	Mean[b]
10–1400	6.15	4.45	10.38	6.99 b	0.83	0.53	0.43	0.60 c
14–1800	7.85	7.80	9.73	8.46 a	2.70	1.43	0.53	1.55 a
18–2200	5.05	7.68	15.50	9.41 a	2.63	1.35	0.40	1.46 a
22–0600	3.10	3.83	5.48	4.14 c	2.53	1.15	0.43	1.37 a
06–1000	2.40	2.50	5.70	3.53 c	1.63	0.98	0.33	0.98 b
Means	4.91	5.25	9.36		2.06	1.09	0.42	

[a]Averages of the four sampling heights.
[b]Means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple range test.

TABLE 4. AVERAGE AIR TEMPERATURES DURING FOUR AIR SAMPLING DAYS[a]

Time period (EDT)	Average air temperature (°C)				
	Sept. 17–18	Sept. 24–25	Oct. 4–5	Oct. 15–16	Mean
10–1400	22.2	17.8	21.1	13.3	18.6
14–1800	19.1	17.5	19.7	13.9	17.6
18–2200	13.9	14.4	17.5	8.6	13.6
22–0600	10.5	13.9	17.2	7.2	12.2
06–1000	13.6	15.4	17.5	9.9	14.1

[a]As measured by thermograph on plot B.

tently highest at midday and that both decreased sharply after 1800 hr. In terms of effect on pheromone vapor concentrations, temperature and wind speed offset each other because emission rate rises with temperature, but the vapors dissipate more rapidly in the greater air turbulence produced by increased winds. The diurnal changes in aerial concentrations shown in Table 3 are apparently the product of the interaction between wind speed and temperature, as modified by other environmental factors such as relative humidity or radiant flux density. Detailed analysis of the contributing factors is a subject for further study.

Distribution under Woodland Canopy. Since concentrations of vapors emanating in a plume from a steadily emitting source are highest in the

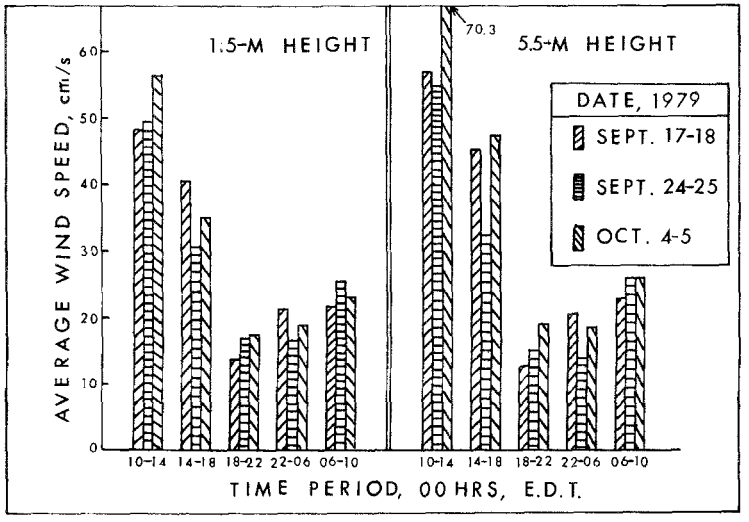

FIG. 2. Average wind speeds on three air sampling days.

TABLE 5. VARIATION IN AERIAL DISPARLURE CONCENTRATIONS WITH HEIGHT ABOVE GROUND

Height above ground (m)	Average disparlure concentration in air[a] (ng/m^3)		
	Plot A, microcapsules	Plot B, flakes	Plot C, fibers
10	13.6 a	5.2 ab	9.8 a
5	10.8 b	4.4 c	8.7 a
2	9.9 b	4.7 bc	8.7 a
0.3	9.4 b	5.5 a	10.6 a
Number of time periods measured	26	27	25

[a]Within single plots, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple range test.

horizontal plane (Aylor, 1976), aerial pheromone concentrations measured at various levels under the canopy will reflect the vertical distribution of the sources. Such measurements made throughout the experiment are shown in Table 5 for each of the formulations applied, along with statistical analysis of the data.

The results show that the hollow fibers, in spite of the difficulty in application, were uniformly distributed throughout the canopy space. By contrast, the microcapsules were deposited in significantly greater numbers at the upper levels of the foliage and in decreasing numbers with approach to the ground, and the laminated flakes were concentrated at both the top of the canopy and near the ground.

The result with the microcapsules is of particular interest because the distribution in this experiment was unlike that in our earlier experiment with microencapsulated disparlure (Plimmer et al., 1978). There, disparlure vapor concentrations decreased with height above ground, whereas the reverse is true in this experiment. The difference is most likely a reflection of canopy density of the forests; both tree stand and foliage density per tree were greater in this later experiment, so the probability that the trajectory of a sprayed microcapsule would intersect a vegetative element was greater. The contrast in the two experiments suggests that the distribution of controlled-release pheromone formulations resulting from an aerial application will very much depend upon the physical characteristics of the area being treated.

CONCLUSIONS

In general, after the first few days after application, the three controlled-release formulations did not differ greatly in their behavior in the field. All

exhibited very low pheromone emission rates after a month or more of exposure, even though most of the applied pheromone was retained in the formulations. In terms of duration of effectiveness, the measurements indicated that, at equal application rates, the hollow fibers would be slightly less persistent than the other formulations. The typical, very high levels of vaporized pheromone occurring in the first days after application of microcapsules or fibers probably have little biological significance because the effect dissipates relatively rapidly.

The general diurnal pattern in aerial disparlure concentrations did not correspond to any single meteorological parameter, but rather reflected a complex of influences. The pattern does, however, match fairly well the requirements for disrupting gypsy moth mating, which is most intense at midday and during the afternoon (Plimmer et al., 1977). Moreover, the gypsy moth flies close to the ground most of the time (Doane, 1976), so that high pheromone vapor concentrations near the ground are favorable for disruption of communication. Our results show that microcapsules sprayed over a very dense canopy would lodge to a large extent among the upper branches of the trees. Consequently, microcapsules in such cases would not be efficiently distributed within the forest canopy.

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ATTRACTION AND REPULSION OF THE APHID, *Cavariella aegopodii*, BY PLANT ODORS

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Abstract—*Cavariella aegopodii* is induced to land on traps by the monoterpene carvone, and the relevance of this to host-finding by the aphid is discussed. Catches are reduced by linalool. The interaction of plant chemicals in natural communities is discussed, and the possibility of using repellent chemicals for crop protection is suggested.

Key Words—Aphid, *Cavariella aegopodii*, Homoptera, Aphididae, plant odor, carvone, attractant, repellent, host-finding.

INTRODUCTION

The aphid, *Cavariella aegopodii* (Scopoli), is common in Britain, occurring on various species of Umbelliferae in the summer and on willows in winter (Dunn, 1965; Dunn and Kirkley, 1966). In this paper we describe the effects of various plant odors, from both host and nonhost plants, on the response of the aphid to water traps.

METHODS AND MATERIALS

The experiments were carried out at Porton Down, Wiltshire, in an area of chalk grassland, about 10 hectares in area, surrounded by beech woodlands, during late May and early June 1979 and 1980. At this time of year the insect is normally moving from its winter host, willow trees, onto various Umbelliferae. There are no willow trees within at least 2 km of the study site. At the site wild parsnip, *Pastinaca sativa*, was in the early stages of vegetative growth in early June; at that time it was already colonized by *C. aegopodii*. The only other umbel present, *Sanicula europaea*, was in flower, but had no aphids. Along

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the hedgerows, within about 1 km of the site, cow parsley, *Anthriscus sativus*, was abundant with a high population of *C. aegopodii* by early June.

The water traps used were smaller versions of the type described by Finch and Skinner (1974), each consisting essentially of a Petri dish 10 cm in diameter mounted on a stout vertical wire 25 cm above the soil surface and with a 5 × 1-cm specimen tube containing the chemical in the center. The chemical evaporated from a dental roll wick projecting 1 cm above the top of the tube. At the start of the experiment each tube was filled with the appropriate chemical to about 1 cm from the top and a rough measure of evaporation rate was obtained by estimating the height of the chemical remaining at the end of the experiment. Most experiments lasted one or two days and only in the case of allyl isothiocyanate was the rate of evaporation so high that it was necessary to refill the tubes. Where combinations of two chemicals were tested, two tubes were placed at the center of the trap; control dishes contained only an empty tube. Great care was taken to avoid cross-contamination of the tubes or dishes with different chemicals. The traps contained water with a few drops of detergent. They were usually painted yellow, but in a few cases uncolored (transparent) or black-painted traps were used.

Traps were arranged in a series of circles with 10 m between adjacent traps. In most cases each circle contained ten traps, two for each of five treatments, and there were five circles separated from each other by at least 30 m. The overall arrangement of traps was such that each trap treatment was adjacent to a treatment similar to itself and also to each of the other trap treatments the same number of times. In this way any possible bias due to interaction between traps was equalized between treatments. The arrangement also ensured that each treatment was in an upwind position in one of the circles irrespective of which way the wind was blowing.

Some experiments were carried out with sticky traps arranged around an odor-releasing tube mounted on a wire without any obvious visual marker. Each sticky trap consisted of two Perspex sheets, 25 cm long by 10 cm deep, fixed at right angles to each other in the form of a cross. Four traps were placed around each odor source, to the north, east, south, and west, with a quadrant facing in each of these directions. The bottom of the trap was 25 cm above the soil surface. Each surface of the trap was coated with tanglefoot and the *C. aegopodii* were collected from them at varying intervals. The traps were placed 0.5 or 1.2 m from the odor source with four sets, two odorless controls and two with odors, separated from each other by 10 m.

Wind speed and direction were recorded with a Porton anemometer and wind vane mounted 1 m above the ground.

The carvone used in 1980 was obtained from Koch Light. Both the (+)-carvone and (−)-carvone were free of contamination by the other isomer. The (−)-carvone used in 1979 was less pure.

RESULTS

Large numbers of *Cavariella aegopodii* were collected in yellow water traps baited with (−)-carvone; the numbers collected in traps baited with linolool, eugenol, and allyl isothiocyanate were not significantly different from the numbers in unbaited control traps (Table 1). Small numbers of *Ovatus crataegarius* were also obtained fairly frequently in the carvone-baited traps. There was no difference in the numbers of *C. aegopodii* collected in traps containing (+)-carvone and (−)-carvone (Table 2).

A circle of colorless traps was set up adjacent to one circle of yellow traps. Similar low numbers of aphids were caught in unbaited traps irrespective of color; carvone enhanced the catch of both colorless and yellow traps, but to a much greater extent with the latter (Table 3).

Only one aphid was caught in five baited black traps over a 48-hr period, although the insects were abundant in nearby baited yellow traps.

In the experiments with sticky traps it is probable that the traps themselves provided a visual target while the odor source in a specimen tube was visually insignificant. The visual target was thus independent of the odor source, unlike the water traps where the two were combined. There was clear evidence of a response to the odor with the traps 0.5 m and 1.2 m from the odor

TABLE 1. TOTAL NUMBERS OF *Cavariella aegopodii* CAUGHT IN YELLOW WATER TRAPS BAITED WITH ODOR OF CARVONE AND OTHER CHEMICALS[a]

	Date				
Chemical	June 21–22, 1979	May 30–June 2, 1980	June 8, 1980	June 9, 1980	June 9, 1980 (*O. crataegarius*)
Control	56	2	5	14	0
Carvone	1120	28	270	256	0
Linalool	24[1]				
Eugenol	90[1]				
Allyl isothiocyanate	36[1]				
Carvone + linalool		12[2]	75[3]		
Carvone + allyl isothiocyanate		22[2]			
Carvone + coumarin		32[2]			
Carvone + eucalyptol			236[2]		
Carvone + eugenol			309[2]		
Carvone + citral				163[2]	2
Carvone + nicotine				282[2]	8
Carvone + lauric acid ethylester				346[2]	5

[a]The statistical significances of the differences observed on different occasions has been examined using analysis of variance. 1, No significant difference compared with control; 2, no significant difference compared with carvone alone; 3, significant reduction compared with carvone alone.

TABLE 2. NUMBERS OF *C. aegopodii* COLLECTED IN YELLOW WATER TRAPS BAITED WITH (+)- AND (−)-CARVONE

	Number of traps/treatment	Total number in traps		
		Control	(+)-Carvone	(−)-Carvone
May 24–26	9	3	19	15
June 8–9	5	—	70	63

source (Table 4). Most insects were caught on the sticky traps at times when the wind speed was low (Figure 1). The positions of the traps relative to the odor source generally had no effect on the number caught, but greater numbers tended to collect on the predominantly downwind sectors of the traps irrespective of whether these were 0.5 m or 1.2 m from the odor source (Figure 2).

The carvone-baited water traps continued to collect large numbers of aphids even when in combination with a range of chemicals representative of different chemical groups (Table 1). Linalool, however, markedly reduced the size of the catch on June 8. The catch with linalool and carvone combined was also lower than with carvone alone over the period May 30 to June 2, but the difference was not significant.

DISCUSSION

The experiments show that *C. aegopodii* is induced to land on certain visual targets in the presence of carvone. They do not provide unequivocal evidence of attraction by the odor since visual attraction with odor-induced settling would produce the same result. The sticky traps show that the insects respond to the odor from over 1 m away. Most insects were caught when the windspeed was less than 1 m/sec and they would be able to make headway

TABLE 3. NUMBERS OF *C. aegopodii* COLLECTED IN YELLOW AND COLORLESS WATER TRAPS

Trap Color	Trap Odor	Number of traps/treatment	Mean number per trap	
			June 9	June 10
Colorless	none	5	0.6 ± 0.5	0.4 ± 0.5
Colorless	carvone	5	8.6 ± 2.6	8.8 ± 3.2
Yellow	none	10	0.5 ± 0.7	1.2 ± 1.2
Yellow	carvone	10	27.0 ± 8.8	25.6 ± 9.9

TABLE 4. NUMBERS OF *C. aegopodii* COLLECTED ON STICKY TRAPS WITH OR WITHOUT ASSOCIATED CARVONE SOURCE

Period (hr and date)	Number collected on traps with		Distance of traps from odor source (m)
	No odor[a]	Carvone odor[a]	
1145–1700 8 June	3, 6	28, 27	0.5
1700–1800 8 June	1, 1	15, 5	0.5
1800 8 June–1100 9 June	1, 0	44, 43	0.5
1100–1530 9 June	11, 9[b]	28, 47	0.5
1530–1800 9 June	13, 7[b]	21, 28	1.2
1800–1820 9 June	1, 3[b]	20, 18	1.2

[a]There were two replicates and hence two numbers for each occasion
[b]These figures are for all aphid species. Approximately one third were *C. aegopodii*.

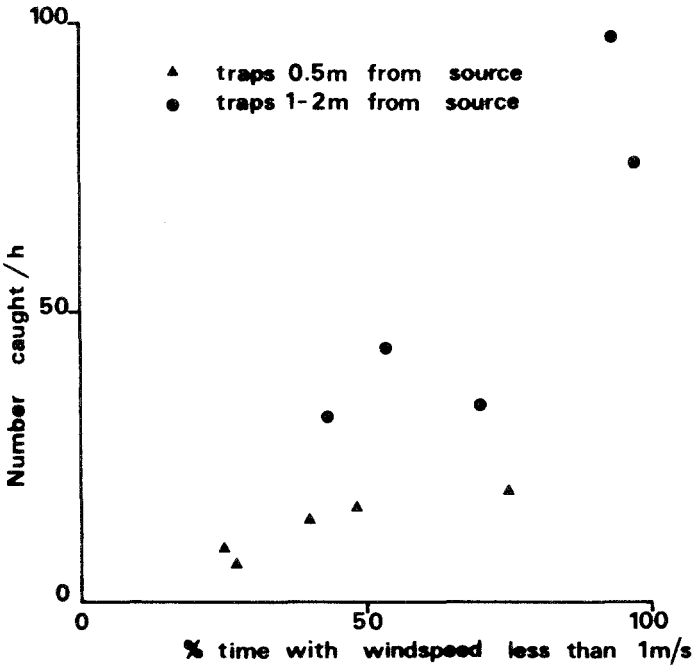

FIG. 1. Relationship between number of aphids caught on sticky traps and the amount of time for which windspeed was less than 1m/sec.

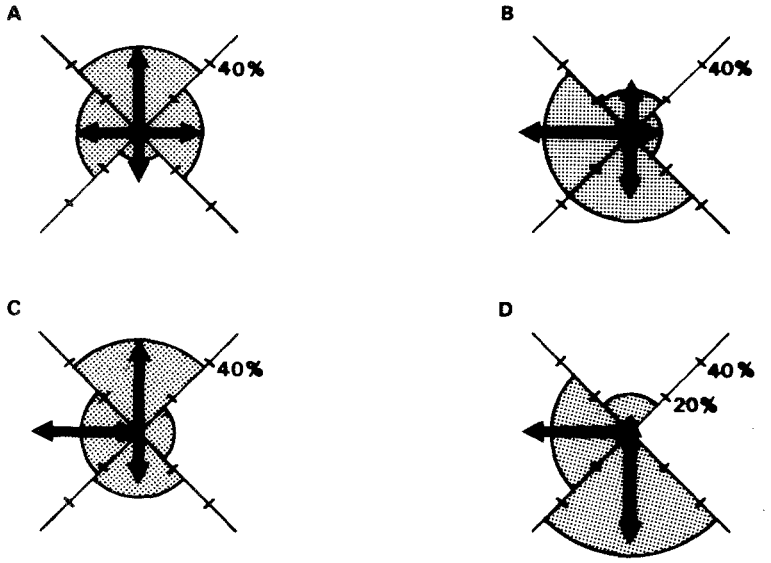

FIG. 2. Relationship between numbers of aphids caught in different sectors of the sticky traps and wind direction over the same periods. Number of aphids in each sector is shown as a percentage of the total number caught in all traps round the carvone attractant source. Superimposed arrows show the percentage of time for which the wind was blowing in each direction with a speed of less than 1 m/sec. (A) traps 0.5 m from odor source, 1145–1800 hr, June 8, $N = 55$; (B) traps 1.2 m from odor source, 1600–1700 hr, June 9, $N = 55$; (C) traps 1.2 m from odor source, 1730–1800 hr, June 9, $N = 49$; (D) traps 1.2 m from odor source, 1800–1830 hr, June 9, $N = 38$.

against it. This, together with the fact that most insects were caught on the downwind side of the traps, suggests that upwind orientation was induced by the odor, although it is possible that preferential landing on the lee sides of the traps occurred regardless of the direction of approach. It is, in any case, not possible to distinguish between an odor-induced visual orientation and anemotaxis in these experiments.

The general view of host-plant selection by aphids is that chemical selection occurs after a visually directed nonspecific landing (Kennedy et al., 1959). Wertheim (1954), however, comments that Fordini appear to be specifically attracted to *Pistacia* since none was seen to land on neighboring trees even when very large numbers of aphids were present. Petterson (1970) has shown that *Rhopalosiphum padi* aggregates in chambers of an olfactometer containing the odor of its winter host *Prunus padus*, although his apparatus was not suitable for demonstrating attraction sensu stricto.

(+)-Carvone, the odor of carraway, has been recorded from a number of Umbelliiferae, and it was present in aphid-infested *Pastinaca sativa* collected

at the study site. Carvone is not, however, characteristic of umbels; it was not present in the heavily infested *Anthriscus sativus*, nor is it restricted to the Umbelliferae. (+)-Carvone has also been recorded from plants of various other families, although these are not present in the European flora. (−)-Carvone, the odor of spearmint, is also found in various plant species including *Mentha crispa*.

It seems probable that carvone can provide a stimulus for host-finding by *C. aegopodii* on umbels and possibly also for *Ovatus crataegarius* on *Mentha*. Clearly it cannot be the only factor influencing the former since some host plants lack the chemical. Whether or not carvone is of particular importance to *C. aegopodii*, the results provide the first clear evidence of odor-induced orientation behavior in aphids. The existence of such a mechanism and the possession by many alate aphids of relatively large numbers of rhinaria on the antennae (Bromley et al., 1979; Dunn, 1978; Shambaugh et al., 1978) suggest that odors may play a role in host selection prior to landing in other aphid species. It might be expected to be important in species with a restricted host-plant range.

There are numerous examples of insects of other orders being attracted by host-plant odors (see, e.g., Brieze-Stegeman et al., 1978; Finch, 1978; Smilanick et al., 1978; Cantelo and Jacobson, 1979) and several more were demonstrated during the current experiments. Yet there can be little doubt that, except for flower odors, the production of such odors by plants has some other function, one of which might be to repel potential herbivores. This probability, and its possible significance in crop protection, is commonly overlooked, partly because the examples of attraction are more obvious and are easy to study.

The reduction in catch of *C. aegopodii* by linalool is an example of a repellent effect of a plant odor, although whether it inhibits landing or affects the orientation response induced by carvone is unknown. Linalool occurs in the foliage of a variety of plants, e.g., *Mentha crispa*, *Citrus bergamia*, and *Cymbopogon citratus*. These plants, growing in the vicinity of the normal hosts, would tend to inhibit the normal host-finding response of the aphid.

Such interaction between host and nonhost odor has been commented on by Atsatt and O'Dowd (1976) and by Tahvanainen and Root (1972) in their concepts of plant guilds or associational resistance. Although repellency was not proved by Tahvanainen and Root (1972), they present clear evidence that the odors of both *Lycopersicon esculentum* and *Ambrosia artemisifolia* reduced the invasion of *Brassica oleracea* by *Phyllotreta crucifera*. Repellency by nonhost plants is also inferred from the experiments of Buranday and Raros (1975) on *Plutella maculipennis* and of Latheef and Irwin (1980) on *Epilachna varivestis*.

The principle of an interaction between host and nonhost plants is often inferred in the practice of intercropping, but the experiments of Latheef and

Irwin (1980) illustrate the dangers of assuming that this interaction is always to man's advantage. The present result with linalool points to the possibility of using synthetic chemicals to repel plant pests and, with the very wide range of plant chemicals available, may offer a more versatile approach than mixed cropping.

Acknowledgments—We are indebted to Miss S. M. Green for advice on experimental design and analysis, Mr. V. Eastop for identifying many aphids and much general advice, and Prof. G. Harborne for checking the purity of our carvone samples and some plant analysis. We are grateful to the authorities of the Chemical Defence Establishment, Porton Down, for allowing us to work unhindered at Porton.

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DEFENSIVE SECRETIONS OF NEW ZEALAND TENEBRIONIDS: I. Presence of Monoterpene Hydrocarbons in the Genus *Artystona* (Coleoptera, Tenebrionidae)

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Abstract—The defensive secretions of four species of the genus *Artystona* endemic to New Zealand differ from those of other tenebrionids in that they contain α-pinene and limonene, as well as the more characteristic quinones and alkenes. Adults and larvae of *A. obscura*, *A. erichsoni*, *A. rugiceps*, and *Artystona* sp. feed on the lichen *Parmotrema reticulatum* (Taylor), but the terpenes are not sequestered from it. The defensive secretions of the four species show some interspecific variation.

Key Words—α-Pinene, defensive secretions, interspecific variation, limonene, monoterpenes, pentadecene, *Artystona* sp., Coleoptera, Tenebrionidae, lichen, *Parmotrema reticulatum*.

INTRODUCTION

Arthropods have the most diverse chemical defense substances among land-dwelling animals, and many of these chemicals have been isolated and identified (Weatherston and Percy, 1970). Except for the defensive secretions of *Platyzosteria novaeseelandiae* (Benn et al., 1977) and six staphylinids, no work has been done on the defensive exudates of arthropods found in New Zealand. Of the staphylinids investigated, the defensive secretions of *Thyreocephalus* species found in New Zealand differed from those of the same species found in Australia (Gnanasunderam et al., 1981a) and *Tramiathaea cornigera* and *Thamiaraea fuscicornis* contained esters which hitherto had not been identified in the defensive secretion of any other staphylinid

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(Gnanasunderam et al., 1981b). However, there is a great deal of diversity in the types of chemicals found in the defensive secretions of staphylinids (Duffey, 1976), and it is difficult to discern whether it is this diversity or the environment that has caused the uncharacteristic chemistry of the defensive secretions of the staphylinids found in New Zealand. The defensive secretions of the tenebrionids, however, are more homogenous than those of the staphylinids despite having variations at generic and specific levels. Of the 147 species of tenebrionids studied by Tschinkel (1975), the defensive secretions were predominantly combinations of quinones and 1-alkenes. The study of the defensive secretions of tenebrionids found in New Zealand was undertaken in order to see if they varied in their chemistry from those studied elsewhere. This is the first report in this series.

METHODS AND MATERIALS

Adults of *Artystona erichsoni* (Auckland district), *A. rugiceps* (Noises Islands off the coast of the North Island), *A. obscura* (Craigieburn State Forest), and *Artystona* sp. (Central Otago) were collected in the late spring. They were all found on the lichen *Parmotrema reticulatum*, but no two species were found in the same locality.

The defensive secretions of all the beetles were collected on small squares of tissue paper which were subsequently immersed in pentane as described in Gnanasunderam et al. (1981b). A portion of the total secretion of each species of *Artystona* was separately chromatographed on 5 × 0.5-cm Florisil columns. The columns were successively eluted with pentane and 5, 10, 20 and 50% (v/v) diethyl ether in pentane (Gnanasunderam et al., 1981b).

A fresh sample (100 g) of the lichen *P. reticulatum* (Taylor) was extracted with ether–pentane (50:50 v/v) and flushed through a (10 × 1-cm) Florisil column with pentane. The pentane eluate was then concentrated under a stream of N_2 and analyzed by gas chromatography.

Gas chromatography was carried out on a Varian 2700 instrument fitted with a flame-ionization detector. Nitrogen was used as the carrier gas. Gas chromatography–mass spectrometry (GC-MS) was performed on a Varian 2700 gas chromatograph coupled via a membrane separator (at 180° C) to an AEI-MS30 mass spectrometer operated at 20 eV for low resolution and 70 eV for high resolution. Helium was used as the carrier gas. Columns used were a 50-m OV-17 SCOT column and 2-m × 2-mm (ID) columns packed with 15% (w/w) OV-17 and 10% (w/w) and Silar 10CP on Chromasorb W-AW-HMDS (100–120 mesh). All columns were temperature programed from 80 to 200° C at 4°/min.

RESULTS

Gas chromatogrpahy of the defensive secretions of the four species of *Artystona* on the OV-17 SCOT column revealed the presence of two major

components (B and F) and four minor components (A, D, E, and G) in all four species (Figure 1). Another minor component, C, was absent from *Artystona* sp. The OV-17 column gave a better separation than the Silar 10CP column. Figure 1 shows differing proportions of the chemicals in each of the four species of *Artystona*.

The gas chromatography of the pentane fraction from the Florisil columns of the defensive secretions revealed the presence of hydrocarbons A, B, F, and G. High-resolution mass spectra of peaks A and B showed a parent ion at m/e 136.1247 ($C_{10}H_{16}$ = 136.1248). An ion at m/e 121.1023 (C_9H_{13} = 121.1014) and the characteristic M-43 of most monoterpenoid spectra at

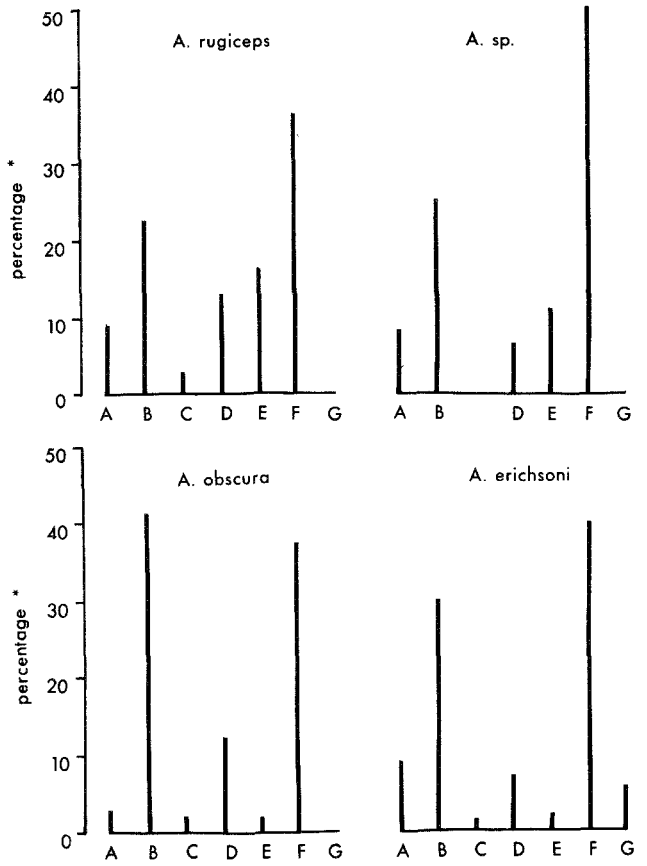

FIG. 1. Composition of the defensive secretions of four species of *Artystona*. *Percentage peak area of the total peak area of the GC trace on a 15% OV-17 column (2 m) temperature programed from 80 to 200° C at 4° /min. (A) α-pinene; (B) limonene; (C) benzoquinone; (D) toluoquinone; (E) ethylquinone; (F) pentadecene; and (G) heptadecene.

93.0708 (C_7H_9 = 93.0702) suggested that A and B were monoterpene hydrocarbons. A peak at *m/e* 68 in the spectra of B further indicated that it was limonene. This fragment is the result of a retro-Diels-Alder rearrangement (Enzel et al., 1972). When A and B were coinjected with a series of monoterpene hydrocarbons the retention time of A coincided with that of α-pinene and that of B with limonene. These compounds gave mass spectra identical to those of authentic α-pinene and limonene, respectively. Mass spectrometry of F and G gave molecular ions at *m/e* 210 and 238 and a fragmentation pattern typical of unsaturated 1-alkene. The mass spectra and retention times of F and G were identical with those of 1-pentadecene and 1-heptadecene.

GC-MS of the quinone fraction (20% ether–pentane) from the Florisil column established parent ions for C, D, and E at *m/e* 108, 122, and 136, respectively. All three components (C, D, and E) were shown to have retention times identical to benzoquinone, toluoquinone, and ethylquinone, respectively, by separate coinjection of the quinone fraction with the individual standards.

When the pentane fraction of the lichen extract was analyzed by gas chromatography, no α-pinene or limonene was detected.

DISCUSSION

The four species of *Artystona* investigated are endemic to New Zealand, and belong to the subfamily Tenebrioninae (Watt, 1974). The genus has usually been included in the tribe Cnodalonini, but it is not closely related to typical members of the tribe (J.C. Watt, personal communication). The adults and larvae have been known to hide during the day under loose bark in dead wood and emerge at night to feed on lichen (Watt, 1969).

The monoterpene hydrocarbons α-pinene and limonene have not previously been reported from tenebrionid defensive secretions. Although terpenoid compounds have been found in four of the families in the order Coleoptera (Weatherston and Percy, 1978), terpene hydrocarbons have not hitherto been identified in the defensive secretions of any beetle belonging to this order. However, these hydrocarbons are found in other insects. α-Pinene and limonene have been isolated from two species of Hemiptera (Aldrich, 1979) and in the frontal gland exudates of termites of the genus *Nasutitermes* (Morre, 1964). α-Pinene has also been isolated from the frontal gland of the sawfly *Neodiprion sertifer* (Eisner et al., 1974). However, the α-pinene in *N. sertifer* is sequestered from conifers such as *Pinus sylverstris* on which the larvae feed (Eisner et al., 1974), but, since neither α-pinene nor limonene are present in the lichen *P. reticulatum* on which the genus *Artystona* feeds, these terpene hydrocarbons must be synthesized by the beetles. The limonene and

α-pinene give the defensive secretion of the *Artystona* species a terpene odor, and the unusual addition of terpenes to the essentially quinone–hydrocarbon defensive secretion is interesting. Tschinkel (1975) suggests that the differences in the defensive secretions of the tenebrionids have evolved in response to different predators and the presence of terpenes in these tenebrionid beetles may also represent an evolutionary response to particular predators. Monoterpene hydrocarbons such as limonene and pinene produced in the frontal gland exudates of termite soldiers are known to form part of a highly effective defensive secretion (Blum, 1978). However, the function of these terpenes in the defensive secretions of the *Artystona* species will remain in doubt until the ecological relationship of the genus *Artystona* has been investigated further.

By peak area, limonene and pentadecene are the two major constituents in the defensive exudate of the genus *Artystona*. Pentadecene is not a common hydrocarbon in the defensive secretion of tenebrionid beetles studied to date as not more than 20 of the 147 species investigated by Tschinkel (1975) contained this C_{15} hydrocarbon. However, pentadecene is the most abundant compound in the defensive secretion of *A. erichsoni*, *A. rugiceps* and *Artystona* sp.

Interspecific variation is not confined to the genus *Artystona* but has been observed in the defensive secretions of other tenebrionids such as in the genera *Platydema*, *Zadenos*, *Argoporis*, and *Blaps* (Tschinkel, 1975). Except for the absence of benzoquinone, the defensive secretion of *Artystona* sp. is very similar to that of *A. rugiceps*, whereas they differ from the defensive secretions of both *A. erichsoni* and *A. obscura* (Figure 1). Taxonomically *Artystona* sp. may be a geographic variant of *A. rugiceps*, and *A. erichsoni* is more closely related to *A. obsura* than either of them are to *A. rugiceps* and *Artystona* sp. (J.C. Watt, personal communication). This is reflected in the differences in the defensive secretions of the four species of *Artystona*. However, despite these differences, there is no great diversity in the basic chemical composition of the defensive exudates within this genus, but the presence of α-pinene and limonene in its defensive secretions sets the genus *Artystona* chemically apart from other tenebrionids.

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MITE PREDATOR RESPONSES TO PREY AND PREDATOR-EMITTED STIMULI

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Abstract—We found that the searching behavior of two acarine predators, *Amblyseius fallacis* and *Phytoseiulus macropilis*, for prey, *Tetranychus urticae*, is affected by the following stimuli: (1) prey silk and associated feces, whose combined physical and chemical properties elicit reduction in the rate of predator movements and longer halts; (2) kairomone extracted from prey silk and associated feces, which, upon contact, elicits frequent predator return to prey-inhabited locales; and (3) predator-emitted marking pheromone, which elicits shorter duration of search in presearched prey locales. We also found that treatment of filter paper with prey kairomone or silk enhanced predator location of prey eggs, leading us to speculate that application of synthetic prey kairomone could be useful in pest management programs.

Key Words—Kairomone, silk, marking pheromone, host finding, *Amblyseius fallacis*, *Phytoseiulus macropilis*, *Tetranychus urticae*.

INTRODUCTION

Amblyseius fallacis (Garman) and *Phytoseiulus macropilis* (Banks) (Acarina: Phytoseiidae) are important predators of spider mites, especially *Tetranychus urticae* Koch. *A. fallacis* occurs in northeastern U.S. commercial apple orchards (Croft, 1975), while *P. macropilis* is undergoing experimentation in greenhouse integrated mite control programs in Florida (Hamlen, 1980). Although reports exist describing various aspects of the biology (Ballard, 1954; Prasad, 1967; McClanahan, 1968; Smith and Newsom, 1970a,b; Rock et al., 1976; Johnson and Croft, 1976; Shih et al., 1979; Hamlen, 1978, 1980) and pesticide resistance (Motoyama et al., 1970; Croft and Nelson, 1972; Hislop and Prokopy, 1981) of these predators, the role of kairomones and marking pheromones in *A. fallacis* or *P. macropilis* host searching behavior

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has received little attention. This is true as well for most other predatory mites, notable exceptions being the work of Farish and Axtell (1966) and Jalil and Rodriguez (1970) demonstrating the role of kairomones in attraction to prey of predacious mites of the families Machrochelidae and Uropodidae.

In spring, *A. fallacis* is normally associated with *T. urticae*, a preferred prey in the orchard understory. In summer, this predator migrates into the trees in search of additional prey, often borne there by wind currents (Johnson and Croft, 1976). In the greenhouse, *P. macropilis* does not usually migrate over long distances in search of new prey but instead tends to remain in close proximity to existing prey. In a related species, *P. persimilis* Athias-Henriot, Mori and Chant (1966) demonstrated changes in predation behavior as a function of humidity and starvation level.

Several factors may influence arthropod predator searching behavior leading to contact with prey: physical substrate characteristics; temperature, humidity, and starvation level (Rasmy and El-Banhawy, 1974, Elsey, 1974; Blommers et al., 1977); and prey silk (Schmidt, 1976; Ohnesorge, 1978). Here we examine for the first time two sorts of factors influencing the host searching behavior of *A. fallacis* and *P. macropilis* (*A. fallacis* was examined in greater detail because of its importance in our apple integrated pest management program): (1) physical and kairmonal cues emitted by *T. urticae*, and (2) predator-emitted stimuli influencing duration of search in presearched areas.

METHODS AND MATERIALS

A. fallacis was collected in Massachusetts apple orchards and cultured in the laboratory on lima bean leaves infested with *T. urticae*. *P. macropilis* was obtained from a colony at the University of Massachusetts Suburban Experiment Station in Waltham.

For collection and bioassay of prey silk, hundreds of *T. urticae* were brushed from bean leaves onto 12-cm-diam glass plates using a mite brushing machine (Henderson and McBurnie, 1943). Pieces of bean leaves ca. 2 cm diam were placed around the edges of each plate to collect the dispersing mites. Leaf pieces containing 300–500 mites were placed individually under 2-cm-diam glass rings for 24 hr, after which the leaf and all mites were removed. Silk spun by the mites across the ring hole was gathered on a 1.5-cm-diam filter paper disk by passing the disk through the ring. After manual removal of eggs and debris from the silk, each disk was bioassayed for predator response. Because we were unable to remove prey feces from the silk, our terminology for silk throughout this paper includes the element of associated prey feces.

For collection and bioassay of kairomone, each of several 12-cm-diam glass plates containing thousands of *T. urticae* brushed from bean leaves was

placed in a petri dish, over which was positioned an acetate cone (12 cm bottom diam, 2 cm top diam). Each was then placed under a fluorescent light, which attracted the mites to the top of the cone. After 24 hr, the mites were removed and the silk was gathered by passing a 1.5-cm-diam preweighed (on a Cahn electrobalance) filter paper disk through the hole in the top of the cone. After manual removal of eggs and debris, the filter paper and silk were weighed and placed into 1 ml of solvent. After 24 hr, the filter paper was removed and the extract centrifuged at 10,000 rpm for 30 min. Final extract concentration was 0.1 mg silk/ml solvent. We used the following four solvents: water, methanol, chloroform, and hexane. Because preliminary assays proved the methanol extract to contain the most active kairomonal components, this extract was chosen for use in the assays reported here.

The assays were conducted in the following manner. One 1.5-cm-diam filter paper disk left untreated (= a control) or containing either silk, 0.02 ml methanol extract of silk (= kairomone), or methanol (= a control) was placed in the center of a 5-cm-diam glass arena surrounded by water to prevent emigration of predators. The surface of the water was ca. 2 mm below the surface of the glass arena. One *A. fallacis* or *P. macropilis* female, starved for 6–8 hr, was placed at the arena edge and allowed to enter the disk at will. Bioassay periods lasted for 90 sec (silk) or 6 min (kairomone) following initial predator contact with the disk. Speed of movement (*A. fallacis* only) was obtained by analyzing the path of predator movement traced for the first 90 sec onto a piece of clear acetate taped to a magnifying glass to enlarge the image. Visual interference of the tracing movements with predator activity was eliminated by reflecting the predator image off two mirrors prior to image magnification. Tracing was performed in dim light to avoid back-reflection.

Influence of prey-emitted stimuli on *A. fallacis* predation efficiency was determined by placing a single *T. urticae* egg midway between the edge and center of a 0.5-cm-diam filter paper disk containing either prey silk, 0.01 ml of methanol solution of kairomone, or methanol alone (= control). The percentage of predators which consumed an egg in a 5-min time period was compared among treatments. The average time *A. fallacis* remained on or within 1 cm of each disk following prey consumption was also measured. *P. macropilis* was not tested.

In examining factors influencing duration of predator searching in presearched areas, we assayed females of *A. fallacis* and *P. macropilis* which had been placed in contact prior to assay with a filter paper disk containing prey silk. In the assay, a filter paper disk (0.5 cm diam) containing *T. urticae* silk was placed in the center of a glass arena. A single predator was introduced and allowed to enter the disk at will. We measured the following: (1) duration of first visit to (within 1 cm of) a disk not previously visited by a predator (= control disks); and (2) duration of first visit to (within 1 cm of) a disk previously searched for ca. 30–40 min prior to assay by 5 conspecific predators.

All experiments were performed under conditions of ca. 24 ± 2° C and 70 ± 10% relative humidity. Data were submitted to analysis of variance and Duncan's multiple range test or a paired *t* test at the 5% level.

RESULTS

Influence of Prey Silk on Searching Behavior. A. fallacis females spent a significantly greater amount of time per visit on disks having fresh or 14-day-old prey silk and associated feces than on control disks. Also, the average duration of stops was significantly greater, and the walking speed significantly less, on the silk disks than on the controls (Table 1). These data suggest that fresh as well as 14-day-old *T. urticae* silk has physical and/or chemical properties which stimulate searching *A. fallacis* females to walk more slowly and stop longer within areas of prey habitation.

Influence of Prey Kairomone on Searching Behavior. The average time spent between *A. fallacis* visits to disks treated with fresh, 3-day-old, or 7-day-old extract of silk and associated feces was significantly less than the time between visits to control disks (Table 2). *A. fallacis* returned to treated disks at an average turning angle of 148° compared with 97° for the controls. In similar fashion, *P. macropilis* returned to disks treated with fresh silk extract in significantly less time (15.3 sec) than to control disks (40.5 sec) (20 replicates). These data clearly demonstrate that a kairomone is present in the extract which stimulates *A. fallacis* and *P. macropilis* females to return to extract disks and that kairomonal activity (for *A. fallacis*) is present for at least 7 days. In contrast to when kairomone was present together with silk (Table 1), the average duration of visits and stops (*A. fallacis*) on silk extract disks was no different from controls, although the speed of movement was significantly less than on the controls (Table 2). Figure 1 shows a typical *A. fallacis* search pattern on silk, silk extract, and methanol control disks.

TABLE 1. INFLUENCE OF *T. urticae* SILK AND ASSOCIATED FECES ON *A. fallacis* SEARCHING BEHAVIOR (20 REPLICATES/TREATMENT)

Disk type	Avg. duration of visit (sec)[a]	Avg. time between visits (sec)	Avg. duration of stops on disk (sec)	Avg. walking speed on disk (cm/sec)
Fresh silk	42.5 a	11.8 a	12.2 a	0.12 a
14-day-old silk	57.0 a	14.0 a	16.3 a	0.15 a
Untreated	12.2 b	24.5 a	0.4 b	0.29 b

[a]Means in each column followed by a different letter are significantly different at the 0.05 level.

TABLE 2. INFLUENCE OF METHANOL EXTRACT OF *T. urticae* SILK AND ASSOCIATED FECES (=KAIROMONE) ON *A. fallacis* SEARCHING BEHAVIOR (20 REPLICATES/TREATMENT)

Disk type	Avg. duration of visit (sec)[a]	Avg. time between visits (sec)	Avg. duration of stops on disk (sec)	Avg. walking speed on disk (cm/sec)
Fresh extract	13.2 a	33.0 a	0 a	0.20 a
3-day extract	14.2 a	33.7 a	0 a	0.19 a
7-day extract	10.9 a	28.0 a	0.1 a	0.18 a
Methanol	9.9 a	66.5 b	0.1 a	0.24 b

[a]See footnote [a] of Table 1.

Data on the influence of visit sequence on *A. fallacis* response to silk extract and control disks are given in Table 3. For the first three visits, the return time to silk extract disks was significantly shorter than the return time to control disks. For visists 4–6 and 7–9 there was no significant difference between treatments in return time. Nor was there at any time a significant difference between treatments in average duration of visits. Initial retention time (i.e., time over which *A. fallacis* was retained within 1 cm of a treatment disk following the first visit) was significantly longer on disks treated with fresh, 3-day-old, and 7-day-old silk extract (51.0, 69.6, and 63.0 sec, respectively) than on control disks (22.8 sec). Thus, contact with the kairomone appeared to elicit initial retention of *A. fallacis* within the vicinity, after which *A. fallacis* may have physiologically adapted or habituated to it. Weseloh (1980) reported habituation of *Apanteles melanoscelus* (Ratzburg) females to silk kairomone of host *Lymantria dispar* L. larvae.

In a test to determine whether the kairomone is perceived by contact or olfactory means, *A. fallacis* exhibited little or no response to wind currents blown over kairomone or control disks at velocities below the threshold eliciting characteristic dispersal behavior (Johnson and Croft, 1976). Wind velocities exceeding this threshold frequently elicited dispersal from both

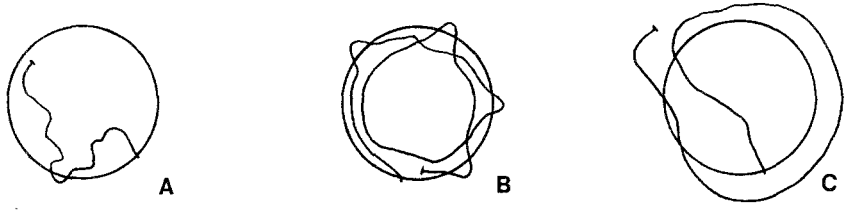

FIG. 1. Typical *A. fallacis* search patterns (90-sec period following first contact with disk) when encountering: (A) *T. urticae* silk and associated feces, (B) methanol extract of silk and associated feces (= kairomone), or (C) methanol control disks.

TABLE 3. INFLUENCE OF VISIT SEQUENCE ON RESPONSE OF *A. fallacis* TO METHANOL EXTRACT OF *T. urticae* SILK AND ASSOCIATED FECES (20 REPLICATES/TREATMENT)

	Avg. duration of visit (sec)[a]			Avg. time between visits (sec)		
Disk type	Visit no. 1–3	Visit no. 4–6	Visit no. 7–9	Visit no. 1–3	Visit no. 4–6	Visit no. 7–9
Fresh extract	15.9 a	14.3 a	8.5 a	24.2 a	32.5 a	28.1 a
3-day extract	18.8 a	10.3 a	9.3 a	29.6 a	28.2 a	24.3 a
7-day extract	18.2 a	9.1 a	8.1 a	24.2 a	24.0 a	21.0 a
Methanol	11.1 a	8.8 a	9.2 a	62.7 b	38.5 a	22.4 a

[a]See footnote a of Table 1.

treatments. These observations suggest that the kairomone is perceived principally or exclusively by contact.

Influence of Prey Silk or Kairomone on Predation Efficiency. A. fallacis achieved maximum predation efficiency when searching for prey (a single *T. urticae* egg) on a disk treated with prey silk, although searching for prey on a disk treated with methanol silk extract also resulted in significantly greater prey consumption compared with searching for prey on control disks which had prey (Table 4). Following prey consumption, *A. fallacis* remained on (within 1 cm of) disks treated with silk significantly longer than on control disks which had prey. They remained on the latter significantly longer than on control disks without prey (Table 4). These data demonstrate that prey consumption elicits retention of *A. fallacis* in areas of prey habitation and that the presence of prey silk or kairomone extends this retention time.

TABLE 4. INFLUENCE OF *T. urticae* SILK AND ASSOCIATED FECES, OR METHANOL EXTRACT OF SILK AND ASSOCIATED FECES, ON *A. fallacis* PREDATION EFFICIENCY AND POST-PREY-CONSUMPTION BEHAVIOR (20 REPLICATES/TREATMENT)

Disk treatment	*A. fallacis* which consumed prey egg in 5 min[a] (%)	Avg. duration of visit to disk after prey consumption (sec)[b]
Silk plus 1 prey egg	88 a	287.7 a
Silk extract plus 1 prey egg	67 b	219.0 b
Methanol plus 1 prey egg	35 c	173.3 b
Methanol		42.1 c

[a]See footnote a of Table 1.
[b]On or within 1 cm of disk.

Influence of Presearched Areas on Predator Searching Behavior. Both *A. fallacis* (N = 14) and *P. macropilis* (N = 14) remained a significantly shorter time during the first visit to prey-silked disks presearched by five different conspecific predators for 30–40 min prior to assay (55.3 and 107.2 sec, respectively) than during the first visit to nonpresearched silked disks (496.1 and 492.3 sec, respectively). A preliminary test revealed that *P. macropilis* females spent ca. 98 sec searching silked disks treated with a methanol extract of presearched disks compared with ca. 129 sec searching silked disks treated with methanol alone ($P = \leq 0.30, t$ test, $N = 12$). We interpret these results as suggesting that both species release marking pheromone during host searching behavior.

DISCUSSION

The literature suggests that host-finding by predatory arthropods is mediated more often by visual or tactile stimuli than by chemosensory stimuli (Rowlands and Chapin, 1978, Greany and Hagen, 1981). However, in predators with limited visual capacity, such as *A. fallacis* appears to have, possession of chemosensory mechanisms for prey detection would be a distinct advantage. Wilbert (1974) and McLain (1979) found that certain predators, with limited visual capabilities, altered their search pattern upon encountering host odors or aqueous solutions of host frass or hemolymph (= kairomones). Similarly, Lewis et al. (1977) discovered a kairomone in *Heliothis zea* (Boddie) moth scales which stimulates larvae of *Chrysopa carnea* Stephens to remain in areas containing moth eggs.

Our results suggest that *A. fallacis* utilizes a combination of physical and chemical stimuli in prey location. Exposure to prey silk and associated feces elicits reduction in the speed of predator movement (inverse orthokinesis; Kennedy, 1977) and elicits longer halts, thus increasing the probability of encountering nearby prey. We do not believe that these influences of silk on predator behavior are the result of silk impeding predator movement because, upon gentle prodding, predators are able to exit from a silked area just as rapidly as from a nonsilked area. Schmidt (1976) likewise found that exposure of *P. persimilis* to *T. urticae* silk resulted in predator arrestment, leading to greater prey consumption. Exposure to a methanol extract of prey silk and associated feces (kairomone) elicits frequent predator return to the stimulus area (direct klinokinesis; Kennedy, 1977). The parasite *Trichogramma evanescens* Westwood is arrested in similar fashion after contact with kairomone separate from or together with scales of ovipositing *Heliothis zea* (Boddie) moths (Lewis et al., 1972).

Our findings suggest a possible disadvantage to *A. fallacis* when encountering old silk of prey. The demonstrated arrestant effect of 14-day-old

silk could conceivably result in waste of predator time and energy when foraging in areas previously but no longer containing prey. Ohnesorge (1978) observed a similar situation in *P. persimilis*.

T. urticae, a principal prey of *A. fallacis*, tends to occur in relatively clumped distributions. *A. fallacis* may achieve maximal predation efficiency by remaining in areas where prey aggregations occur. Our findings suggest that the same host cues (silk in combination with kairomone) which elicit predator retention also lead to increased predation efficiency. In fact, encounters with prey eggs alone, even in the absence of additional prey stimuli, retain searching *A. fallacis* in prey areas for a greater time than in areas devoid of prey. Such a response may be of substantial benefit in discovering additional prey within areas of prey aggregation. Similar alterations in predator searching behavior following prey encounter or consumption have been demonstrated in *Anthocaris nemoralis* (F.) (Brunner and Burts, 1975), *Anthocaris confusis* (Evans, 1976), *Coccinella septempunctata* (Marks, 1977), and *Hippodamia convergens* (Rowlands and Chapin, 1978).

Prey kairomone influencing the searching behavior of *A. fallacis* or *P. macropilis* could be of significant value to pest management programs. For example, if the kairomone secreted by *T. urticae* could be identified, synthesized, and applied together with artificial food substances (such as honeydew or pollen; e.g., Hagen et al., 1970), the result might be greater retention of *A. fallacis* or *P. macropilis* on the foliage in times of low natural prey densities. Such artificially retained predators could function to "guard" against possible spider mite outbreaks.

Finally, our data suggest that during searching of prey-silked areas, *A. fallacis* and *P. macropilis* deposit pheromone which marks such sites as already having been recently explored. Marking pheromones have been discovered in predatory insects (Marks, 1977), parasitoids (Vinson, 1976), and phytophagous insects (Prokopy, 1981), but this appears to be the first such case in a predatory acarine.

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Journal of Chemical Ecology, Vol. 7, No. 5, 1981

Letter to the Editors

DEFENSIVE SECRETION OF STICK INSECTS

To The Editor

In a recent paper, Smith et al. (1979) presented the chemical composition of the defensive secretion of the stick insect *Graeffea crouani* Le Guillo. I found the paper very interesting, but incomplete in the following points.

Even if only some odd chemical structures of the defensive secretion of *Paradoxomorpha crassa* (Blanch) have been published (Schneider, 1934; Moreno, 1940), they should at least have been mentioned.

Furthermore, the chemotaxonomy of the defensive secretion within the insect order Phasmida was never discussed. It has been pointed out that the defensive compounds in general are characteristic at higher levels, e.g., the families Carabidae and Tenebrionidae (Coleoptera) (Blum 1978). For comprehensive reviews see Bettini (1978).

In the two species of Phasmida in which the defensive compounds have been detected with modern techniques, viz., *G. crouani* (Smith et al., 1979) and *Anisomorpha buprestoides* (Stoll) (Meinwald et al., 1962, Happ et al., 1966), the following discussion can be made. The insect order Phasmida is divided into two suborders (Bradley and Galil, 1977), and *G. crouani* and *A. buprestoides* belong to different suborders. It is therefore very interesting to see that anisomorphal (from *A. buprestoides*) is very similar in structure to the different isomers of iridodial (from *G. crouani*). This could indicate that closely related chemical compounds are used within this insect order, even in such distantly related species as *G. crouani* and *A. buprestoides*.

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Conference Summary

REGULATION OF INSECT DEVELOPMENT AND BEHAVIOR

An International Conference on Regulation of Insect Development and Behavior was held in Karpacz, Poland, June 23–28, 1980. The conference was a unique attempt to bring together entomologists, physiologists, biochemists, and chemists to discuss and present in an integrated manner the state of the science governing life and behavioral processes in insects. While previous topical conferences specialized primarily in research on juvenile hormones (JH), this conference dealt with many aspects of chemical communication, both within the insect (hormones) and between insects (pheromones).

Biochemical papers were presented describing the discovery of JH "0" in eggs of *Manduca sexta* and regulation of hormone levels of biosynthetic enzymes in the corpus allatum, and the role of other factors such as esterase inactivation, binding and carrier proteins. New prototypes of JH antagonists working primarily on biosynthetic mechanisms underscored the renewed interest in this means of insect control. Also revealed were new kinds of feedback mechanisms regulating output of the endocrine glands, including the regulation of JH output by ovarian ecdysone in *Nauphoeta.*

In the hormonal regulation of insect development, the conferees heard how the degradation of JH by the individual tissues as well as by the hemolymph is important for their reprograming response to ecdysteroids at metamorphosis. This reprograming was shown to occur in a tissue-, and even intracellular-, specific pattern. The hormonal regulation of insect reproduction by the same two hormones, ecdysone and JH, was shown to be much more diverse among the insect groups. For instance, yolk protein synthesis occurs not only in fat body (controlled by ecdysone in Diptera and by JH in locusts and cockroaches), but also in the ovaries of some species such as *Drosophila* (controlled by JH) and *Cecropia* (no known hormonal control). On the molecular level, the most exciting findings reported were the isolation by several laboratories of cytosolic receptors for JH. Beginning studies on the action of both ecdysone and JH on the regulation of transcription of specific genes and on posttranscriptional processes were presented.

The role of peptide neurohormones in insect development and behavior was significantly extended. Discussed were their control of insect ecdysis, pupariation behavior, egg development, and carbohydrate and lipid metab-

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olism. The importance of aminergic neurotransmitters such as 5HT controlling the release of PTTH, and octopamine modulating various aspects of insect behavior in regulation and control of insect function were disclosed.

Pheromones also modulate insect behavior, and aspects of both the control of their production by environmental factors and this influence on the recipient's behavior were presented. At the receptor level, selectivity of specific components of a pheromone mixture by the individual cells was shown, as well as the role of binding proteins and degradative enzymes in pheromone action. The importance of the stereochemical features of insect sex pheromones has become increasingly apparent, and the challenge to the synthetic chemist to devise stereoselective syntheses was elegantly demonstrated in several papers.

Coevolution of plants and insects was discussed with emphasis on plant natural products serving as antifeedants and on their ability to disrupt insect development.

Many other new findings were presented ranging from the role of insect intestinal fungi in the reduction of JHs in the gut of insects to announcement of new juvenoids as potentially practical insect control agents in plant protection.

It was evident from the scope of scientific information presented at the conference that interdisciplinary research is an essential requisite for continued advances in our understanding of how insects function. This knowledge is essential in our continuing quest to discover selective, biodegradable insecticidal or insectostatic chemicals.

This conference was dedicated to the memory of the pioneer Polish endocrinologist, Stefan Kopeć. Fittingly, it was held in Kopeć's land, where it was organized and sponsored by the Technical University of Wroclaw under the auspices of the Research Programme "Environment Protection."

Generous financial support was provided by the Technical University through the efforts of its vice president, Professor Alfred Dziendziel, and Professor Andrzej Zabźa.

The conference owes its success to the dedication of the Polish organizing committee whose efforts brought together participants from over 20 countries. Their contributions to the scientific content and the warm fellowship characterized this memorable conference in the Silesian mountains of Poland.

The conference proceedings will be published by the Publishing Office of the Technical University of Wroclaw.

J.J. MENN
F. SEHNAL

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS.
XII. The Stimulatory Effects of Host Eggs and the Importance of Host-Egg Density to the Effective Use of Kairomones for *Trichogramma pretiosum* Riley[1-3]

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Abstract—*Trichogramma pretiosum* Riley females exhibit success-motivated searching after oviposition. The stimulatory effect of contact with host eggs makes host-egg density an important factor in determining the appropriate strategy for behavioral manipulation, using kairomones, that simulate host seeking, in biological control programs. Host eggs can be used, in conjunction with kairomones or by themselves, to improve the performance of these important beneficial insects.

Key Words—*Trichogramma pretiosum*, Hymenoptera, Trichogrammatidae, biological control, kairomone, *Heliothis zea*, Lepidoptera, Noctuidae, host density.

INTRODUCTION

With the renewed emphasis on biological control, interest in *Trichogramma* spp., as biological control agents, has grown. Recent work with these parasitoids has increasingly involved studies of their host-selection behavior. Laing (1937) investigated the host-selection behavior of *Trichogramma evanescens* Westwood and demonstrated that while visual cues may be

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important at very close range (less than 2–3 cm), chemical residues [kairomone(s)] left by adult moths play an important role in host finding. The source of this kairomone was shown later to be the scales of adult moths left behind at oviposition sites (Lewis et al., 1971, 1972; Jones et al., 1973). Subsequent studies demonstrated that the kairomone functions by releasing and maintaining an intensified host-search behavior rather than as an attractant or orienting factor (Lewis et al., 1975b, 1979; Beevers et al., 1981). This kairomone can be used to increase rates of parasitization in the field, and its application does not have to be limited to the immediate vicinity of the host egg (Lewis et al., 1975a; Gross et al., 1975).

Laing (1937) showed that the host egg itself is a stimulus for an intensified host-searching behavior by *T. evanescens*, and Beevers et al. (1981) demonstrated the same for *Trichogramma pretiosum* Riley. This phenomenon, termed success-motivated searching (Vinson, 1977), has been reported for several other parasitoids (Edwards, 1954; Chabora, 1967; Gerling and Schwartz, 1974). Gross et al. (1981) found that prerelease parasitization experience improves the retention of *T. pretiosum* females in laboratory assays and improves their efficiency in the field. Lewis et al. (1979) demonstrated that the appropriate distribution of kairomone is dependent, to a great extent, on host density. While previous studies in this series concentrated on various aspects of the kairomones themselves, this study was undertaken to further decipher the importance of the interaction between host density and kairomone distribution.

METHODS AND MATERIALS

The hexane extract of *Heliothis zea* (Boddie) moth scales (MSE) (1 : 1000 dilution) used in this study was formulated according to the procedures of Jones et al. (1973). Some experiments employed simulated moth scales (SMS), which consisted of moth-scale extract impregnated particles. Two particle types were used: Anakrom U (Analabs, North Haven, Connecticut) (diatomaceous earth), 40/50 mesh, which were white (WSMS), and Anakrom C-22 (diatomaceous earth), 40/50 mesh, which were red (RSMS). These particles were impregnated with moth-scale extract by mixing 5 g of either particle type with 1 ml of crude moth-scale extract and 50 ml of hexane in a boiling flask and vacuum-evaporating the solvent (Lewis et al., 1979).

The *H. zea* eggs used in this study (and for the rearing of the *Trichogramma*) were obtained from laboratory cultures, processed with a sodium hypochlorite wash (as described by Burton, 1969), irradiated with 25 krad (^{60}Co source) when 8–36 hr old, to prevent eclosion, and stored at ca. 10° C. The *T. pretiosum* stock used in the study was the same as that used in the studies reported in previous papers in this series. The parasitoids were

reared in *H. zea* eggs at ca. 26° C and 70% relative humidity, according to the method of Lewis and Redlinger (1969). To determine the percentage of parasitization, host eggs were dissected according to the procedure described by Lewis and Redlinger (1969). Paired *t* tests or Duncan's new multiple-range test were used for statistical analyses of the data. Arcsin transformations were conducted on percentages prior to analysis.

RESULTS

Expanded Laboratory Universe. The expanded laboratory universe (ELU) system of Lewis et al. (1979) was used in this series of experiments. The control ELU consisted of sheets of white butcher paper (Schrier Brothers, Inc., Mamaroneck, New York) (117.5 × 30.4 cm) placed on a laboratory table. The butcher paper sheets were separated at each end by 30 cm and on the sides by 60 cm. Three eggs were attached to each sheet with a camel's hair brush moistened with a Plantgard® (Polymetrics Intl., New York, New York) solution (Nordlund et al., 1974). Eggs were placed in a line along the long axis of the sheet. One egg was located in the center, i.e., 15.2 cm from the sides and 58.75 cm from each end, while the other two eggs were placed 45.72 cm to either side of the center egg, 15.2 cm from the sides, and 13.03 cm from the respective ends of the sheet. Eggs were positioned in the centers of 7.6-cm-diam circles that were treated (over the circumscribed area) with 0.2 ml of MSE, using a syringe. These locations are referred to as simulated oviposition sites (SOS). Two vials of 5 *T. pretiosum* females were released, one each midway between the center egg and the eggs on each end of the ELU. The parasitoids were allowed to search for 30 min, after which the eggs from the SOS were collected and dissected to determine percentage of parasitization. Three paired comparisons of two treatment patterns were conducted at a time. The means of these three readings of each treatment constituted one replication.

A series of comparisons of various treatments or treatment combinations, applied to the above described control universe, was conducted. Treatments were as follows:

SHE = 40 supplemental host eggs (*H. zea*) distributed across the universe and applied as described above;

RSMS = 144 RSMS evenly distributed over the universe, attached with a camel's hair brush moistened with water;

SS = Solid sprayed with 25 ml MSE, using a chromatographic sprayer.

Five different comparisons were conducted.

The results of these experiments are given in Table 1, and the increased rate of parasitization resulting from the application of supplemental host eggs

TABLE 1. MEAN PERCENTAGE PARASITIZATION (± 1 SE) OF EGGS FROM SIMULATED OVIPOSITION SITES (SOS) IN EXPANDED LABORATORY UNIVERSE (ELU) EXPERIMENTAL DESIGN WITH DIFFERENT TREATMENT COMBINATIONS[a]

Comparison	No. of replications	Treatment combinations and percentage parasitization		
I	16	Control	vs.	SHE[b]
		31.2 (±4.1)a		46.5 (±3.7)b
II	8	SHE	vs.	SHE + RSMS
		27.8 (±5.9)a		48.0 (±7.3)b
III	24	RSMS[c]	vs.	RSMS + SHE
		52.7 (±3.4)a		49.2 (±3.2)a
IV	12	SS[d]	vs.	SS + SHE
		20.4 (±5.3)a		45.3 (±6.5)b
V	8	SS + SHE	vs.	RSMS + SHE
		37.5 (±9.1)a		40.2 (±8.4)a

[a] Means in each comparison followed by different letters are significantly different at the $P < 0.05$ level as determined by the paired t test.
[b] Supplemental host eggs.
[c] Red simulated moth scales.
[d] Solid sprayed with MSE.

(Comparison I, Table 1) demonstrates conclusively that *T. pretiosum* females do exhibit success-motivated searching. Beevers et al. (1981) also demonstrated success-motivated searching in *T. pretiosum* females by showing that the parasitoid's behavior, after contact with a host egg, is similar to that of females exposed to a kairomone source. Thus, contact with a host egg can contribute significantly to the probability that another host egg, located near the first, will be discovered and parasitized. Addition of simulated moth scales (RSMS) to ELUs with supplemental host eggs (SHE) (Comparison II, Table 1) increased parasitization of the eggs on the SOS, but the addition of SHE to ELUs with RSMS (Comparison III, Table 1) did not. One possible explanation for this is that at a density of 3 SOS/0.36 m^2, 144 RSMS/0.36 m^2 provides approximately optimum stimulation. Addition of supplemental host eggs to ELUs treated with a solid application of MSE (Comparison IV, Table 1) did increase rates of parasitization. Also, no significant differences in the rates of parasitization of eggs on the SOS of ELUs treated with RSMS + SHE and ELUs treated with a solid application of MSE + SHE (Comparison V, Table 1) was demonstrated. Although as Lewis et al. (1979) demonstrated, a solid application of MSE to the ELUs is considerably less than the optimum method for application of kairomone, these results demonstrate that supplemental host eggs can provide the environment in which solid application is or at least approaches optimum, i.e., a simulated high host density. Without the

addition of supplemental host eggs, some form of intermittent contact system is necessary to provide the optimum distribution of kairomone.

Greenhouse Experiment. Experiments using arenas that consisted of twelve 22.8-cm pie pans of cotyledonous stage, pink-eyed, purple hull cowpeas, arranged in a 3 × 4 pan design (ca. 0.7 m^2 of foliage) were conducted in the greenhouse (Figure 1). Two *H. zea* eggs, one on each of a pair of cotyledons, were placed in the center of 4 pans, as shown in Figure 1. These cotyledons were sprayed lightly (>0.2 ml) with MSE, using a chromatographic sprayer and a cardboard screen to restrict the distribution of MSE to the target leaves. These treated and egged spots are also referred to as simulated oviposition sites (SOS). Two vials of 6 female *T. pretiosum* each were released in the center of each arena.

Four different arena treatments were applied to the above described arena and compared: (I) 5 WSMS applied to each of the 12 pans; (II) 15 WSMS applied to each of the 12 pans; (III) 5 WSMS applied to each of the 12 pans and 2 *H. zea* eggs applied to each of the 8 pans which did not have SOS; and (IV) 15 WSMS applied to each of the 12 pans and 2 *H. zea* eggs applied to each of the 8 pans which did not have SOS.

The eggs from the four SOS and those from the eight untreated pans, in treatments III and IV, were collected and dissected separately. The mean of four ca. 2-hr readings from one arena of each treatment constituted one replication.

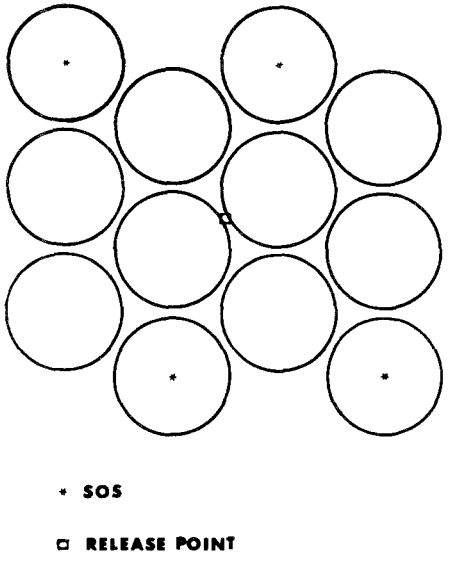

FIG. 1. Pan arrangement for greenhouse experiment.

TABLE 2. PERCENTAGE PARASITIZATION IN 12 PAN ARENAS, TREATED WITH WHITE SIMULATED MOTH SCALES (WSMS) AND SUPPLEMENTAL HOST EGGS (SHE), BY *T. pretiosum*[a]

5 WSMS/Pan		15 WSMS/pan	
SOS[b]	SHE	SOS	SHE
Arenas with WSMS only			
48.6 a		35.1 b	
Arenas with 2 SHE added to pans without SOS			
14.6 c	29.2 b	29.2 b	57.0 a

[a]Means of 15 replications. Means followed by different letters are significantly different ($P < 0.05$) as determined by Duncan's new multiple-range test.
[b]Simulated oviposition site.

The results of this experiment (Table 2) show that at the low host density, the rate of parasitization was increased more by the low rate of WSMS. However, at the higher egg density, the rate of parasitization was increased more by the higher rate of WSMS. This demonstrates again that host-egg density is an important factor in the optimum distribution of kairomone for *T. pretiosum*. As host density increases, the density of kairomone sources can be increased.

Field Experiment. Plots, 4 row × 4.6 m with 15-m separation, were set up in a field of pink-eyed, purple hull cowpeas. SOS were applied at 0.9-m intervals (5/row) by spraying individual leaves with MSE and applying one *H. zea* egg with a camel's hair brush, using Plantgard® as an adhesive. SHE and WSMS were applied in a Plantgard–H_2O (1:2) solution using a pneumatic sprayer (Nordlund et al., 1974). Eight cups of ca. 50 *T. pretiosum* were

TABLE 3. MEAN PERCENTAGE PARASITIZATION OF EGGS ON SIMULATED OVIPOSITION SITES (SOS) IN PLOTS OF PINK-EYED, PURPLE-HULL COWPEAS RECEIVING TREATMENTS OF WHITE SIMULATED MOTH SCALES (WSMS) AND SUPPLEMENTAL HOST EGGS (SHE)[a]

Treatment	Mean
I. Control	33.8a
II. 54 SHE/m	57.7b
III. 54 SHE and 16 WSMS/m	66.9b
IV. 54 SHE and 82 WSMS/m	59.3b
V. 54 SHE and 244 WSMS/m	75.7c

[a]Means of 24 replications. Means followed by different letters are significantly different at the $P < 0.05$ level as determined by Duncan's new multiple-range test.

TABLE 4. MEAN PERCENTAGE PARASITIZATION OF SUPPLEMENTAL HOST EGGS (SHE) IN PLOTS OF PINK-EYED, PURPLE-HULL COWPEAS RECEIVING TREATMENTS OF WHITE SIMULATED MOTH SCALES (WSMS) AND SUPPLEMENTAL HOST EGGS (SHE)[a]

Treatment	Mean
II. 54 SHE/m	28.0a
III. 54 SHE and 16 WSMS/m	32.3a
IV. 54 SHE and 82 WSMS/m	31.0a
V. 54 SHE and 244 WSMS/m	47.2b

[a]Means of 24 replications. Means followed by different letters are significantly different at the $P < 0.05$ level as determined by Duncan's new multiple-range test.

released in each plot. Five treatment patterns were compared: (I) SOS only (control); (II) SOS + 54 SHE/m; (III) SOS + 54 SHE and 16 WSMS/m; (IV) SOS + 54 SHE and 82 WSMS/m; and (V) SOS + 54 SHE and 244 WSMS/m.

T. pretiosum were released in each plot (8 cups of ca. 50/plot). The eggs from the SOS and samples of the SHE were collected after ca. 24 hr. This experiment was replicated 24 times.

Results (Tables 3 and 4) demonstrate the interaction between host-egg density and density of kairomone sources. Once the host-egg density is high enough for success-motivated searching to increase rates of parasitization effectively, simulated moth scales must be applied at high rates to produce any additional increase.

DISCUSSION

It is obvious that host-egg density is an important factor in the host-selection behavior of *T. pretiosum* and must be considered when attempting to utilize *Trichogramma* spp. as biological control agents, particularly when attempting to manipulate their behavior with kairomones. Much work remains to be done before we know enough about the interaction to make clear-cut recommendations as to what action to take at any particular host density.

It does appear, however, that application of nonviable or factitious host eggs to target fields in biological control programs using *Trichogramma* spp. may offer several benefits, particularly if used early in the season when naturally occurring host densities are low. One is that these additional host eggs will stimulate success-motivated searching and thus increase the rate of parasitization of the target pest. The additional host eggs will serve as

alternate hosts for these parasitoids, thereby contributing to an increase in the *Trichogramma* population early in the season and lessening the need for releases later in the season when the pest populations tend to increase (Lewis and Nordlund, 1980). Another possible benefit is the possible simplification of kairomone applications, since at a sufficiently high host density (natural or simulated), solid-spray application apparently results in the highest rate of parasitization. As the season progresses and natural host densities increase (as a result of immigrating adults), the need for supplemental eggs and/or kairomone may decrease. Also, if the *Trichogramma* population has increased sufficiently, possibly no additional control measures will be necessary (Gross, 1981, Knipling and McGuire, 1968). Parker et al. (1971) reported suppression of *Pieris rapae* (L.) by mass releases of both the pest and its parasitoids. This suppression undoubtedly resulted from the maintenance of a high host population density, which can be provided artificially as described above.

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ANTENNAL OLFACTORY RESPONSE OF BOLL WEEVIL TO GRANDLURE AND VICINAL DIMETHYL ANALOGS[1]

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Abstract—Electroantennogram techniques were used to elucidate antennal olfactory response of male and female boll weevils to a dilution series of grandlure, its components, and some vicinal dimethyl analogs. At higher concentrations, response to the mixture of the two aldehyde components of grandlure was significantly higher than to the two alcohol components. Only one vicinal dimethyl analog elicited a significantly higher response than the control. There were no significant differences in response due to sex over all compounds.

Key Words—electroantennogram, boll weevil, grandlure, pheromone, *Anthonomus grandis* Boh., Coleoptera, Curculionidae.

INTRODUCTION

Grandlure, the synthetic sex and aggregation pheromone of the cotton boll weevil, *Anthonomus grandis* Boheman, consists of two alcohols {compound I [(+)-(*Z*)-2-isopropenyl-1-methylcyclobutaneethanol] and compound II [(*Z*)-3,3-dimethyl-Δ-1-beta-cyclohexaneethanol]} and two aldehydes {compound III [(*Z*)-3,3-dimethyl-Δ-alpha-1-cyclohexaneacetaldehyde] and compound

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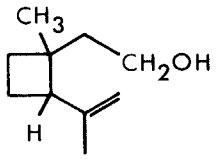
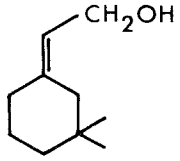
GRANDLURE COMPONENTS

I



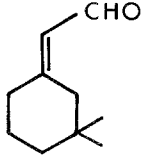
(+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol

II



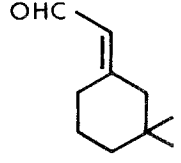
cis-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol

III



cis-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde

IV



trans-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde

FIG. 1. Structures of the grandlure components.

IV[(*E*)-3,3-dimethyl-Δ-alpha-1-cyclohexaneacetaldehyde]} (Tumlinson et al., 1969) (Figure 1). A mixture of 40% I + 30% II + 30% III + IV was found to be effective in aggregating weevils in the field (Hardee et al., 1974) and has been incorporated in pest management programs (Perkins, 1980).

Although a number of laboratory and field studies have been carried out on the behavioral responses of weevils to the pheromone and its individual components (Hardee, 1972), there have been no investigations at the antennal olfactory perception level. Therefore, we undertook the following investigation to elucidate antennal olfactory response of the boll weevil to grandlure and its components. To provide some insight into the structure–activity relationships of the pheromone, certain vicinal dimethyl analogs were also tested.

METHODS AND MATERIALS

Adult boll weevils used in the study were obtained from traps baited with grandlure and kept refrigerated until use. Electroantennograms (EAGs) (Schneider, 1957) were used as a measure of antennal olfactory responsiveness. The methods used for obtaining EAGs from beetle antennae are described in detail elsewhere (Payne, 1970, 1975). Electroantennograms were recorded with glass capillary electrodes filled with 3 M KCl. The recording and indifferent electrodes were implanted in the distal end of the antenna and in the head of the weevil, respectively. EAGs were obtained from dilution series of grandlure (mixture of 40% I + 30% II + 30% III + IV), compounds I, II, and a mixture of III + IV. Also tested were four vicinal dimethyl analogs of grandlure component II {compound 4 [(±)-(*Z*)-2-(*cis*-3,4-dimethylcyclohexyliden)ethanol]; compound 4a [(±)-(*E*)-2-(*cis*-3,4-dimethylcyclohexyliden)-ethanol]; compound 5[(±)-(*Z*)-2-*trans*-3,4-dimethylcyclohexyliden)ethanol]} and two vicinal dimethyl analogs of a 1:1 mixture of components III and IV{compound 6[(*Z*) + 7(*E*), (±)-(*cis*-3,4-dimethylcyclohexyliden) acetaldehyde]; compound 8[(*Z*) + 9(*E*), (±)-*trans*-3,4-dimethylcyclohexyliden) acetaldehyde]} (Figure 2). All compounds had 99% purity by GLC.

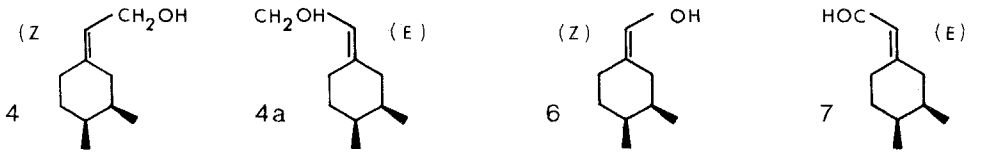
Grandlure and its components were prepared in dilution series of 10^{-3} to 10^{1} μg of compound/μl of *n*-pentane. The dimethyl analogs were prepared in series of 10^{-3} to 10^{2} μg compound/μl *n*-pentane.

Each insect preparation was exposed to *n*-pentane as a control, 10 μg/μl of grandlure as a standard, and no more than one dilution series of a compound or mixture in increasing concentrations. A 5-min interval between each stimulation was sufficient to allow the olfactory receptors to recover to the initial response level. Ten μl of a given sample (or the control) were placed

GRANDLURE COMPONENTS

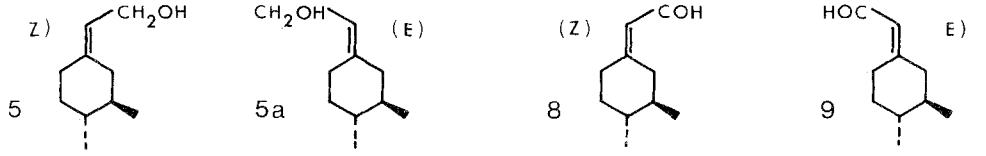
II III IV

VICINAL DIMETHYL ANALOGUES



(±)-2-(*cis*-3,4-dimethylcyclohexyliden) ethanol

(±)-(*cis*-3,4-dimethylcyclohexyliden) acetaldehyde



(±)-2-(*trans*-3,4-dimethylcyclohexyliden) ethanol

(±)-(*trans*-3,4-dimethylcyclohexyliden) acetaldehyde

FIG. 2. Structures of vicinal dimethyl analogs of grandlure components II, III, and IV.

on a piece of Whatman No. 1 filter paper contained in a glass tube and delivered via a 2 l/min airflow through a three-way valve (Payne, 1975). For all derivatives and their respective controls and standards, only 5 μl was used due to scarce supply. Although it is the amount of compound that evaporates from the filter paper that is involved in stimulating the antenna, later discussion refers to stimulation in terms of the amount of compound on filter paper.

Differences in antennal olfactory response to behavioral chemicals has been reported between the sexes for another coleopterous pest (Dickens and Payne, 1977), therefore, male and female boll weevils were tested separately.

Five replicates were obtained for each sex to grandlure and its components; three replicates were obtained for each sex to all analogs. All data were converted to mean percentage of EAG response to the standard (10 μg and 5 μg of grandlure, respectively) $\pm$ standard error and subjected to analysis of variance and Duncan's multiple-range test (Ott, 1977).

RESULTS AND DISCUSSION

Unlike laboratory or field bioassays, where all four grandlure components have to be present for optimum behavioral response (Tumlinson et al., 1969; Hardee et al., 1974), both male and female weevils responded electrophysiologically to single components of the mixture at the antennal olfactory level (Figure 3). A general linear models procedure (Helwig and Council, 1979) showed no significant differences between sexes, over all compounds and all concentrations within compounds. Thresholds of response were lower for grandlure and component I than for component II and the mixture of III + IV (Table 1). At the higher concentrations, response to the alcohols was significantly lower than to the aldehydes. No differences in response were found between grandlure and component I at the threshold concentration, and grandlure and the mixture of the aldehydes at 100 μg. In fact, the results indicate that the threshold of response to grandlure could be accounted for by component I, whereas the levels of response at the higher concentrations of grandlure could be attributed to components III + IV. Since the intensity of the EAG is reported to be a relative measure of the number of available receptors for a given stimulus (Payne, 1975; Payne and Dickens, 1976), it appears that the boll weevil has more receptors for the aldehydes as compared to the alcohols.

Receptor specificity for pheromone structure was such that structural modifications (vicinal dimethyl analog) generally elicited less response than the pheromone components. Only vicinal dimethyl analog 4 elicited a response significantly higher than *n*-pentane (Figure 4).

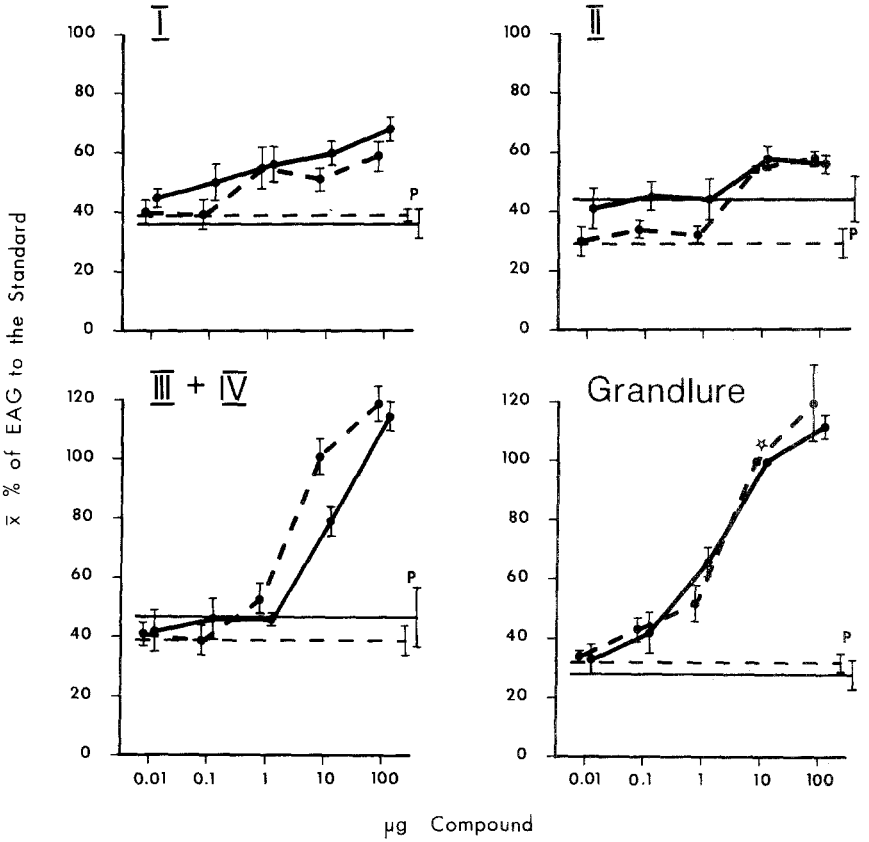


FIG. 3. Mean percent EAG response of boll weevils to grandlure and its components. Data presented as percent of response to the standard (grandlure 10 μg). Data points represent five replicates. Solid lines = females; broken lines = males; vertical bars = standard errors; P = *n*-pentane control; open star = standard.

TABLE 1. MEAN PERCENT EAG RESPONSE OF BOLL WEEVILS TO THREE CONCENTRATIONS OF GRANDLURE COMPONENTS[a]

Compound	$\bar{X}$ Response (%)[b]		
	1	10	100
Grandlure	58.8 a	100.0 a	115.8 a
Compound I	55.3 a	55.6 c	63.4 b
Compund II	[c]	56.2 c	57.0 b
Compounds III + IV	[c]	89.7 b	116.8 a

[a] Mean percent of EAGs to the standard (10 μg grandlure).
[b] Reading vertically, numbers with same letters are not significantly different at the 5% level according to Duncan's multiple-range test.
[c] Only means significantly higher than the control are listed in the table.

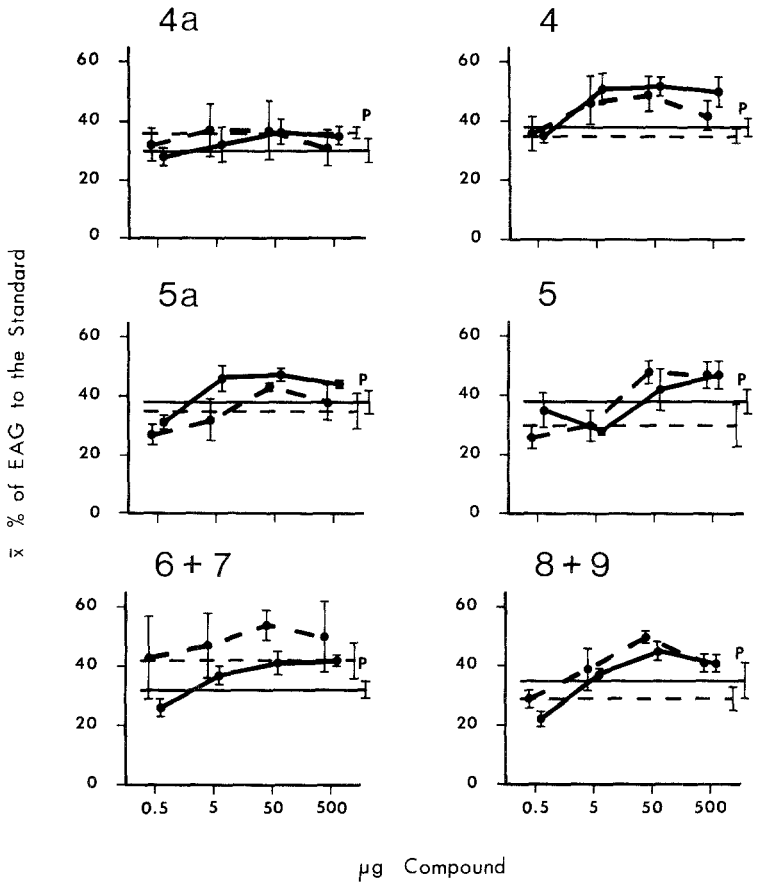


FIG. 4. Mean percent EAG response of boll weevils to vicinal dimethyl analogs of grandlure components II and III + IV. Data presented as percent of response to the standard (grandlure 5 μg). Data points represent four replicates for females, and three replicates for males. Solid lines = females; broken lines = males; vertical bars = standard errors; P = *n*-pentane control.

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ALLEVIATION OF α-TOMATINE-INDUCED TOXICITY TO THE PARASITOID, *Hyposoter exiguae*, BY PHYTOSTEROLS IN THE DIET OF THE HOST, *Heliothis zea*

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Abstract—The solitary, endoparasitic ichneumonid, *Hyposoter exiguae* (Viereck) is a parasitoid of the tomato fruitworm, *Heliothis zea* (Boddie). However, the parasitoid is deleteriously affected by the tomato glycoalkaloid, α-tomatine, ingested from hosts (*H. zea*) fed on artificial diets or semipurified extracts of tomato plants that contained α-tomatine. α-Tomatine causes prolonged larval development; disruption or prevention of pupal eclosion; deformation of antennal, abdominal, and genital structures; and reduction in adult weight and longevity of the parasitoid. These toxic effects are exacerbated when the dietary dose of α-tomatine is increased from 12 μmol to 20 μmol/g dry wt of diet. However, the toxicity of α-tomatine is attenuated in parasitoids reared from hosts fed on artificial diets that contain equimolar or supramolar amounts of 3β-OH-sterols admixed with α-tomatine. Further, the toxicity of extracts from the foliage of different cultivars of tomatoes to *H. exiguae* is contingent upon the composite levels of α-tomatine and total phytosterol (free and esterified). Cultivars with a low total sterol–tomatine ratio are more toxic to *H. exiguae*. The possible mode of action of α-tomatine toxicity to *H. exiguae* by disruption of sterol metabolism is discussed.

Key Words—Host-plant resistance, α-tomatine, sterols, sterol esters, *Lycopersicon esculentum*, *Heliothis zea*, Lepidoptera, Noctuidae, parasitoids, *Hyposoter exiguae*, Hymenoptera, Ichneumonidae, secondary compounds.

INTRODUCTION

The larval stage of the noctuid, *Heliothis zea* (Boddie), is a major pest of the commercial tomato (*Lycopersicon esculentum* Mill.) in many areas through-

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out North America (Wilcox et al., 1956; Wyman et al., 1979; Metcalf and Flint, 1962). In California, the solitary, endoparasitic ichneumonid, *Hyposoter exiguae* (Viereck), commonly parasitizes young larvae of *H. zea* and hence is a promising biological control agent of *H. zea* (Michelbacher and Essig, 1938; Wilcox et al., 1956; Puttler 1961; Campbell and Duffey, 1979b).

Alternatively, the increasing costs of pesticides and concern over their potential detrimental impact on the environment has encouraged research that places more emphasis on the breeding of tomato cultivars which will be resistant to *H. zea* (Canarday et al., 1969; Fery and Cuthbert, 1973, 1974; Cosenza and Green, 1979). As an extension of efforts to breed insect-resistant tomatoes, there have been attempts to isolate and identify natural products in the foliage and fruit that may contribute to resistance of tomato plants to insects via chemical antibiosis. A volatile, 2-tridecanone, from the foliage of the wild tomato, *L. hirsutum* f. *glabratum* C.H. Mull, (Williams et al., 1980) and certain flavonoids, in particular rutin, isolated from commercial varieties of tomatoes (Duffey et al., 1980) are toxic to larvae of *H. zea*. However, α-tomatine, the major glycoalkaloid found in the foliage and unripened fruit of tomatoes, is toxic to the parasitoid *H. exiguae* (Campbell and Duffey, 1979a). α-Tomatine was acquired by the parasitoid from hosts which ingested the alkaloid from artificial diets. However, toxicity was alleviated by addition of equi- or supramolar levels of cholesterol to diets containing α-tomatine.

In this paper the results of more definitive investigations on the effects of mixtures of plant sterols and α-tomatine on the development of *H. exiguae* are described. These investigations included the use of selected genotypes of *L. esculentum* as natural sources of these compounds. Also, a possible mode of action of α-tomatine toxicity to *H. exiguae* is discussed based on features of the life history of the parasitoid, manifestations of α-tomatine toxicity in the parasitoid, and physical–chemical interactions between different sterols and α-tomatine.

METHODS AND MATERIALS

Rearing of Insects. Larvae of *H. zea*, hatched from eggs provided by the Shell Developmental Co., Modesto, California, were reared as previously outlined (Campbell and Duffey, 1979b). Wasps of *H. exiguae* were reared from pupae procured from Dr. E.R. Oatman, Department of Entomology, University of California, Riverside, and Ms. P.G. Hotchkin, Department of Entomology, University of California, Davis. The wasp colony was maintained as previously described (Campbell and Duffey, 1979b).

Methods of Parasitization. Cohorts of 20 second-instar larvae of *H. zea* were placed with one *H. exiguae* wasp for 24 hr in a 0.95-liter container. After exposure to the wasps, the host larvae were transferred to individual cups (30

ml) containing 1-g pellets of experimental diet. Each larva was inspected every 12 hr to determine the time of pupation of the parasitoid. Cocoons of parasitoids were inspected daily to assess time of emergence of wasps. After emergence, each wasp was transferred to a 237-ml container supplied with an excess of honey. Wasps were examined daily and fresh weight was recorded on day of death. Each cohort was replicated 5 times per diet treatment.

Preparation of Artificial Diets with Supplements. Experimental diets were based on the Custom Southern Corn Borer Diet #1107 (Bio-Serv, Frenchtown, New Jersey). The dry components of the Bio-Serv diet and the natural product(s) to be tested were mixed for 5 min in a pulverizer (Wig-L-Bug®, Crescent Dental Mfg. Co., Chicago, Illinois) and then added to the agar, water, and vitamin mixture to complete the diet. The natural products of *Lycopersicon* tested were: α-tomatine, tomatidine, cholesterol, sitosterol, and cholesterol oleate (Sigma Chemical Co.). Equimolar mixtures of the following chemicals were also added to diets: α-tomatine and cholesterol, α-tomatine and cholesterol oleate, and α-tomatine and sitosterol. Diets were made at 12 μmol/g (3.0%) and 20 μmol/g (5.0%) on a dry weight basis for each supplement and equivalent to moderate and high levels, respectively, of α-tomatine in foliage of *Lycopersicon* (see Roddick, 1974).

The above preparative method was tested for its ability to distribute water-soluble and -insoluble supplements homogeneously throughout the diet. Subsamples of a test diet incorporated with [^{3}H]digitoxin (New England Nuclear) (water insoluble) and salicylic acid (water soluble) were taken with a spatula from the top, center, bottom, and edges, and then each was weighed. [^{3}H]Digitoxin was extracted with $CHCl_3$ and quantified via scintillation counting. Salicylic acid was extracted using EtOH and quantified colorimetrically using the ferric chloride test for phenols (Wesp and Brode, 1934). Results showed that both [^{3}H]digitoxin and salicylic acid were evenly distributed in the subsamples with the coefficient of variation $<1.0\%$. We assumed that α-tomatine and the sterols were, likewise, homogeneously distributed.

Preparation of Diets Containing Extracts of Leaf Powders. Leaf powders were prepared by grinding lyophilized foliage pooled from 6–8 plants (grown in outdoor plots) of each of the following varieties of *L. esculentum:* VFN Bush, VF 198, VF 315, UC 134, and Royal Flush. Leaf powder of each variety equivalent to the dry weight of the artificial diet (i.e., approx. 2.5 g dry wt/20 g wet wt) was leached individually for 12 hr in 200 ml 95% EtOH (acidified with AcOH; pH 4), filtered, decanted, and the residue reextracted for 1 hr in boiling 95% EtOH and refiltered. Both extracts were combined and evaporated to dryness in vacuo at 40° C. The gum was extracted with 20 ml of hot H_2O (pH 5, 80° C), cooled, and defatted by partitioning against 20 ml *n*-hexane. The remaining water-insoluble residue of the gum was dissolved in

minimal Et_2O and added to the *n*-hexane fraction. The hexane fraction was evaporated to dryness in vacuo, redissolved in 50 ml Et_2O, and poured onto the dry ingredients of the artificial diet. This slurry was stirred gently at room temperature until all Et_2O evaporated. A given diet was completed by adding the defatted H_2O fraction (see above). Control diets were prepared by evaporating 50 ml of Et_2O from the dry ingredients and using distilled H_2O. Both water and hexane fractions were combined in preparing diets of extracts of VF 198, VF 315, UC 134, and Royal Flush. For VFN Bush, one diet was prepared using only the water fraction, one diet using only the hexane fraction, and one diet using the combined fractions.

Extraction and Quantification of α-Tomatine. Lyophilized tomato foliage (approx. 5.0g) was ground with acid-washed sand in hot MeOH, the homogenate filtered, and the residue similarly reextracted and refiltered. The pooled filtrates were diluted to 25% MeOH with H_2O, acidified with AcOH (pH 4), and partitioned against an equal volume of *n*-hexane followed by Et_2O. The water fraction was dried in vacuo, and the residue dissolved in 50% MeOH and stored at 0° C. α-Tomatine was later quantified using a blood hemolysis assay for saponins as outlined previously (Campbell and Duffey, 1979a).

Extraction, Identification, and Quantification of Sterols. Free sterols and sterol esters were identified and quantified from extracts of the artificial diet, 3rd-instar larvae of *H. zea*, 3rd-instar larvae and wasps of *H. exiguae*, and the foliage of the five varieties of *L. esculentum*. Extraction of lipids followed procedures of Rouser et al. (1963). Lyophilized tissue or diet was extracted in 50 ml of $CHCl_3$–MeOH (2:1, v/v) by grinding in a glass tissue homogenizer. The extract was filtered, the residue reextracted in 50 ml $CHCl_3$ and refiltered and the filtrates pooled and partitioned against 50 ml basified H_2O (pH ~ 8). The $CHCl_3$ fraction was evaporated to dryness and stored at 0° C under N_2 gas.

Next, free sterols and sterol esters were purified by column chromatography on Florisil®, as described by Carroll (1961). Individual, semipurified $CHCl_3$ extracts (see above) were dissolved in *n*-hexane and applied to a column of Florisil (Sigma Chemical Co.) (3 g, 35 × 0.5 cm). The lipid classes were serially eluted via the following solvents: 5 ml *n*-hexane, 15 ml 5% Et_2O in hexane, 30 ml 15% Et_2O in hexane, 25 ml 25% Et_2O in hexane, and 15 ml 50% Et_2O in hexane. Sterol esters and free sterols were eluted in the 15% and 50% Et_2O–hexane fractions, respectively. The fractions containing sterol esters and free sterols were further purified by TLC on silica gel using *n*-hexane–Et_2O–AcOH (90:10:1; v/v/v) as the mobile phase (Mangold and Malins, 1960). After purification by TLC, sterol esters were eluted and subsequently saponified in 33% KOH at 37–40° C for 1 hr (Abell et al., 1952) and the resulting free sterols semipurified via TLC (see above).

Each TLC-purified sterol was identified as a TMS ether by GLC according to its relative retention time (RRT) to cholestane. Quantification of each known sterol was based upon GLC peak heights, in comparison to its purified reference sterol (Supelco, Inc., Bellefonte, Pennsylvania). TMS ethers were made using *N*-methyl-*N*-trimethylsilyl-trifluoracetamide (MSTFA) with trimethylchlorosilane (TMCS) added as a catalyst (Pierce Chemicals, Rockford, Illinois). GLC was performed using the following specifications on a Varian 3700 gas chromatograph: column, 2 m × 3.2 mm (stainless steel) with a precolumn 12 cm × 2 mm (glass), 5% OV-101 on Chromsorb W-HP 80/100 mesh; temperatures, injector 250° C, detector (FID) 300° C, column 250° C isothermal. GLC peaks which did not correspond to available reference sterols were designated as "unknown 1, unknown 2, . . . , etc." An estimate of quantity of each unknown sterol was based upon equivalents of peak height referenced to cholesterol.

Determination of Relative Uptake of Free Sterol and Sterol Ester by Heliothis zea *Larvae*. Artificial diets (20 g) were prepared with supplements of ~60 μmol of [^{14}C]cholesterol palmitate (specific activity 9.7 μCi/mmol) with equimolar amounts of α-tomatine (at 20 μmol/g dry wt diet). [^{14}C]cholesterol palmitate was synthesized from [4-^{14}C]cholesterol (9.7 μCi/mmol) (New England Nuclear) and analytical grade palmitic acid (Nutrional Biochemical Corp., Cleveland, Ohio) as outlined by Vogel (1967), then purified by TLC. The control diet contained only [^{14}C]cholesterol (60 μmol/20 g diet). Twenty larvae of *H. zea* were reared on each diet for 8 days, and the larvae then starved for 24 hr. On the 10th day, individual larvae were weighed and extracted for total lipids (see above). Crude extracts of individual larvae were transferred to scintillation vials, dried, and then monitored for radioactivity.

RESULTS

Effects of Sterols on α-Tomatine toxicity to H. exiguae. Pupal eclosion of *H. exiguae* is significantly reduced when parasitoids are reared from hosts fed on diets containing either 12 μmol or 20 μmol of α-tomatine/g dry wt diet (Campbell and Duffey, 1979a). However, addition of equimolar amounts of cholesterol or cholesterol oleate to the 12-μmol or 20-μmol α-tomatine diets of the host resulted in improved pupal eclosion of the parasitoid (Figure 1). The pupal eclosion of parasitoids reared from hosts fed on a control diet was >85%. When parasitoids were reared from hosts fed on diets containing 12 μmol or 20 μmol α-tomatine/g dry wt diet, pupal eclosion of the parasitoids declined significantly ($F_{5,32} = 5.62, P < 0.05$) to ~60%. In contrast, equimolar amounts of cholesterol and α-tomatine in a diet resulted in the alleviation of toxicity as shown by a normal percentage of pupal eclosion of *H. exiguae* at 12 μmol (i.e., ~85%). At 20 μmol, cholesterol was less effective in alleviating

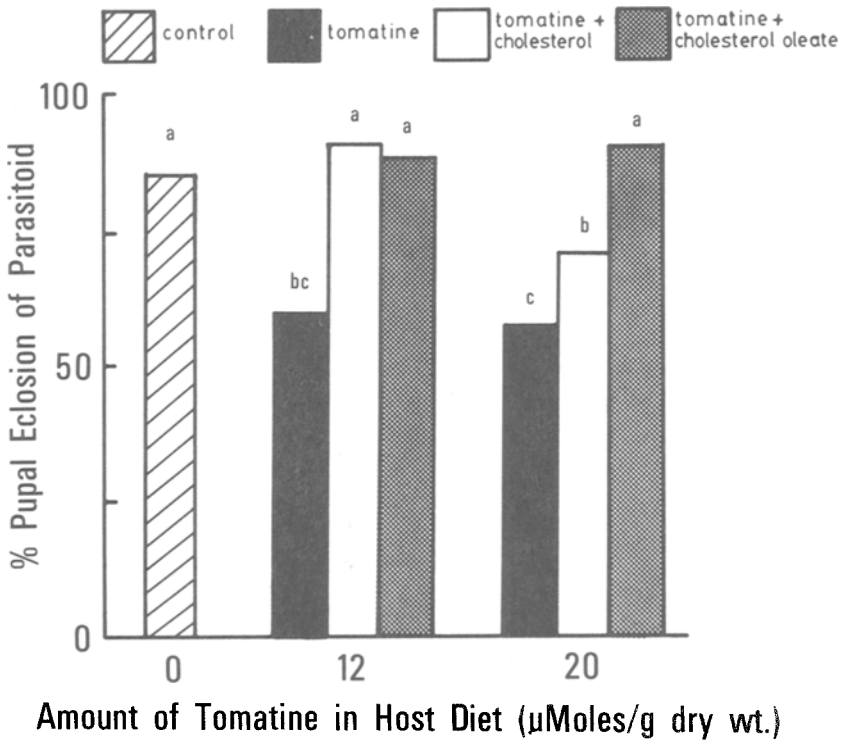

FIG. 1. Histograms representing the percent pupal eclosion of *H. exiguae* reared from larvae of *H. zea* fed on control diets, diets containing 12 or 20 μmol/g (dry wt) α-tomatine, or diets containing either cholesterol or cholesterol oleate admixed with equimolar amounts of α-tomatine. Histograms with different letters are significantly different ($P < 0.05$). (Percent pupal eclosion is retranslated from arcsin-transformed percentages.)

toxicity, but pupal eclosion still improved to ~72%. Similarly, the addition of equimolar amounts of the sterol ester, cholesterol oleate, and α-tomatine to diets resulted in the alleviation of toxicity and hence, normal percentages of pupal eclosion by *H. exiguae* in both the 12-μmol and 20-μmol diet cohorts. Experiments with diets containing sitosterol, cholesterol, cholesterol oleate, and cholesterol plus sitosterol at 32 μmol/g dry wt diet without α-tomatine showed that these sterols, by themselves, had no effect on parasitoid development in comparison to a control diet.

Approximately 20% of the wasps that emerged from hosts fed the 20-μmol α-tomatine diet exhibited malformations in antennal and genital structures (Figure 2). Wasps reared from hosts fed on a 12-μmol α-tomatine diet did not show these deformities. The chief antennal deformities in wasps were invagination (collapse) and convolution of the distal flagellomeres

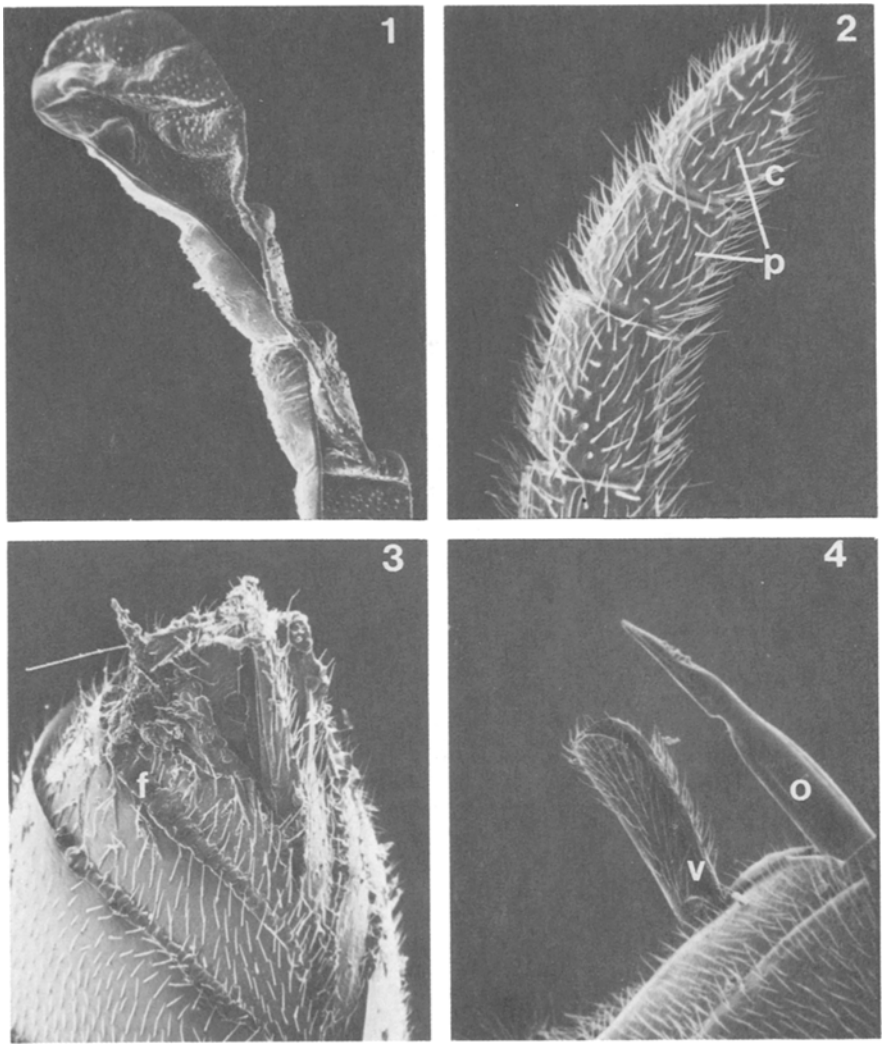

FIG. 2. Scanning electron micrographs of the distal portion of the antennae and abdominal terminalia of wasps of *H. exiguae*: (1) distal flagellomeres of antennae of wasp reared from *H. zea* fed on a 20-μmol α-tomatine diet; (2) distal flagellomeres of a wasp reared from *H. zea* larvae fed on a control diet (c = sensilla chaetica; p = sensilla placodea); (3) terminal abdominal segment of a wasp reared from α-tomatine-treated host (f = fused abdominal sternites); (4) terminal abdominal segments of a control female wasp (o = ovipositer; v = valvifers).

(Figure 2-1), which were terete in normal wasps (Figure 2-2). Additionally, the deformed flagellomeres lacked sensilla chaetica (c, Figure 2-2) and sensilla placodea (p, Figure 2-2). The major genital deformities (Figure 2-3) in wasps reared from hosts fed the 20-μmol diet were an unextruded ovipositor and a lack of valvifers. Also, sclerotization of the intersegmental membrane caused fusion of the last three abdominal sternites (f, Figure 2-3). In healthy wasps the valvifers (v, Figure 2-4) and ovipositor (o, Figure 2-4) are plainly visible. Furthermore, addition of equimolar amounts of cholesterol or cholesterol oleate to 20-μmol α-tomatine diets of the host fully eliminated the development of deformities.

Absorption of Free Sterols and Sterol Esters by Heliothis zea *Larvae.* The homogeneity of artificial diets incorporated with α-tomatine and [^{14}C]cholesterol or [^{14}C]cholesterol palmitate and the respective amounts of either of these isotopes absorbed by larvae of *H. zea* is represented in Figure 3. Larvae reared on the diet containing only [^{14}C]cholesterol showed the highest level of absorption with 2.4 ± 0.3 μmol of labeled sterol/g body wt, while larvae reared on the [^{14}C]cholesterol + α-tomatine diet showed the lowest levels of absorption at 1.0 ± 0.1 μmol/g. Larvae reared on the [^{14}C]cholesterol palmitate + α-tomatine diet showed a level of absorption at 1.3 ± 0.2

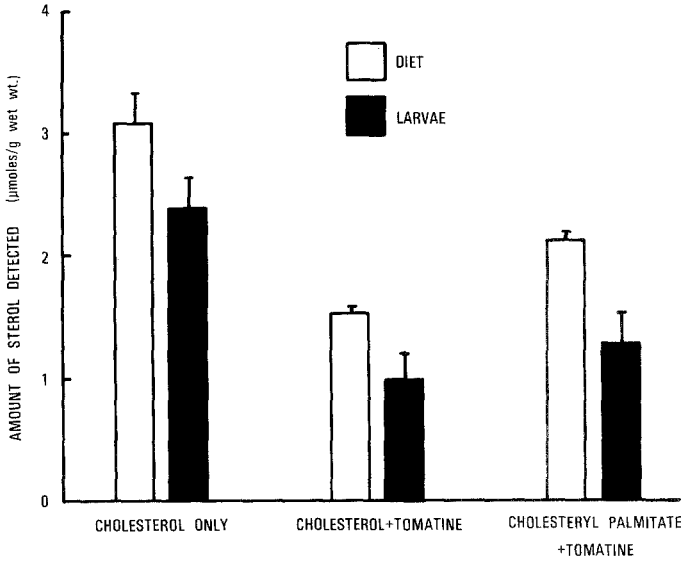

FIG. 3. Histograms showing the relative mean amounts of sterol detected in diets and larvae of *H. zea* reared for 8 days on artificial diets. [^{14}C]cholesterol and [^{14}C]cholesterol palmitate (specific activity = 9.7 μCi/mmol) were added to diets at 3 μmol/g wet wt (= ~24 μmol/g dry wt). α-Tomatine was added at 20 μmol/g dry wt. Bars above histograms represent 1 standard error.

μmol/g, which was not significantly different ($P > 0.10$) than the [^{14}C]cholesterol + α-tomatine cohort. Even though host larvae reared on the [^{14}C]cholesterol-only diet showed a significantly greater ($F_{2,52} = 21.38, P < 0.05$) absorption of labeled sterol than host larvae in the other cohorts, this is probably pharmacologically insignificant in that the absorption of sterols by larvae in all cohorts were of the same order of magnitude. Also, the proportion of labeled sterol in the diet to the amount absorbed by the larvae was similar for all cohorts, and it appeared that α-tomatine had little effect on sterol absorption by the host.

Comparison of Free Sterols and Sterol Esters in Artificial Diets, Heliothis zea *Larvae, and* Hyposoter exiguae *Larvae and Adults.* The retention times (RT) and relative retention times (RRT) to cholestane-TMS of the sterols (including unknown sterols) quantified by GLC are listed in Table 1. Because our GLC peaks were not critically identified by mass spectroscopy, our identification of the sterols present is subject to error by the possibility that some of our peaks represented sterols contaminated with fatty alcohols or solely fatty alcohols (the unknowns?). The identity and amount of sterols, free and esterified from the artificial diet of *H. zea*, 3rd-instar larvae of *H. zea*, and 3rd-instar larvae and adults of *H. exiguae* are outlined in Table 2. Only six sterols were present in the free sterol and sterol ester fractions of the artificial diet. These sterols were cholesterol, campesterol, stigmasterol, sitosterol, and unknowns 3 and 4. Sitosterol was the most abundant free sterol

TABLE 1. RETENTION TIMES (RT) AND RELATIVE RETENTION TIMES (RRT) TO CHOLESTANE-TMS OF TMS-DERIVATIZED STEROLS[a] AND UNKNOWNS DETECTED BY GLC ANALYSIS

Sterol-TMS	RT (sec)	Mean RRT
Cholestane	260–70	—
Cholesterol	505–15	1.92
Desmosterol	550–60	2.10
Campesterol	630–40	2.40
Stigmasterol	680–90	2.58
Sitosterol	770–80	2.92
Fucosterol[b]	770–80	2.92
Ergosterol[b]	605–15	2.30
Unknown 1	430–50	1.65
Unknown 2	460–70	1.73
Unknown 3	940–50	3.50
Unknown 4	845	3.15
Unknown 5	577	2.17

[a]Based upon known sterol standards.
[b]Not detected in any of the extracts of lipids of the diet, insects, or tomato foliage.

TABLE 2. AMOUNTS OF INDIVIDUAL FREE STEROLS AND STEROL ESTERS IN ARTIFICIAL DIET OF *H. zea*, 3RD INSTAR LARVAE OF *H. zea*, 3RD INSTAR LARVAE OF *H. exiguae*, AND ADULTS OF *H. exiguae* (IN μMOL/G DRY WT) AS ANALYZED BY GLC

Artificial Diet		*H. zea* (3rd instar)		*H. exiguae* (3rd instar)		*H. exiguae* (adult)	
Sterol	μmol/g	Sterol	μmol/g	Sterol	μmol/g	Sterol	μmol/g
Free sterols							
Cholesterol	0.5	Cholesterol	11.5	Cholesterol	11.3	Cholesterol	3.4
Campesterol	0.2	Campesterol	0.1	Campesterol	0.3	Campesterol	0.1
Stigmasterol	0.6	Stigmasterol	2.2	Stigmasterol	0.1	Stigmasterol	0.4
Sitosterol	1.3	Sitosterol	6.3	Sitosterol	1.6	Sitosterol	0.5
Unknown 3	Trace	Unknown 1	4.6[a]	Unknown 1	0.9[a]	Desmosterol	0.1
Unknown 4	Trace	Unknown 2	1.0[a]	Unknown 3	1.0[a]		
				Unknown 4	0.3[a]		
Sterol esters[b]							
Cholesterol	3.1	Cholesterol	7.5	Cholesterol	2.7	Cholesterol	7.5
Campesterol	0.8	Campesterol	1.4	Stigmasterol	0.6	Campesterol	0.2
Stigmasterol	5.5	Sitosterol	1.4	Sitosterol	0.1	Stigmasterol	0.1
Sitosterol	0.2	Desmosterol	1.6	Desmosterol	0.4	Sitosterol	1.2
Unknown 3	Trace	Unknown 1	14.5[a]	Unknown 4	0.2[a]	Desmosterol	0.1
Unknown 2	Trace	Unknown 3	6.5[a]			Unknown 3	0.9[a]
		Unknown 4	2.9[a]			Unknown 4	0.3[a]
		Unknown 5	Trace			Unknown 5	0.3[a]

[a]Quantities based upon GLC peak heights to cholesterol standards.
[b]Saponified.

(at 1.3 μmol/g dry wt) and stigmasterol was the most abundant sterol (a total of 6.1 μmol/g in both free and ester fractions).

Ten sterols were identified in 3rd-instar larvae of *H. zea* (Table 2). The same phytosterols detected in the artificial diet were found in the host larvae with the addition of a number of other sterols (desmosterol and unknowns 1–5) that were probably intermediates in sterol metabolism (see Svoboda et al., 1978). The parasitoid larvae also accumulated sterols as indicated by a total of 61.4 μmol sterols/g dry wt in comparison to the 12.1 μmol/g in the diet. The more abundant sterols in the larvae were cholesterol (a total of 19 μmol/g) and unknown 1 (at 19.1 μmol/g). The greater proportion of cholesterol, over other sterols, in larvae of *H. zea* indicated that phytosterols ingested from the diet were either metabolically converted to cholesterol or that cholesterol was selectively absorbed from the diet and/or preferentially incorporated into tissues. Evidence of this is seen in the case of stigmasterol, which was the major sterol in the diet but only a minor sterol in host larvae (at 2.2 μmol/g).

There were fewer sterols in the larvae of the parasitoid than in its host (7 vs. 10, respectively, Table 3). Also, in the tissues of the larvae of *H. exiguae*, the level of total sterol was lower than that of its host (19.4 μmol/g vs. 61.4 μmol/g, respectively). Some of the phytosterols detected in the artificial diet were found in the larvae of the parasitoid, indicating that these sterols from the diet had not been completely degraded or metabolized by the host larvae. However, cholesterol was >2.5 times more in abundance in the tissues of the larvae of the parasitoid than all other sterols combined (Table 3). The phytosterols, campesterol, stigmasterol, sitosterol, and unknowns 3 and 4, occurred in the wasp of *H. exiguae*, indicating that these sterols persisted through the pupal stage of the parasitoid (Table 2). However, cholesterol was

TABLE 3. TOTAL NUMBER OF DIFFERENT STEROLS, RATIOS OF CHOLESTEROL TO OTHER STEROLS, AND RATIOS OF TOTAL FREE STEROL TO STEROL ESTER IN ARTIFICIAL DIET OF *H. zea*, 3RD INSTAR LARVAE OF *H. zea*, AND 3RD INSTAR LARVAE AND ADULTS OF *H. exiguae*

	Diet	Larvae of *H. zea*	Larvae of *H. exiguae*	Adults of *H. exiguae*
Total number of sterols[a]	6	10	7	8
Total cholesterol: other sterols[b]	0.42	0.45	2.59	2.73
Free sterol: sterol ester	0.26	0.72	2.85	0.43

[a]Based on total number in both free sterol and sterol ester fractions.
[b]Based on total number of μmol/g in both free sterol and sterol ester fractions.

by far the dominant sterol at being >2.7 times more abundant in the wasp than all other sterols combined (Table 3).

Lastly, there was a notable difference between the ratio of free sterol to sterol ester in the larvae of *H. exiguae* and this ratio in the diet, host, and wasp (Table 3). In the larvae of the parasitoid the amount of free sterol was >3 times that of the sterol esters, whereas in the artificial diet, larvae of the host, and wasps of the parasitoid, the amount of sterol ester was ~2–3 times that of the free sterols.

Effects of Extracts of Foliage of Various Tomato Cultivars on Development and Longevity of Hyposoter exiguae. The effects of the combined water and hexane extracts of foliage of tomato cultivars VF 315, UC 134, VF 198, and Royal Flush on the larval and pupal development, pupal eclosion and adult weight and longevity of *H. exiguae* are outlined in Table 4. Extracts of foliage of the different cultivars affected dry weight ($F_{4,27} = 4.32$, $P \sim 0.05$) and longevity ($F_{4,27} = 4.87$, $P < 0.025$) of the wasps. The largest difference in wasp weight occurred between the control and VF 315-treated cohorts (1.4 vs. 1.0 mg, respectively). Wasps reared from hosts fed the control diet or diets containing extracts of UC 134 or Royal Flush lived significantly longer than wasps reared from hosts fed on the diet containing the extract of VF 315 (~18–20 days vs. ~6 days, respectively, see Table 4). Wasps reared from hosts fed on the diet containing the extract of VF 198 had intermediate longevities (~16 days). None of the extracts of any of these cultivars affected duration of larval or pupal period. There were no significant differences between the cohorts in percentage pupal eclosion nor were any physical deformities seen in any of the wasps reared from hosts fed on diets containing these extracts.

The duration of larval and pupal periods, percent pupal eclosion, and wasp weight and longevity of *H. exiguae* reared from hosts fed on the

TABLE 4. EFFECTS OF COMBINED HEXANE AND WATER EXTRACTS OF DIFFERENT VARIETIES OF TOMATO PLANTS (*L. esculentum*) INCORPORATED INTO ARTIFICIAL DIETS OF *H. zea* ON BIOLOGICAL PARAMETERS OF *H. exiguae* (MEANS ± 1 SE)[a]

Tomato variety	Larval period (days)	Pupal period (days)	Pupal eclosion (%)	Adult weight (mg)	Adult longevity (days)
Control	8.2 ± 0.7 (NS)	8.5 ± 0.2 (NS)	93.5	1.4 ± 0.1 b	18.7 ± 1.3a
VF 315	8.2 ± 0.3 (NS)	8.7 ± 0.3 (NS)	86.1	1.0 ± 0.1 a	6.3 ± 1.5b
UC 134	7.8 ± 0.5 (NS)	8.7 ± 0.3 (NS)	89.4	1.3 ± 0.1 ab	21.3 ± 1.2a
VF 198	7.7 ± 0.3 (NS)	8.3 ± 0.2 (NS)	87.3	1.3 ± 0.1 ab	16.3 ± 3.2ab
Royal Flush	8.3 ± 0.3 (NS)	8.5 ± 0.3 (NS)	93.5	1.2 ± 0.2 ab	21.3 ± 2.1a

[a]Means followed by different letters within a column are significantly different ($P = 0.05$); NS = not significant.

TABLE 5. EFFECTS OF PARTITIONED EXTRACTS OF FOLIAGE OF VFN BUSH INCORPORATED INTO ARTIFICIAL DIETS OF *H. zea* ON BIOLOGICAL PARAMETERS OF PARASITOID *H. exiguae*. (MEANS ± 1 SE)[a]

Foliage extract	Larval period (days)	Pupal period (days)	Pupal eclosion (%)	Adult weight (mg)	Adult longevity (days)
Control	6.8 ± 0.1ab	8.5 ± 0.2 (NS)	89	1.4 ± 0.0 (NS)	14.7 ± 2.1ab
Water + hexane	7.2 ± 0.2bc	8.3 ± 0.2 (NS)	90	1.4 ± 0.1 (NS)	18.3 ± 1.9ab
Water	7.6 ± 0.2c	9.8 ± 0.2 (NS)	88	1.1 ± 0.1 (NS)	10.7 ± 1.9b
Hexane	6.4 ± 0.2a	8.3 ± 0.2 (NS)	89	1.7 ± 0.3 (NS)	23.6 ± 4.8a

[a]Different letters within a column denote significant differences ($P < 0.05$); NS = no significant difference.

partitioned extracts of foliage of VFN Bush are summarized in Table 5. The duration of the larval period of wasps reared from hosts fed on the control diet or diet with the hexane extract was ≃6.5 days. The larval period of wasps reared from hosts fed on the diet containing the water extract was significantly ($F_{3,32} = 7.9, P < 0.005$) longer at ≃7.6 days. The larval period of the cohort of wasps from the diet containing the water + hexane extracts was intermediate to that of the control hexane-extract cohorts and water-extract cohort at ≃7.2 days (Table 5.)

There were significant differences ($F_{3,28} = 4.0, P < 0.025$) in the mean longevities between cohorts of wasps reared from hosts fed the different extracts of VFN Bush (Table 5). The longest-lived wasps (≃24 days) were reared from hosts fed on the hexane-extract diet. Wasps reared from hosts fed on the water-extract diet had significantly shorter life spans (≃11 days). The wasps reared from hosts fed on the control or water + hexane-extract diets had life spans intermediate to those of the wasps from the hexane-extract and water-extract cohorts. None of the different extracts of VFN Bush had a significant effect on duration of pupal period, percent pupal eclosion, or mean weight of wasps, although the mean weight of wasps from the water-extract cohort was almost significantly less ($F_{3,28} = 2.0, P \simeq 0.10$) at 1.1 mg than the cohort of wasps from the hexane-extract cohort which had a mean weight of 1.7 mg. Lastly, none of the wasps had the antennal or genital deformities as seen in wasps reared from hosts fed artificial diets containing 20 μmol α-tomatine.

Comparison of Quantities of Free Sterols, Sterol Esters, and α-Tomatine from the Foliage of Different Lycopersicon esculentum *Cultivars*. The identities and their quantities of free sterols and sterol esters in the different cultivars of *L. esculentum* are presented in Table 6. In all cultivars, cholesterol and stigmasterol were the most abundant free sterols with foliar amounts

TABLE 6. AMOUNTS OF INDIVIDUAL FREE STEROLS AND STEROL ESTERS IN FOLIAGE OF VARIOUS CULTIVARS OF *L. esculentum* (μMOL/G DRY WT) AS ANALYZED BY GLC

Cultivars									
VF198		VF315		UC 134		VFN Bush		Royal Flush	
Sterol	μmol/g	Sterol	μmol/g	Sterol	μmol/g	Sterol	μmol/g	Sterol	μmol/g
Free sterols									
Cholesterol	0.17	Cholesterol	0.15	Cholesterol	0.12	Cholesterol	0.12	Cholesterol	0.17
Desmosterol	0.01	Desmosterol	0.01	Desmosterol	0.01	Desmosterol	0.02	Desmosterol	0.01
Campesterol	0.13	Campesterol	0.02	Campesterol	0.02	Campesterol	0.02	Campesterol	0.04
Stigmasterol	0.19	Stigmasterol	0.12	Stigmasterol	0.12	Stigmasterol	0.12	Stigmasterol	0.19
Sitosterol	0.08	Sitosterol	0.06	Sitosterol	0.06	Sitosterol	0.06	Sitosterol	0.10
Unknown 1	0.04[a]	Unknown 1	0.04[a]	Unknown 1	0.04[a]	Unknown 1	0.03[a]	Unknown 1	0.04[a]
Unknown 2	0.12[a]	Unknown 2	0.03[a]	Unknown 2	0.03[a]	Unknown 2	0.03[a]	Unknown 2	0.04[a]
Unknown 3	0.01[a]	Unknown 3	0.01[a]	Unknown 3	0.01[a]	Unknown 3	0.03[a]	Unknown 3	0.01[a]
Unknown 4	0.01[a]	Unknown 4	0.02[a]					Unknown 4	0.02[a]
Sterol Esters[b]									
Cholesterol	0.07	Cholesterol	0.16	Cholesterol	0.45	Cholesterol	0.10	Cholesterol	nd[c]
Desmosterol	0.26	Desmosterol	0.10	Desmosterol	0.53	Desmosterol	0.19	Desmosterol	0.35
Stigmasterol	0.13	Stigmasterol	0.05	Stigmasterol	0.17	Stigmasterol	0.08	Stigmasterol	0.14
Sitosterol	0.95	Sitosterol	0.16	Sitosterol	1.12	Sitosterol	0.33	Sitosterol	0.50
Unknown 1	0.30[a]	Unknown 2	1.00[a]	Unknown 2	6.04[a]	Unknown 1	0.15[a]	Unknown 1	0.35[a]
Unknown 2	4.66[a]	Unknown 4	0.10[a]	Unknown 3	0.06[a]	Unknown 2	1.79[a]	Unknown 2	4.10[a]
Unknown 5	0.38[a]	Unknown 5	0.11[a]					Unknown 4	0.12[a]
								Unknown 5	0.29[a]

[a]Quantities based upon GLC peak heights to cholesterol standards.
[b]Saponified.
[c]nd = not detectable.

individually in each cultivar being ~0.2 μmol/g dry wt. Sitosterol was slightly less abundant as a free sterol than either cholesterol or stigmasterol, with foliar amounts averaging 0.1 μmol/g. The remaining free sterols (i.e., desmosterol, campesterol, and unknowns 1–4) were in between at 0.01–0.04 μmol/g dry wt of foliage, with the exception of unknown 2 in VF 198 at 0.12 μmol/g.

A total of nine sterol esters were detected in the foliage of the cultivars, with no one cultivar having all nine (Table 6). Desmosterol, stigmasterol, sitosterol, and unknown 2 were found in all cultivars, and cholesterol not detected only in Royal Flush. In all cultivars, unknown 2 was highest in abundance, with sitosterol and desmosterol next highest. Unknown 3 was only found in UC 134; unknown 1 in VF 198, VFN Bush, and Royal Flush; unknown 4 in VF 315 and Royal Flush; and unknown 5 in VF 198, VF 315, and Royal Flush. While unknown 2 was only moderately present in comparison to other free sterols, it was the most abundant sterol ester.

On an absolute molar basis, there are only minor differences between the cultivars in amounts of total free sterol (Table 7). However, there are large differences between certain cultivars in the amounts of total sterol ester (Table 7). For example, the foliage of UC 134 and VF 198 was highest in total amounts of sterol ester and, consequently, highest in total amount of sterols overall.

There was no relationship between the amount of free sterol or sterol ester and the amount of α-tomatine in the foliage of the cultivars (Table 7). For example, the foliage of VF 198 had high amounts of total sterol and relatively low amounts of α-tomatine. In contrast, the foliage of UC 134 had high amounts of both total sterol and α-tomatine. In comparing amounts of total sterol to α-tomatine within a cultivar, the foliage of Royal Flush had >9 times more total sterol than α-tomatine (highest) while VFN Bush and VF 315 had <2 times more total sterol to α-tomatine (lowest).

TABLE 7. RELATIONSHIP OF TOTAL FREE STEROL, STEROL ESTER, AND α-TOMATINE CONTENT IN FOLIAGE OF VARIOUS CULTIVARS OF *L. esculentum* (μMOL/G DRY WT)

Cultivar	Free sterol (μmol/g)	Sterol ester (μmol/g)	Total sterol (μmol/g)	α-Tomatine (μmol/g)	Index total sterol: tomatine
VF 198	0.7	6.8	7.4	1.0	7.7
VF 315	0.5	1.7	2.1	1.2	1.8
UC 134	0.4	8.4	8.8	1.7	5.2
VFN Bush	0.4	2.6	3.1	1.7	1.8
Royal Flush	0.6	5.9	6.5	0.7	9.2

DISCUSSION

Possible Mode of Action of α-Tomatine-Induced Toxicity to Hyposoter exiguae. The manifestations of α-tomatine-induced toxicity to *H. exiguae* were: prolonged larval development; disruption or prevention of pupal eclosion; deformation of antennal, abdominal, and genital structures; and reduction of adult weight and longevity. Each of these manifestations was exacerbated when the dosage of α-tomatine in the diet of *H. zea* (the host) was increased from 12 μmol to 20 μmol/g. However, the effect of toxicity by α-tomatine to *H. exiguae* was obviated in parasitoids reared from hosts fed on artificial diets that contained equimolar or supramolar amounts of sterols admixed with α-tomatine.

Furthermore, the toxicity of the foliage of any particular cultivar was dependent upon the composite foliar level of both α-tomatine and total sterol. For example, the pooled water + hexane extracts of UC 134 were relatively nontoxic to *H. exiguae*, although they contained one of the highest concentrations of α-tomatine (at 1.7 μmol/g) compared with the other cultivars, but UC 134 also contained the highest level of total sterol. In contrast, despite the slightly lower level of α-tomatine in extracts of VF 315 (at 1.2 μmol/g) than in UC 134, VF 315 was the most toxic cultivar to *H. exiguae*. However, the extracts of VF 315 contained 4 times less total sterol than UC 134, and this difference in composite levels of sterols and α-tomatine may account for the relative differences in toxicities between these cultivars. Further evidence of the "composite" effect was that the water extract of VFN Bush (containing α-tomatine) was toxic to *H. exiguae* and that this toxicity was alleviated by the hexane extract (which contained all the foliar sterols). Despite the fact that our identification of sterols solely by RRT values does not exclude the possibility of contamination by fatty alcohols (an overestimation of total foliar sterol content), this fact does not detract from the observation that alleviation of α-tomatine-induced toxicity occurred. The presence of antidotal levels of foliar sterols is the most reasonable cause for this alleviation.

The toxicity of α-tomatine to *H. exiguae* may have been the result of a depletion of cholesterol in the parasitoid. Unlike phytophagous insects, predaceous and parasitic insects studied to date cannot dealkylate C_{28} and C_{29} phytosterols to C_{27} sterols; therefore, these zoophages rely on their phytophagous hosts for a source of cholesterol or utilizable sterols (Svoboda et al., 1978). It is essential for parasitic insects (as for all insects) to procure an adequate supply of cholesterol or utilizable sterols from their hosts so that requirements are met for the inclusion of cholesterol into cellular/subcellular membranes, the use of cholesterol as a precursor for the synthesis of the steroidal molting hormones (the ecdysones), and the incorporation of sterols

into sperm and ova (see Robbins et al., 1971). Previous studies have found that in vitro, α-tomatine forms a 1:1 molecular complex with 3β-OH-sterols (Schultz and Sander, 1957) and causes the lysis of cells whose membranes contain 3β-OH-sterols (Wilson et al., 1961; McKee, 1959; Fowlkes et al., 1967).

Hence, there is a likelihood that α-tomatine ingested by *H. exiguae* from its host had some negative effect on normal sterol supply, utilization, and/or metabolism in the parasitoid. Such a negative effect would have been at a peak in the parasitoid during its late larval or pupal stages when bioaccumulation of α-tomatine would have been highest (*H. exiguae* does not excrete until after emergence to an adult); the predominant sterol accumulated was cholesterol. These conditions may have been especially conducive for the formation of a cholesterol–tomatine complex in light of the fact that of all sterols tested, α-tomatine has the highest affinity for cholesterol (Roddick, 1979). Expression of this negative effect at relatively high doses of α-tomatine was possibly represented by the deformations in antennal and genital structures and the decline in pupal eclosion of *H. exiguae* reared from hosts fed on diets containing 20 μmol/g α-tomatine. These abnormalities in the development of *H. exiguae* resemble those observed to occur in other insects when inhibition of sterol metabolism by azasterols or triparanol disrupted the synthesis of steroidal molting hormones which resulted in abnormal metamorphosis (see Svoboda et al., 1972).

An additional possibility is that α-tomatine is "teratogenic" to developing *H. exiguae*. "Teratogenesis" could occur if α-tomatine is capable of blocking steroid hormone sites in the parasitoid during certain critical periods of metamorphosis. This has, analogously, been proposed as the mechanism for certain solanaceous steroidal alkaloids causing teratogenesis in mammals (Brown and Keeler, 1978).

Lastly, it was not likely that α-tomatine caused an indirect depletion of sterols to *H. exiguae* by reducing the amount of sterols available to the host. Such a reduction in uptake of sterols by the host could have occurred if α-tomatine formed a complex with certain sterols in the diet or in the gut of the host during digestion. However, the amount of either [^{14}C]cholesterol or [^{14}C]cholesterol palmitate assimilated by the host was dependent upon the dietary dose of either isotope, whether or not α-tomatine was present in the diet (Figure 3).

Implications to Host-Plant Resistance and Evolutionary Ecology. Since Fraenkel (1959) first proposed his original concepts on the "raison d'etre" for plant secondary compounds, researchers in the field of plant–herbivore interactions have searched for natural products in plants that would account for the resistance of any given plant to a given herbivore. Often, conclusions about a particular plant's ability to protect itself against herbivores have been

made solely on the basis of whether a putative antibiotic is present or absent in the plant or whether it is toxic to herbivores when incorporated into artificial diets, e.g., tannins in oak leaves (Feeny, 1968), L-canavanine in *Dioclea* (Janzen, 1971), 2-tridecanone in tomato foliage (Williams et al., 1980), phenolics in tropical vegetation (McKey et al., 1978), to mention a few. Also, the effects of other natural products within the plant in aggravating or alleviating the toxicity of these antibiotics have largely been neglected.

The results of our study show a plant–herbivore interrelationship wherein the role of the putative antibiotic (α-tomatine) as a protectant for the plant is ambiguous. Because the parasitoid (*H. exiguae*) is poisoned by purified α-tomatine, the compatibility of α-tomatine as a chemical basis of host-plant resistance with biological control was questioned (Campbell and Duffey, 1979a). Hence, the practicality of breeding into cultivars high levels of this antibiotic to effect insect control needs to be thoroughly considered. A more thorough consideration of the effect of natural products upon insects involves providing evidence that the candidate toxin is operative against the insect in planta. As we have demonstrated, the toxicity of a candidate (α-tomatine) to an insect (*H. exiguae*) is alleviated by antidotal products in the foliage (sterols). This antidotal effect is also operative for larval *Heliothis zea* (unpublished data). The presence of natural antidotal chemicals obviously complicates breeding programs designed to enhance resistance to pests via elevated levels of candidate toxin(s), as in the case of tomatoes where two factors at a minimum (tomatine and sterol levels) would have to be monitored. It follows then that in studying the impact of plant allelochemicals upon insects one must be cautious about extrapolating data on the biological activity of a chemical in isolation to biological activity in planta. It remains to be determined how many putative allelochemical systems are subject to antidotal or exacerbating effects by co-occurring natural products.

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EFFECT OF VOLATILES COLLECTED ABOVE FECAL PELLETS ON BEHAVIOR OF THE RABBIT, *Oryctolagus cuniculus*, TESTED IN AN EXPERIMENTAL CHAMBER. II. Gas Chromatographic Fractionation of Trapped Volatiles

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Abstract—Gas chromatographic techniques were used to trap and fractionate the volatile substances emanating from the fecal pellets of wild rabbits, *Oryctolagus cuniculus*, which incorporate the odor of the anal gland secretion. The volatiles were bubbled through water, trapped on Chromosorb 105, transferred to Silicone SF96 traps, passed through a GC capillary column, and retrapped on SF96. The behavioral effect of trapped and fractionated volatiles was determined in four series of bioassays involving 51 adult, male, wild-type rabbits in 112, ten-minute tests. The bioassay was based on the demonstration of territorial confidence by the rabbits under the influence of their own odor. In the bioassays of the unfractionated volatiles eluted from Chromosorb 105 and SF96 traps the animals were significantly more confident in the presence of the volatiles from their own fecal pellets. One of the two fractions of the total volatiles was ineffective while the effect of the other was less than that of the unfractionated odor. The results demonstrate that the gas chromatographic techniques can be used to manipulate complex mammalian odors with precision. Combined with a discriminative bioassay, this opens up the possibility of identifying the specific combinations of volatile substances involved in the formation of olfactory signals.

Key Words—Rabbit, *Oryctolagus cuniculus*, anal gland secretion, feces, headspace volatiles, gas chromatographic fractionation, bioassay, territorial confidence.

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INTRODUCTION

In our previous efforts to determine the chemical composition of anal gland secretions of wild rabbits, *Oryctolagus cuniculus*, with which feces become coated when passing through the end portion of the rectum, changes in the heart rate of rabbits located to sniff the effluent of a gas chromatographic capillary column were used to pinpoint compounds of likely behavioral importance. Many zones in the complex chromatograms were then found to induce heart-rate responses (Goodrich et al., 1978, 1981). As previously emphasized, however, changes in heart rate of experimentally restrained animals merely indicate their interest in olfactory cues. Overt behavioral changes, which are far more informative than physiological ones, were observed by using an experimental chamber containing two rabbits into which the total odor of fecal pellets and chemically extracted fractions were introduced (Hesterman et al., 1981).

Since in our future investigations we plan to use gas chromatographic fractionation techniques to locate the active compounds, it became imperative to determine whether the behaviorally active components would survive gas chromatography.

The present paper describes the results of experiments designed to test the effect on a rabbit's territorial behavior of the total odor from its own fecal pellets, and of fractions of that odor, trapped from the effluent of a gas chromatographic capillary column.

METHODS AND MATERIALS

General Approach

Odors were collected from above fecal pellets, and fractionation of the trapped volatiles was carried out by means of gas chromatography.

Behavioral testing followed the same general scheme and employed the same test chamber as described in detail in a previous study (Hesterman et al., 1981). In each test a randomly selected pair of adult male rabbits was introduced into a test chamber containing the odor derived from the fecal pellets of one of them. The behavior of the pair was observed. At a later time the same pair of rabbits was again tested but in the presence of the odor derived from the other individual. The degree to which the odor influenced the territorial confidence of the rabbits was assessed by comparing their behavior in the donor and nondonor situations.

Preparation and Presentation of Odor for Testing

Collection of Fecal Pellets. Overnight accumulations of fecal pellets were collected onto wire mesh trays placed under the individually caged rabbits.

Pellets obviously contaminated with urine were discarded. The pellets were stored in sealed glass containers at −20° C for no longer than 28 days.

Collection of Headspace Volatiles. Volatile components from above the fecal pellets of individual rabbits were collected on Chromosorb 105 (Johns-Manville, USA). Oxygen-free nitrogen (40 ml/min) was passed over 30 g of fecal pellets at 40° C for 8 hr, and the effluent bubbled through 30 ml of distilled water contained in a gas scrubbing tube. Volatile constituents present in the emerging gas stream were collected in a single preconditioned trap containing a bed of Chromosorb 105 (50–60 mesh, 100 mg) packed into a stainless-steel tube (90 mm, 3.2 mm OD). Each trap contained the equivalent of 19.2 liters of headspace volatiles. The trap was then purged with dry nitrogen to remove residual water, sealed with Teflon end caps, and stored over solid carbon dioxide until required for further fractionation or for bioassay.

Desorption of Volatiles from Chromosorb 105 onto SF96 Traps. Volatiles trapped on Chromosorb 105 were desorbed using the method of Williams and Strauss (1977). The procedure was as follows: (1) The Chromosorb 105 trap was coupled with Teflon tubing to another trap packed with 10% Silicone SF96 on 30–40 mesh Chromosorb A. (2) A flow of nitrogen gas at 40 ml/min was passed through the Chromosorb 105 trap, and a liquid nitrogen cooled probe was applied to the SF96 trap. (3) To complete the desorption of the volatiles, the temperature of the Chromosorb 105 trap was raised to 170° C for 30 min using a heated aluminium block. The SF96 trap was then disconnected, sealed with Teflon end caps, and stored over solid carbon dioxide.

Gas Chromatographic Fractionation of Volatiles. The Silicone SF96 traps were introduced at 170° C over a period of 5 min onto a glass SCOT column (60 m, 0.5 mm ID) coated with FFAP, in a Hewlett-Packard series 7600A gas chromatograph which was modified as previously described (Goodrich et al., 1981). The column was temperature programed at 20 min at 60° C followed by 1° C/min rise to 80° C, then 2° C/min rise to 190° C.

The flow from the capillary column was split between the flame-ionization detector and an outlet collection port in the ratio 1:8. Volatile compounds were collected at the outlet port onto SF96 traps as above. The amount of volatiles contained in each trap was equivalent to that in 17.1 liters of fecal pellet headspace. The collection technique has been described elsewhere (Murray, 1977).

In the first stage of experimentation the total effluent from the capillary column was collected onto a single SF96 trap which was then bioassayed to establish whether all behaviorally important components of the odor were still present. Subsequently the total effluent was divided into two parts (fraction 1 and fraction 2), which were similarly collected on separate SF96 traps (see Table 1). The trapped volatiles were stored over solid carbon dioxide until

TABLE 1. MANIPULATIONS INVOLVED IN COLLECTION, TRAPPING, AND GAS CHROMATOGRAPHIC FRACTIONATION OF VOLATILES FROM FECAL PELLETS OF RABBITS FOR BIOASSAY

Volatiles collected on Chromosorb 105	Volatiles collected on SF96	Fractionated volatiles
fecal pellet headspace	fecal pellet headspace	fecal pellet headspace
scrubbed by water	scrubbed by water	scrubbed by water
trapped on Chromosorb 105	trapped on Chromosorb 105	trapped on Chromosorb 105
desorbed into test chamber	transferred to Silicone SF96	transferred to Silicone SF96
	desorbed onto GC column	desorbed onto GC column
	effluent	effluent
	to flame ionization detector — 1 part	to flame ionization detector — 1 part
	8 parts	8 parts
	trapped on Silicone SF96	fraction 1 trapped on Silicone SF96
	desorbed into test chamber	desorbed into test chamber
		fraction 2 trapped on Silicone SF96
		desorbed into test chamber

required for bioassay. The selection of the point on the chromatogram at which the total effluent was divided to form the two fractions (see Figure 2) was based on earlier observations of the heart-rate responses of rabbits to the gas chromatographic effluent components (Goodrich et al., 1981). Fraction 2 contained the components which elicited the most numerous heart-rate responses.

Introduction of Trapped Volatile Components into Test Chamber. The fecal pellet volatiles contained in traps were introduced into the test chamber through a heated (170° C) ball valve of similar design to that used in the modified Hewlett-Packard gas chromatograph. The valve was located in the

inlet head of the test chamber. Desorption of volatiles from Chromosorb 105 traps was carried out for 30 min and from Silicone SF96 traps for 5 min using nitrogen (40 ml/min). The concentrations of volatiles attained in the test chamber were 25.6 liters headspace equivalents/kl for the Chromosorb 105 traps and 22.8 liters headspace equivalents/kl for the SF96 traps.

Animals

In the four series of behavioral tests 51 rabbits were used. All were wild-type adult males and were normally kept in mixed sex groups of 3 or 4 individuals in large open-air pens. For the duration of the tests they were accommodated in separate cages in an animal house and fed ad libitum on a standard rabbit food.

Experimental Design

Tests on Unfractionated Volatiles. The total, water-scrubbed volatiles contained on Chromosorb 105 or SF96 traps were tested at separate times using the same design as described in detail in an earlier report (Hesterman et al., 1981). For each test series, 14 animals were randomly assigned into 14 pairs, each rabbit being used in two different pairings. Within each pairing they were tested twice, once in the presence of the volatiles derived from one animal and again in the presence of the volatiles derived from the other. Each series therefore consisted of 28 tests. Tests employing the same animals were separated by a period of at least 4 days. All traps were tested within 7 days of being collected.

Tests on Fractionated Volatiles. The two series of tests on fractions 1 and 2 of the fecal pellet volatiles were run concurrently. In each series the same 28 rabbits were used but in different pairings. Within each series the animals were paired once and tested with the volatiles derived from their own fecal pellets and with those derived from their opponents, giving 28 tests for each series.

Since both fractions of the fecal pellet volatiles from individual animals were trapped-out during the same fractionation run, one of them had to be stored for 4 days longer than the other. To balance the two series of tests for the storage time factor, and for the order of testing, 14 of the donor rabbits were tested first with fraction 1 traps and 14 were tested with fraction 2 traps first. Storage times for both types of traps were 1–5 days when tested first and 6–10 days when tested second. An abridged version of the scheme used for these tests is presented in Table 2.

Test Procedure

Tests were carried out in the same way as described in detail elsewhere (Hesterman et al., 1981). The behavioral parameters recorded for each animal

TABLE 2. SCHEDULE USED IN TESTING BEHAVIORAL EFFECTS OF FRACTIONS 1 AND 2 OF VOLATILES FROM FECAL PELLETS OF RABBITS[a]

Day 0	Day 3	Day 4	Day 7	Day 11	Day 15	Day 17	Day 21
A_1-I	E_2-M	A_2-J	E_1-N	J_2-A	N_1-E	I_1-A	M_2-E
B_1-J	F_2-N	B_2-I	F_1-M	I_2-B	M_1-F	J_1-B	N_2-F
C_2-K	G_1-O	C_1-L	G_2-P	L_1-C	P_2-G	K_2-C	O_1-G
D_2-L	H_1-P	D_1-K	H_2-O	K_1-D	O_2-H	L_2-D	P_1-H

[a]The schema shown is an abridged version involving 16 animals represented by the letters A–P. The donor animal for each test is shown first with a subscript to indicate the fraction tested.

were: time spent exploring; incidence and duration of sniffing at the other rabbit; incidence and duration of aggressive behavior; incidence and duration of sexual mounting; animal making the first approach. All of these parameters, when quantified, reflect the confidence of the animals. Apart from these easily quantifiable variables, characteristic postures and patterns of movement are also related to the level of confidence (Mykytowycz and Hesterman, 1975). Therefore at the conclusion of each test the two observers made subjective judgments as to which of the two rabbits was dominant or more confident.

Statistical Procedures

The statistical techniques used to analyze the data were similar to those described elsewhere (Hesterman et al., 1981). Analyses of the data for incidences and durations of the various behavioral components were carried out on the transformed variables:

$$\text{Log}_e\,[(\text{donor score} + 1)/(\text{nondonor score} + 1)]$$

where donor and nondonor scores were for the same animal matched against the same opponent. Before proceeding with the tests on the fractionated volatiles, the results from the two total headspace series were examined separately. For these analyses *t* tests were performed on the transformed variables for each behavioral component, i.e., the differences between the paired scores. Subsequently the results from all four series were analyzed collectively by means of multivariate analysis of variance (MANOVA) supplemented by canonical variate analysis (Morrison, 1976).

The frequency data from the subjective judgments and first-contact observations were analyzed by means of binomial probabilities and chi-square tests for independence.

RESULTS

Behavioral Tests. The results of the two series of tests on unfractionated, water-scrubbed volatiles are presented in Table 3. In both series the mean differences between donor and nondonor situations for all behavioral parameters were positive and, except for mounting, were statistically significant (one-tailed t tests; $P < 0.05$). Donor animals also made initial contact and were judged to be dominant or more confident in significantly greater numbers of tests than the nondonors (binomial probability; $P < 0.01$) in both series.

Table 4 summarizes the results of the tests carried out on the two fractions of the fecal pellet volatiles. Multivariate analysis of variance was carried out on the data from all four series of tests and the eight behavioral variables. This analysis shows that there was a significant effect due to the type of volatiles used in the different series (Wilk's lambda test, $P < 0.05$). Further analysis was performed to indicate which of the compound means differed, and these results are shown in Figure 1 together with the results of a canonical variate analysis of the data. In Figure 1 the positions of the canonical means are shown in relation to the first and second canonical variates which account for 95.8% of the total variation.

The canonical means for the Chromosorb 105 and SF96 series are grouped together in the canonical space and do not differ significantly. The fraction 1 mean is separate and differs significantly from the Chromosorb 105 and SF96 means. The fraction 2 mean lies in an intermediate position and does not differ significantly from any of the other three canonical means.

The frequency data for first contacts and subjective assessments of confidence clearly do not differ between donor and nondonor animals in the fraction 1 series. In the tests on fraction 2 volatiles the donor animals scored higher in both measures, although the differences between donor and nondonor scores were not statistically significant (binomial probability; $P > 0.05$).

Analysis of the frequency data from all four series of tests by means of 4×3 contingency tables, in which all three categories of results were used, demonstrated significance between series differences for subjective assessment scores ($\chi^2_6 = 17.06, P < 0.01$) but not for first contacts ($\chi^2_6 = 7.50, P \simeq 0.30$).

There were no significant differences between the results obtained from the traps of the fractionated volatiles used 1–5 days after collection and those stored for 6–10 days.

Gas Chromatographic Results. A gas chromatogram of the volatiles trapped from the GC column effluent onto a Silicone SF96 trap is presented in Figure 2. The total effluent from above fecal pellets was bubbled through distilled water, collected onto a Chromosorb 105 trap, and transferred to the

TABLE 3. MEAN VALUES OF VARIOUS BEHAVIORAL PARAMETERS FOR RABBITS PLACED IN PAIRS INTO TEST CHAMBER CONTAINING NON-WATER-SOLUBLE VOLATILES FROM FECAL PELLETS OF ONE OF THEM (DONOR), DESORBED FROM CHROMOSORB 105 OR SF96 GC TRAPS

	Chromosorb 105 traps (28 tests)					SF96 traps (28 tests)				
	No. of tests in which behavior occurred	Mean score		Mean difference ± SD (donor–nondonor)	Significance[a]	No. of tests in which behavior occurred	Mean score		Mean difference ± SD (donor–nondonor)	Significance[a]
		Donor situation	Nondonor situation				Donor situation	Nondonor situation		
Exploration duration (sec)	28	61.8	32.6	29.2 ± 49.3	**[b]	28	45.1	19.5	25.6 ± 36.8	***
Approach, incidence	28	11.0	3.9	7.1 ± 16.0	***	28	6.9	3.0	3.9 ± 6.6	***
Sniffing other rabbit										
Incidence	28	24.0	16.1	7.9 ± 16.0	***	28	22.3	13.9	8.4 ± 18.1	***
Duration (sec)		44.7	24.9	19.8 ± 35.0	***		45.3	15.3	30.0 ± 38.7	***
Aggression										
Incidence	17	7.7	1.7	6.0 ± 17.1	**	18	19.7	11.2	8.5 ± 50.5	*
Duration (sec)		5.1	0.9	4.2 ± 12.8	**		10.9	5.4	5.5 ± 25.2	*
Mounting										
Incidence	1	0.4	0	0.4	+	8	1.9	0.2	1.7	+
Duration (sec)		3.5	0	3.5			14.9	0.1	14.8	
Numbers of tests in which donor or nondonor rabbits made initial contact										
Donor	16**					17***				
Nondonor	4					3				
Simultaneous	8					8				
Numbers of tests in which donor or nondonor rabbits were judged to be most confident										
Donor	21***					21***				
Nondonor	2					2				
Equal	5					5				

[a] *t* tests were carried out on the transformed variables (see text).
[b] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; + insufficient data.

TABLE 4. MEAN VALUES OF VARIOUS BEHAVIORAL PARAMETERS FOR RABBITS PLACED IN PAIRS INTO TEST CHAMBER CONTAINING ONE OF TWO DIFFERENT FRACTIONS OF NON-WATER-SOLUBLE VOLATILES FROM FECAL PELLETS OF ONE OF THEM (DONOR), SEPARATED BY MEANS OF GAS CHROMATOGRAPHY

	Fraction 1 (28 tests)				Fraction 2 (28 tests)			
	No. of tests in which behavior occurred	Mean score		Mean difference ± SD (donor–nondonor)	No. of tests in which behavior occurred	Mean score		Mean difference ± SD (donor–nondonor)
		Donor situation	Nondonor situation			Donor situation	Nondonor situation	
Exploration, duration (sec)	28	47.6	43.7	3.9 ± 47.1	28	44.8	29.7	15.1 ± 55.8
Approach, incidence	28	8.9	4.8	4.1 ± 14.7	28	5.8	5.1	0.7 ± 8.3
Sniffing other rabbit								
Incidence	28	20.1	12.1	8.0 ± 21.3	28	17.6	10.4	7.2 ± 13.2
Duration (sec)		31.2	8.4	22.8 ± 49.3		18.7	7.5	11.2 ± 13.5
Aggression								
Incidence	20	18.0	19.7	−1.7 ± 41.8	16	19.9	9.7	10.2 ± 28.0
Duration (sec)		9.8	8.9	0.9 ± 18.0		10.8	4.0	6.8 ± 16.0
Mounting								
Incidence	12	3.8	5.0	−1.2 ± 8.6	12	5.7	3.4	2.3 ± 12.1
Duration (sec)		41.1	25.7	15.4 ± 107.9		50.3	13.0	37.3 ± 98.8
Numbers of tests in which donor or nondonor rabbits made initial contact								
Donor	11	NS[a]			15	NS		
Nondonor	10				8			
Simultaneous	7				5			
Numbers of tests in which donor or nondonor rabbits were judged to be most confident								
Donor	10	#			13	NS		
Nondonor	11				8			
Equal	7				7			

[a]NS = not significant; # = test inappropriate.

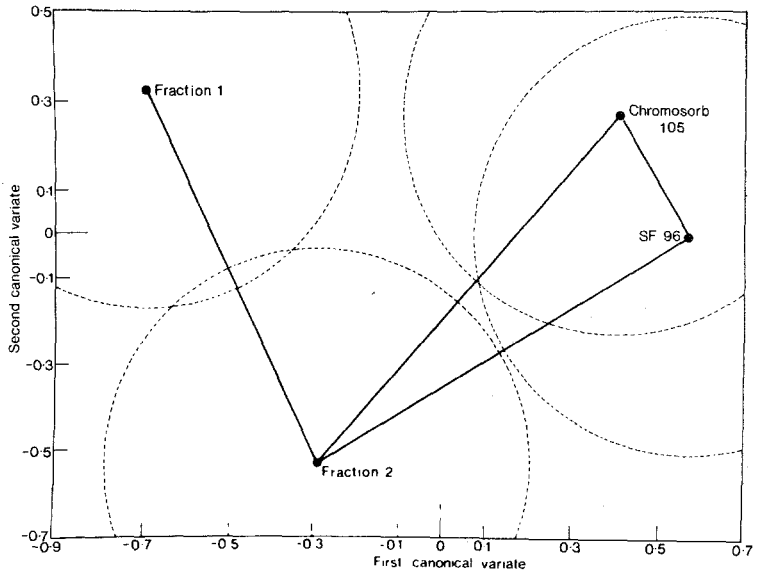

FIG. 1. The relationship between the data for the four series of tests as indicated by canonical variate analysis. Means joined by lines are not significantly different ($P > 0.05$). Broken circles indicate the 95% confidence limits for the mean values.

SF96 trap as described earlier. The figure also shows the position at which the volatiles were divided to form fractions 1 and 2.

DISCUSSION

The results of the first two series of bioassays reported in this paper show that the volatiles derived from the anal gland secretion which is carried by the fecal pellets can be manipulated by gas chromatographic techniques without reducing their specific effect on the rabbit's territorial confidence. In this study the volatiles were trapped on Chromosorb 105, transferred to a Silicone SF96 trap, desorbed onto an FFAP-coated glass SCOT column, and retrapped onto a SF96 trap. Under test conditions rabbits displayed a level of behavioral response to the volatiles similar to that seen in earlier experiments in which fecal pellets and the total headspace volatiles of fecal pellets were used (Mykytowycz et al., 1976; Hesterman et al., 1981). Thus one can conclude that none of the behaviorally important constituents of the odor signal are lost during the gas chromatographic procedures employed.

In an earlier study (Hesterman et al., 1981) fecal pellet odors which were scrubbed three times through distilled water (3 × 30 ml) and tested at a

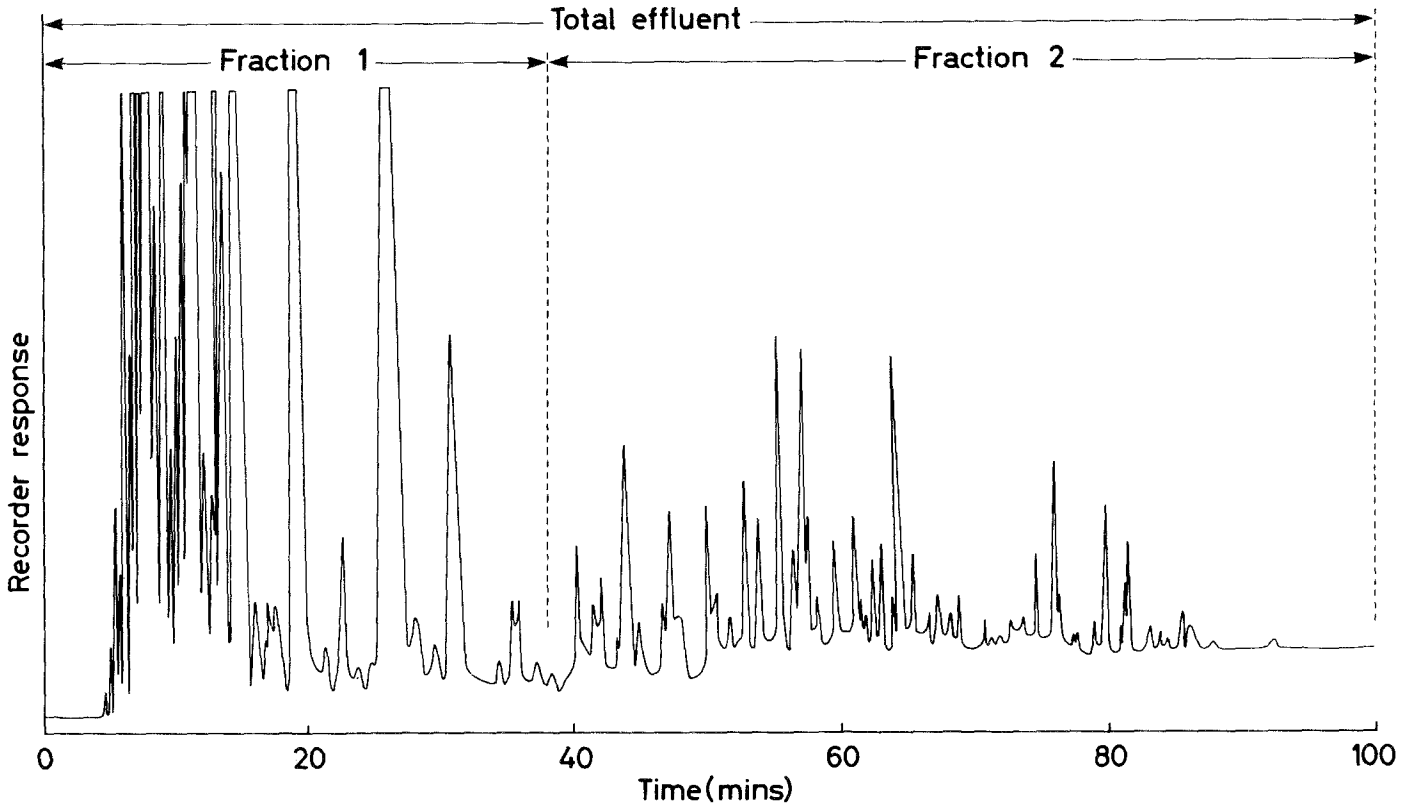

FIG. 2. Gas chromatogram of the non-water-soluble volatiles from the fecal pellets of a rabbit, *Oryctolagus cuniculus*, desorbed from a Silicone SF96 trap. The zones included in fractions 1 and 2 are indicated.

concentration of 3.2 liters headspace equivalent/kl were less effective than the total odor in influencing the rabbit's territorial confidence. In the present experiments in which the volatiles were less thoroughly scrubbed through water (1 × 30 ml) and tested at concentrations of 22.8 or 25.6 liters headspace equivalent/kl their behavioral effect appears to be as strong as that of the total odor.

A superficial examination of the results for the four series of tests carried out so far on the total or water-scrubbed volatiles from fecal pellets suggests that the strength of the behavioral effect may be directly related to the concentration of volatiles present in the test chamber. This can most easily be illustrated by relating the concentrations of volatiles used in the test chamber in the different series to the percentages of donor animals which were judged to be most confident. Thus the concentrations of 3.2 and 12.0 liters headspace equivalents/kl used in the previous study (Hesterman et al., 1981) resulted in the donor animals being more confident than nondonors in 62 and 69% of tests, respectively. In the two series reported in this paper in which the concentrations were 22.8 and 25.6 liters headspace equivalents/kl, the donor animals were more confident in 75% of tests.

Changes in the level of a rabbit's territorial confidence in relation to the concentration of its own marking odor would be compatible with its observed behavior in the field. The readiness of rabbits—and many other animals—to attack strange conspecifics appears to depend on their distance from the homesite or burrow, around which odor-marking activity is most intense.

Fraction 1 of the fecal pellet volatiles clearly had no significant influence on the results of the tests. The results obtained with fraction 2, although statistically equivocal, strongly suggest that it did have some behavioral effect but not of the same magnitude as the unfractionated volatiles. These results indicate that fraction 2 may contain a large proportion, but not all, of the components of the odor signals.

It is interesting to note in this regard that the zones of the total effluent covered by the fractions were selected on the basis of heart-rate response data gathered in an earlier study (Goodrich et al., 1981). Fraction 2 contained the volatiles from the portion of the total effluent which elicited the most numerous heart-rate responses. Thus the results of this study support the usefulness of the heart-rate technique as a method of screening complex odors for components of possible behavioral importance.

The fractionation studies will be continued in an effort to delineate more precisely the areas in the chromatogram of the total effluent in which the components of the territorial marking signals occur. Further manipulations of the odor which are currently being carried out include adding parts of fraction 1 to fraction 2, subdividing all active fractions produced, and recombining volatiles selected-out from different zones of the chromatogram.

Information on the general chemical composition of the anal gland secretion and of the volatiles from fecal pellets is available and many compounds associated with specific peaks of their gas chromatograms have been identified (Goodrich et al., 1978, 1981). Ultimately the results from fractionation studies should make it possible to compile a list of the compounds specifically involved in the formation of the signals related to territorial possession.

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A HIGH-EFFICIENCY COLLECTION DEVICE FOR QUANTIFYING SEX PHEROMONE VOLATILIZED FROM FEMALE GLANDS AND SYNTHETIC SOURCES

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Abstract—A high-efficiency collection device for sex pheromones volatized from forcibly extruded female glands is described. Filtered nitrogen gas is the carrier and glass wool the adsorbent. Small quantities of distilled carbon disulfide are used to rinse the glass wool. Recovery efficiency of synthetic compounds was usually 90–100%, and a mean of 2.4 ± 0.65 SD ng/min of (*Z*)-7-dodecenyl acetate was recovered in emissions from individual *Trichoplusia ni* (Hubner) glands.

Key Words—Volatilized pheromone, glass adsorption, *Trichoplusia ni*, cabbage looper, quantifying pheromone emission.

INTRODUCTION

Sex pheromone research has progressed steadily toward the point where quantification of component release rates from both natural and synthetic sources is essential. It is necessary not only during implementation programs, such as for formulation of disruptants, but also during the initial isolation and identification process. For instance, aldehydes sometimes can be found in abundance by aeration of glands but not in glandular extracts (Hill et al., 1975; Cross et al., 1976; Weatherston et al., 1971; Sanders and Weatherston, 1976), and their contribution to optimal behavioral responses can be underestimated or missed entirely. Although a variety of collection devices have been reported for quantifying synthetic compound emissions (see Weatherston et al., 1981), few can be used efficiently for female emissions

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because of: (1) extremely low quantities of emitted pheromone relative to background peaks from solvent, adsorbent, or extracted scales; (2) adsorption of pheromone onto female bodies, reducing recovery; and (3) degradation of pheromone through oxidation or body surface enzymes. Even for quantifying emissions from controlled-release formulations, it is difficult (Weatherston et al., 1981) to account for all the pheromone that has left an emission surface, i.e., to obtain a mass balance.

In a previous report (Baker et al., 1980), it was determined that glass surfaces were excellent adsorbers of pheromone, and also were easily desorbed of pheromone by solvent rinses. This static-air–glass adsorption method had several disadvantages, however, which limited its usefulness. First, in the collection chamber ca. 90% of collectable pheromone was lost due to adsorption onto calling females' bodies, and although it could be accounted for and used as a release rate correction factor, this loss seemed undesirable. Second, as with any collection device using air as a carrier, oxidation of labile compounds could readily occur and cause miscalculations and loss of otherwise recoverable materials such as aldehydes, when they are left on the adsorbing surface too long (Weatherston et al., 1981). These factors, plus the goal of having a more dynamic system capable of quantifying very low emission rates in a variety of flow regimes, prompted us to investigate further the use of glass as an adsorbent and to try to develop a system that would be useful for both female- and synthetic-emitted pheromone for collecting high-lability compounds with nearly 100% efficiency.

METHODS AND MATERIALS

General Procedure. The collection device is illustrated in Figure 1. Charcoal- and glass wool-filtered nitrogen (N_2) was introduced from a ground glass connector at an ambient temperature 21–23°C and a flow rate of 0.5 ml/sec ± 10%. Volatiles were adsorbed onto ca. 0.25 g of moderately packed glass wool, eluted with distilled carbon disulfide (CS_2) (ca. 1.5 ml in 0.5-ml aliquots), and an internal standard was added immediately. The solution was condensed to ca. 5 μl, using a Snyder column apparatus with a small Teflon boiling chip, and analyzed by gas–liquid chromatography (GLC) on a column of 10% XF-1150 (100–120 mesh Ch W, AW DMCS, 1.394 g, 1.0 m × 4.0 mm OD). Oven temperature was 150°C, and N_2 flow was 25 ml/min. All glassware, pipets, and syringes were rinsed thoroughly with distilled acetone and dried in an oven at 125°C between uses. GLC peak areas were estimated using peak height × retention time. (*Z*)-7-Dodecenyl acetate (*Z*7-12:Ac) and (*Z*)-11-tetradecenyl acetate (*Z*11-14:Ac), the internal standards, were formulated gravimetrically, then serially diluted to the desired concentration.

Determiniation of Breakthrough Time. Ninety nanograms of each of seven model pheromone compounds, decyl acetate (10:Ac), decyl alcohol

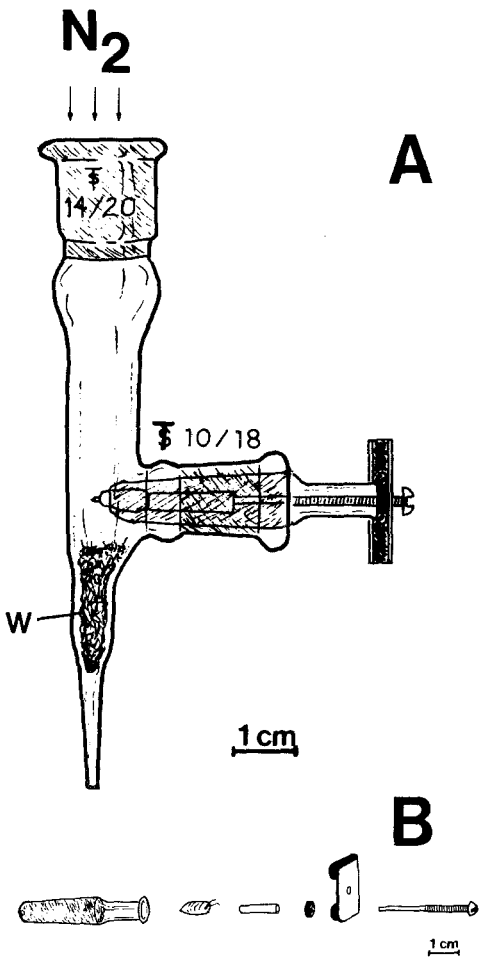

FIG. 1. (A) The one-receptacle collection device. Charcoal- and glass-wool-filtered N_2 gas at 0.5 ml/sec flows through the lumen and passes over pheromone emitter such as (pictured) a forcibly extruded female gland held in an extrusion tube. A volume of packed glass wool (W) then adsorbs the pheromone. (B) Expanded view of the extrusion tube components, with (from left to right) the extrusion tube, ligated abdomen with gland unextruded, Teflon plunger, GLC-conditioned silicone rubber gasket, steel clip, and screw for exerting pressure.

(10:OH), dodecyl acetate (12:Ac), (*Z*)-9-tetradecenal (*Z*9–14:ALD), tetradecyl acetate (14:Ac), (*Z*)-11-hexadecenal (*Z*11–16:ALD), and hexadecyl acetate (16:Ac), were injected onto the tip of an artificial, glass gland (a modified gland extrusion tube, Figure 1B), in 9 μl hexane. The gland was placed within 10 sec of impregnation into the collection device with the N_2 stream already flowing. Compounds "breaking through" the glass wool

collection area were captured in 20-min fractions in glass capillaries cooled by dry ice and connected by Teflon to the distal end of the apparatus. The capillaries were rinsed with ca. 30 μl CS_2, and 50 ng internal standard was added immediately. Pressure was equalized in this system by drawing a vacuum on the end of each capillary to match the N_2 flow. The glass wool and glass gland were rinsed with CS_2 as above, and 50 ng internal standard was added immediately. The total quantity collected from glass wool, gland, and breakthrough fractions was compared to the amount introduced into the collector. The latter was determined by injecting the gland with compounds as above, waiting ca. 10 sec to mimic time normally elapsed before insertion into the collector, then washing the gland surface immediately with CS_2 and adding internal standard.

Recovery Efficiency. Efficiency was measured not only in the breakthrough study, but also in two separate experiments with different emission quantities. Nine μl of a nine-component solution [the seven compounds used above, plus hexadecyl alcohol (16:OH) and (*Z*)-11-hexadecenyl alcohol (*Z*11-16:OH)], were applied with a syringe to the tip of the glass gland. Solution concentrations of 10 ng/μl and 1 μg/μl of each component in CS_2 were used to determine the system's efficiency of recovery as well as the evaporation rates from the glass gland for each component. The volatiles carried by the N_2 were adsorbed by the glass wool and subsequently eluted with CS_2. Fifty ng and 5 μg of internal standard, respectively, were added. Quantities recovered from glass wool and gland were compared to quantities actually introduced into the collector, as above, for each replicate, and percent recovery of each compound was calculated.

Pheromone Collection from Female Moths. Trichoplusia ni (Hübner) females in their fourth scotophase were placed in a freezer (−20° C) for 5–10 min. Abdominal scales were removed with a gentle vacuum. A ligature was tied about the abdomen near the thorax and the abdomen then severed just anterior to the ligature. The abdomen was placed in the gland extrusion tube (Figure 1B) so that the distal end protruded through the small opening in the tube. A cylindrical Teflon plunger was placed behind the abdomen and a silicone rubber gasket placed at the opening of the tube (Figure 1B). A machine screw was inserted through a steel clip and the rubber gasket. Pressure was applied behind the Teflon plunger with the screw to extrude the gland. Five-minute collections were made using the one-receptacle collection apparatus. Sixty-minute collections were made using a four-receptacle apparatus similar to the device in Figure 1 except that the four receptacles opened into the N_2 stream at 90° angles to each other. At the end of the collection period, the glass wool was eluted with CS_2 and 50 ng 14:Ac added as an internal standard. Each collection was condensed and analyzed as described above.

RESULTS AND DISCUSSION

The recovery efficiency of nanogram quantities of model pheromone compounds was nearly 100% (Tables 1 and 2). The glass wool adsorbed and retained for 2 hr nearly all the materials except 10:Ac without appreciable breakthrough (Table 1). Importantly, the two aldehydes suffered no apparent oxidation or other degradation in either the 2- or 1-hr (Table 2) collections as evidenced by their high rate of recovery. Compounds of greater molecular weight were collected in increasing quantities on the glass gland and, consequently, in decreasing quantities from the glass wool, as compared to lower-molecular-weight compounds. The recovery of microgram quantities also appeared to be nearly 100%, although only lower-molecular-weight compounds such as 10:OH and 12:Ac had been volatilized and collected from the glass wool in μg amounts after 1 hr.

Collection of pheromone from ligated *T. ni* female abdomens appeared quite successful. For the first 5 min of gland extrusion a mean of 2.4 ($\pm$ 0.65 SD) ng/min *Z*7–12:Ac and 0.25 ($\pm$ 0.07 SD) ng/min 12:Ac was collected. The *Z*7–12:Ac quantity is somewhat lower than that reported by Bjostad et al. (1980) from intact, calling females using Porapak-Q® as the adsorbent in an air stream. They collected a mean of 21.8 ($\pm$ 3.7 SD) ng/min *Z*7–12:Ac. One reason for the discrepancy between the two studies may be the large difference in flow rates over the gland. Their rate, 37.5 ml/sec, was nearly 100-fold greater than ours and could have substantially increased volatilization of pheromone from the gland surface. Also, unlike their technique which collected pheromone during a female's initial gland extrusion, our method collected from females already calling for an unknown period of time. Alternatively, the differences may reflect a reduced ability of ligated female abdomens to produce pheromone compared to intact females. If this is so, an adaptation of our system could be used for intact females, in which a vacuum just matching N_2 flow is drawn on the end of the apparatus to equalize pressure, eliminating the need for a sealed receptacle for abdomens.

The advantages of the N_2–glass wool device are many, not the least of which is the obviation of extensive precollection treatment to clean up the adsorbent such as Porapak-Q (Byrne et al., 1975). Because small quantities of solvent are used and the glass wool is free of impurities, interfering background GLC peaks are reduced, and nanogram quantities can be quantified. Therefore, quantification of pheromone emitted from individual females can be performed for many lepidopterous species. A further advantage is that the glass wool can be used repeatedly hundreds of times because during each rinsing it is stripped of all compounds. Scales are not shed into the glass wool, and, therefore, are not extracted to add to background impurities, and the pheromone does not have a chance to readsorb onto

TABLE 1. BREAKTHROUGH TIMES AND RECOVERY EFFICIENCY OF 7 MODEL PHEROMONE COMPOUNDS USING N_2–GLASS WOOL COLLECTING DEVICE WITH GLASS "GLAND" LOADED WITH 90 NG OF EACH COMPOUND[a]

	$\overline{X}$ percent compound recovered (± SD) ($N = 6$)								
			Breakthrough						
	From glass wool	From glass gland	0–20 min	20–40 min	40–60 min	60–80 min	80–100 min	100–120 min	Total (%)
10:Ac	80 ± 34	0 ± 0	10 ± 13	3 ± 1	7 ± 4	6 ± 3	6 ± 3	14 ± 10	127
10:OH	82 ± 25	1 ± 3	0 ± 0	0 ± 0	0 ± 0	4 ± 6	4 ± 6	1 ± 3	89
12:Ac	89 ± 10	0 ± 0	1 ± 1	1 ± 1	0 ± 0	1 ± 2	1 ± 2	2 ± 2	95
Z9-14:ALD	76 ± 11	11 ± 4	0 ± 0	0 ± 0	0 ± 0	1 ± 2	1 ± 2	2 ± 3	91
14:Ac	74 ± 12	22 ± 11	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	96
Z11-16:ALD	56 ± 11	30 ± 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	86
16:Ac	26 ± 4	72 ± 6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	98

[a]Collection time: 2 hr. N_2 flow at 0.5 ml/sec. The percent recovery is as a percent of compound actually introduced into the apparatus measured by controls.

TABLE 2. PERCENT COMPOUND RECOVERED FROM COLLECTING DEVICE DURING 1-HR COLLECTION BEGINNING WITH EITHER 90 NG OR 9 μG EACH COMPOUND, NO BREAKTHROUGH COLLECTION MADE

	$\bar{X}$ percent compound recovered[a]					
	90 ng			9 μg		
	From glass wool	From glass gland	Total (%)	From glass wool	From glass gland	Total (%)
10:Ac	85 ± 44	0 ± 1	85	6 ± 3	63 ± 8	69
10:OH	94 ± 13	0 ± 0	94	16 ± 3	76 ± 9	92
12:Ac	92 ± 9	4 ± 3	96	8 ± 2	97 ± 5	105
Z9-14:ALD	78 ± 21	13 ± 5	91	4 ± 1	97 ± 4	101
14:Ac	52 ± 21	41 ± 24	93	2 ± 2	103 ± 7	105
Z11-16:ALD	37 ± 19	45 ± 28	82	1 ± 1	102 ± 6	103
16:Ac	12 ± 8	80 ± 21	92	0 ± 0	101 ± 9	101
16:OH	0 ± 0	73 ± 12	73	0 ± 0	105 ± 13	105
Z11-16:OH	0 ± 0	85 ± 26	85	0 ± 0	93 ± 6	93

[a] $N = 4$ for 90 ng, $N = 3$ for 9 μg.

females' bodies as in some other systems (Baker et al., 1980). Most importantly, more labile compounds such as aldehydes do not oxidize in the N_2 stream, and hence collection and quantification of these compounds can proceed for long periods without jeopardizing the high efficiency. A device using filtered air and glass beads instead of glass wool has recently been described for quantifying emission rates of synthetic pheromone from controlled release formulations (Weatherston et al., 1981).

Our four-receptacle device allows for collections from four females at once and newly extruded glands can be continually rotated into the device to replace used glands. Thus, for those species emitting at very low rates or for collection of minor components even in high-rate emitters, mass collection and quantification can be performed more easily. In addition, flow rate may be varied to study its effects on emission rate, a characteristic lacking in a static-air–glass adsorption device.

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BEHAVIORAL RESPONSES OF MALE *Periplaneta americana* TO PERIPLANONE B, A SYNTHETIC COMPONENT OF THE FEMALE SEX PHEROMONE

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Abstract—Bioassays were performed on male *Periplaneta americana* L. using concentrations of (±)-periplanone B of 10^{-9}–10^{-3} μg and a serial dilution of natural female sex pheromone extract. Synthetic (±)-periplanone B stimulated the complete repertoire of behavior released by the extract: increased rate of locomotion, upwind orientation, and the wing-raising component of courtship display. Quantitative behavioral comparisons of the activity of natural extracts prepared by this and other laboratories are possible by the standardization of the stimulus in terms of μg of (±)-periplanone B applied per assay.

Key Words—American cockroach, *Periplaneta americana*, Orthoptera, Blattidae, sex pheromone, periplanone B, locomotion, orientation.

INTRODUCTION

Periplanone B, a major component of the female sex pheromone of the American cockroach, *Periplaneta americana* L., was recently synthesized by Still (1979), based on the chemical characterization of Persoons (1977), Persoons et al. (1976, 1979), and Talman et al. (1978). Adams et al. (1979) established the absolute configuration of the pheromone. Persoons (1977) demonstrated the presence of a total of six active compounds in the pheromone extract, all of which elicited courtship behavior in males, yet only two of them occurred in sufficient quantities to allow a complete chemical identification (Persoons and Ritter, 1979).

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Previously published studies have reported the effects of the natural female sex pheromone extract on the behavior of male *P. americana*. Sex pheromone extracts were shown to increase the rate of locomotion (Wharton et al., 1954a,b; Block and Bell, 1974; Rust, 1976), release positive anemotaxis (Rust and Bell, 1976; Bell and Kramer, 1980; Tobin, 1981), stimulate local search and chemotaxis (Hawkins, 1977, 1978; Rust et al., 1976; Bell et al., 1977; Bell and Tobin, 1981), and stimulate courtship behavior (Roth and Willis, 1952; Wharton et al., 1954a,b; Takahashi and Kitamura, 1972; Rust, 1976; Silverman, 1977; Schafer, 1977). The purpose of this paper is to analyze the behavioral responses of male *P. americana* to synthetic (±)-periplanone B to determine if (±)-periplanone B releases the behavioral repertoire in the same quantitative and qualitative sequence as the natural pheromone which is known to contain additional components.

METHODS AND MATERIALS

Adult male *P. americana*, aged less than 2 months postemergence, were isolated from females for approximately 7 days prior to testing, with access to lab chow and water at 24 ± 3° C, and a 12:12-hr, dark–light photocycle. Only individuals with intact antennae and legs were used in assays.

Pheromone Sources. Two pheromone sources were employed: (1) sex pheromone extract prepared by an acetone–hexane extraction of approximately 1000 whole virgin females by the research group of Prof. J. Boeckh, University of Regensburg, and (2) (±)-periplanone B, synthesized by Prof. Clark Still, Columbia University. Dilution series were prepared for both pheromone sources from stock solution. The amount of sex pheromone on a weight per volume basis in the Regensburg extract was not quantified, and so concentration values given in the text are relative to the stock solution. Concentration values for (±)-periplanone B are given in micrograms applied per sample.

Locomotory and Wing-Raising Activity Assays. Males were tested in twenty groups of five individuals in plastic cages with lids (12.5 × 18.5 × 29 cm). Samples of (±)-periplanone B or extract were applied onto 1-cm^2 filter paper squares and positioned either on the floor of the cage or suspended from the cage lid 18 cm above the cockroaches. Locomotory activity was measured by recording the total number of times the cockroaches crossed a line drawn across the center of the bottom of the cage, and expressed as ACPM, the total activity counts per minute for the five insects. The courtship display of wing-raising was measured by the total number of seconds that the five cockroaches held their wings raised per minute (WRPM). Males were not retested the same day since habituation reduces responsiveness unless a 24-hr period is allowed between tests (Rust, 1976).

Persoons Assay. Persoons (1977) devised a bioassay technique that

employs a 5.0-liter container fitted with two parallel plastic tubes (3.8 cm diam × 30 cm) which open at one end into the container. At the opposite end the tube is fitted to a stopper holding a disposable pipet containing the sample. A vacuum pump draws air at 50 cm/sec through the pipet and the plastic tube into the container. Pheromone was placed either on filter paper or directly onto the glass inside the pipet. If the cockroaches resting inside the container were stimulated by the sample, they rapidly oriented to the tube openings and entered the tube containing pheromone. Occasionally one or several individuals also entered the control tube during a test. Rarely were all of the 20 individuals in the jar responsive to the pheromone. Each test was performed for 6 min, during which the number of cockroaches in each tube was counted each successive minute. The largest number of cockroaches attracted to the pheromone tube minus the number of cockroaches in the control was calculated as an attractiveness index.

Wind Tunnel Assay. Males were tested individually in a low-air-speed wind tunnel to determine the threshold concentrations of sex pheromone required to elicit orientation and allow a comparison of the orientation pathways observed.

A sample was placed on filter paper held 2.0 cm off the floor of the wind tunnel (2.4 × 1.2 × 0.6 m) with an air speed of 22 cm/sec as described by Tobin (1981). A male was allowed to adjust to the wind tunnel in a wire cage at the downwind end for 20 min prior to the test. The cage was opened to allow free movement of the male before introducing the pheromone sample. The success of orientation to the pheromone source upwind was recorded to (±)-periplanone B dilutions of 10^{-2}–10^{-5} μg and extract dilutions of 10^{-1}–10^{-3}.

Trapping. A dilution series of (±)-periplanone B of 10^{-2}–10^{-4} μg was used to bait previously unused 21.5 × 28-cm cardboard boxes which also contained a food additive (fenugreek seed extract, Penick Corp.) and adhesive. Colonies of 150 cockroaches with approximately equal proportions of nymphs, adult males, and adult females were established in a 2.4-m-diam circular arena along with cardboard shelters from colony cages, food, and water, 24 hr before testing.

Two traps, a control and one with (±)-periplanone B, were positioned near the center of the arena near the end of the photophase of the photocycle. The cockroaches normally remain inside the shelters during the photophase and are active primarily close to the wall of the arena during the scotophase. After 24 hr the traps were removed, and the number of individuals captured in the adhesive was counted.

RESULTS

Locomotory and Wing-Raising Activity. Pheromone threshold values for a significant increase in locomotory activity are one to two orders of

magnitude lower than those for eliciting wing-raising activity. The pattern of locomotory activity over the 5-min test period depends in part on whether the cockroaches contact the pheromone source. The temporal sequence of responses to the pheromone is similar for the extract and synthetic material.

The threshold for locomotory activity is between 10^{-4} and 10^{-3} for the extract and between 10^{-7} and 10^{-6} μg for (±)-periplanone B (Figure 1). The peak activity rates were reached during the first min when either sample was suspended from the cage lid (abbreviated as UP) so that antennal contact could not be made. A significant decrease in activity occurred after the second

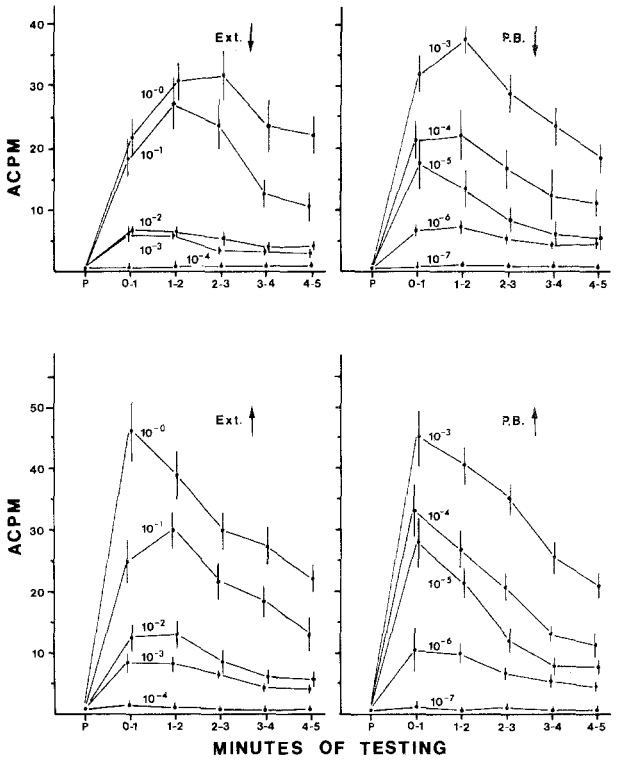

FIG. 1. Locomotory activity expressed as activity counts per min (ACPM) for five males stimulated by (±)-periplanone B (P.B.) or sex pheromone extract (Ext). Arrows indicate position of pheromone source in test cage; arrow upwards indicates a pheromone source suspended above the cockroaches (UP), arrow downward indicates a pheromone source placed on the floor of the test cage (DN). Mean ACPM values for solvent controls: extract-UP = 4.6 ± 4.8, DN = 2.6 ± 3.3, (±)-periplanone B-UP = 5.0 ± 3.7, DN = 3.6 ± 3.6. Vertical lines are standard errors of means, P, activity prior to testing with solvent control or pheromone sample. Each point represents the mean of 20 runs, except 10^{-4} extract and 10^{-7} μg (±)-periplanone B which is based on 5 runs.

min for 10^{-0} extract-UP and 10^{-3}, 10^{-4}, and 10^{-5} μg (±)-periplanone B-UP ($P < 0.005$; Mann-Whitney U test).

Peak activity values at the highest concentrations were larger with pheromone sources positioned above than on the floor (extract, $P < 0.01$; periplanone B, $P < 0.05$). No significant differences were observed between the values for the UP and DN experiments during the second and third minute of testing. At the higher concentrations with pheromone samples DN, the peak locomotory activity values tended to be delayed until the second minute. Total activity values for both compounds (first and second minutes combined) are proportional to the log of the pheromone concentration (regression values: periplanone B-UP, $r = 0.986$; periplanone B-DN, $r = 0.988$; Extract-UP, $r = 0.969$; Extract-DN, $r = 0.937$).

The threshold values for eliciting the wing-raising courtship display are 10^{-5} μg for (±)-periplanone B and the 10^{-1} dilution for the extract (Figure 2).

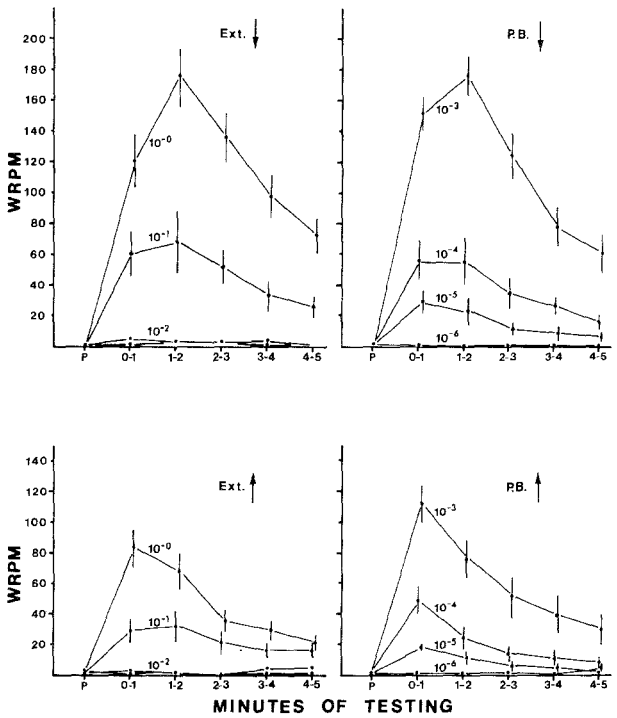

FIG. 2. Wing-raising activity expressed as the number of seconds that the five male cockroaches held their wings raised per minute of testing (WRPM) stimulated by (±)-periplanone B or sex pheromone extract. Mean WRPM values for controls: extract-UP = 0.0, DN = 0.4 ± 1.2, (±)-periplanone B-UP = 2.0 ± 4.9, DN = 0.6 ± 1.5. Symbols are the same as in Figure 1.

The threshold for wing-raising compared with locomotory activity is apparently only a single order of magnitude higher for (±)-periplanone B, whereas it is two orders of magnitude higher for the extract. As with locomotory activity, when the sample was placed UP, the wing-raising activity reached a maximum during the first minute and then declined. In contrast to locomotory activity, antennal contact with the pheromone sample DN increased wing-raising activity. The effect is greatest at the highest concentration of (±)-periplanone B and at the two highest concentrations of extract. The maximum activity was reached during the second min at both of the highest concentrations with the sample DN.

Pheromone samples in the DN position stimulated male components of courtship (in addition to wing-raising) that are normally released by a sex pheromone-secreting female, including orientation to the sample, abdominal extension and copulatory motions toward the sample.

To determine if the quantity of pheromone released from filter paper samples used in tests remained constant over the testing period, the following experiment was performed. Samples of 10^{-6}, 10^{-5}, and 10^{-4} μg of (±)-periplanone B were pipetted onto filter papers and allowed to remain in the open air with ventilation for 0, 1, 5, and 10 min before testing with males in the locomotory activity assay (above). No diminution in activity was apparent over the 10-min period. These data suggest that decline in locomotory activity after 2 min of testing is due to habituation or sensory adaptation and not a decrease in the quantity of pheromone emitted from test samples.

Persoons Assay. The threshold value for (±)-periplanone B applied to the glass pipet is between 10^{-9} and 10^{-8} μg/sample (Figure 3). These values are the same as those recorded by Persoons (1977) using natural pheromone and by Adams et al. (1979) for synthetic (−)-periplanone B. Good agreement between the laboratories indicates that standardization of the experimental system and conditioning of the males can produce comparable results even though different stock cultures of *P. americana* were used and environmental conditions probably differed.

As indicated in Figure 3, the threshold and relative responses are shifted about two orders of magnitude lower if the sample is applied to a 1-cm^2 filter paper positioned inside the pipet instead of directly onto the glass. Differences in the behavioral responses must reflect the relative release rates of (±)-periplanone B from paper as compared to glass substrates.

Wind Tunnel Assay. Orientation to the pheromone placed 2 m upwind in the tunnel was elicited by as low as 10^{-5} μg (±)-periplanone B per sample. Of 30 males tested individually, 16 found the source at 10^{-5} μg; of the remaining 14, 7 responded at 10^{-4} μg (±)-periplanone B. With the female extract, 11 of 24 responded to the 10^{-2} dilution and 7 of the remaining 13 responded to the 10^{-1} dilution. The threshold dosages are ten times higher than that required to elicit

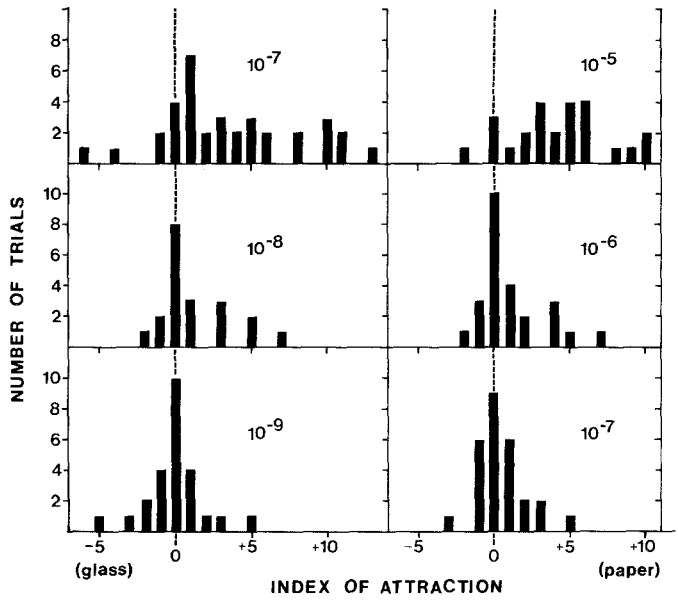

FIG. 3. Attractiveness of (±)-periplanone B in Persoons assay. Values on abscissa represent the number of cockroaches in the control tube subtracted from the number of cockroaches in the pheromone tube. Number of replicates for each concentration is 25 tests of 20 cockroaches per test. Chi-square analysis was performed to test for differences from an equal distribution of values to the left (−) and right (+) of zero: glass, $10^{-7} = P < 0.001$, $10^{-8} = P < 0.025$, 10^{-9} = NS; paper, $10^{-5} = P < 0.001$, $10^{-6} = P < 0.025$, 10^{-7} = NS.

a locomotory response in the locomotory and wing-raising assays. The measurements are not directly comparable, however, since the relative release rates and molecular concentration in the air are not known.

The males were able to locate the sample within 1 min of its introduction by orienting directly upwind. Photographic analysis of the pathway (Figure 4) shows that a male generally exhibits a zig-zag movement pattern and stays within the boundaries of the pheromone plume in the center of the tunnel (Tobin, 1981). As observed in the locomotory and wing-raising assays, the males show an increased and sustained locomotory rate. Orientation in the wind tunnel, however, rarely includes wing-raising or other courtship actions.

Traps Baited with (±)-Periplanone B. Table 1 shows that (±)-periplanone B significantly enhanced the attractiveness of traps to nymphs, males, and females at concentrations of 10^{-2} and 10^{-3} μg. Because the pheromone is known in the literature exclusively as a male stimulant, the trapping experiment was repeated with only nymphs and females in the arena. The resulting trap catches were not significantly different from controls, suggesting

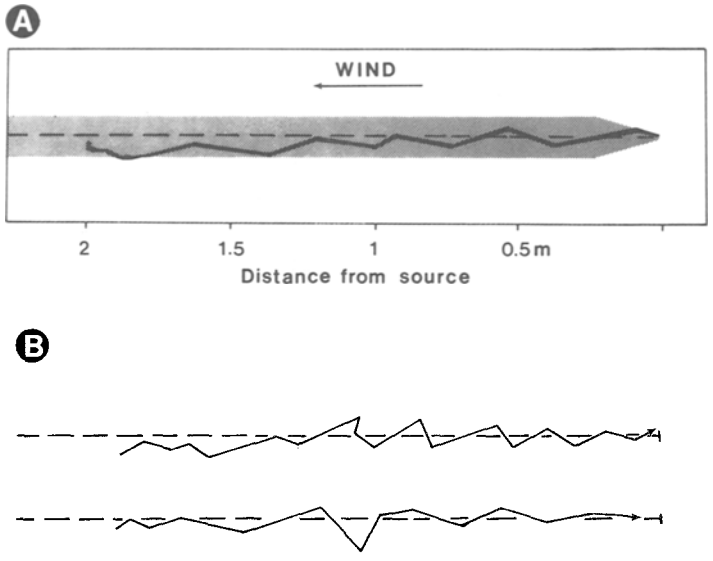

FIG. 4. Upwind orientation in a plume of (±)-periplanone B. (A) Dimensions of wind tunnel and plume, showing the pathway of one male. (B) Pathways of two other males.

that the presence of males or an odor they secrete draws females and nymphs into (±)-periplanone B-baited traps.

DISCUSSION

Tests comparing the natural female sex pheromone extract and synthetic (±)-periplanone B strongly suggest that they elicit equivalent responses. If the other components of the sex pheromone such as periplanone A, isolated by Persoons (1977), play a qualitatively significant role in releasing male behavior, such a function is not evident from the tests reported here. The behavior released by both the extract and the synthetic compound increase quantitatively in the same manner over several orders of magnitude of concentrations tested. Since the absolute concentration or the relative percentage of periplanone B in the extract is not known, we are unable to determine if the secondary pheromone components quantitatively affect the behavioral threshold.

An estimate can be made of the quantity of pheromone which the female secretes each day by comparing the level of behavioral activity released to that of known quantities of (±)-periplanone B. Hawkins and Rust (1977) reported testing of filter paper exposed to virgin females for a 24-hr period using a

TABLE 1. MEAN NUMBER OF COCKROACHES CAPTURED IN TESTS COMPARING (±)-PERIPLANONE B-BAITED TRAPS WITH CONTROLS[a]

Concentration of (±) periplanone B		Mean number captured (± SE)[b]					
		Males		Females		Nymphs	
μg	N	PB[c]	Control	PB	Control	PB	Control
10^{-2}	15	15.1 ± 1.3a	0.2 ± 0.1	8.8 ± 1.4a	0.9 ± 0.7	14.1 ± 2.1b	2.8 ± 2.6
10^{-3}	5	12.5 ± 1.6b	0.3 ± 0.3	6.7 ± 2.2c	1.3 ± 0.3	10.0 ± 1.8c	4.0 ± 1.7
10^{-4}	5	3.8 ± 4.4	0.1 ± 0.1	2.3 ± 1.9	0.8 ± 0.3	4.3 ± 4.4	1.7 ± 0.9

[a]Both (±)-periplanone B-baited traps and controls contain fenugreek seed extract.
[b]Probability values based on Student's *t* test: a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.05$ for comparison of means to the left and right of symbol letters. Chi-square analysis of percent captures at different concentrations shows significance for males, females, and nymphs ($P < 0.05$).
[c]PB = periplanone B.

similar locomotory activity assay as in this study. The range of values of 13–40 ACPM for the first minute indicates a similar level of activity as 10^{-6}–10^{-4} μg pheromone per 24 hr. Interestingly, Persoons (1977) purified 10 μg of natural pheromone [(−)-periplanone B] from fecal material of 6000–7000 virgin females collected during a 4-week period. This figures out to about $6\text{–}5 \times 10^{-5}$ μg/day/female. Experiments are presently underway to directly measure the emission rates from virgin females.

Standardization of the bioassay conditions and of the method for applying the pheromone sample is the first necessary step to determine the exact quantitative concentration of pheromone molecules per volume of air. As demonstrated in the Persoons assay, the release rate of the pheromone depends upon the substrate to which it is applied. Direct comparison of the thresholds for locomotory, wind tunnel, or trapping assays requires knowledge of the release rate over time and how it disperses within the assay container. Work is currently underway to establish standard curves for the release rate from filter paper.

The actual thresholds for a particular assay also depend upon the context of the stimulus presentation. As noted in the locomotory and wing-raising assays, locomotion may be released at low concentrations of pheromone, but wing-raising can be elicited by using either a higher concentration or by allowing the males to make antennal contact with the pheromone source.

The ability to perform quantitative behavioral experiments is now markedly improved by the availability of synthetic female sex pheromone. The practical uses of (±)-periplanone B might include trapping to monitor and reduce populations of *P. americana*. Although it has been known for some time that the natural sex pheromone improves trap-catches (Bell et al., 1977; Chow et al., 1976), commercial potential was not realistic using pheromone extracts. The unexpected findings that nymphs and females are attracted to male-visited (±)-periplanone B-baited traps may indicate that other chemicals, such as an aggregation pheromone, are involved in the attraction process.

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SEASONAL AND INTRAPLANT VARIATION OF CARDENOLIDE CONTENT IN THE CALIFORNIA MILKWEED, *Asclepias eriocarpa*, AND IMPLICATIONS FOR PLANT DEFENSE[1]

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Abstract—Root, stem, leaf, and latex samples of *Asclepias eriocarpa* collected from three plots in one population at 12 monthly intervals were assayed for total cardenolide content by spectroassay and for individual cardenolides by thin-layer chromatography. From May to September mean milligram equivalents of digitoxin per gram of dried plant were: latices, 56.8 >> stems, 6.12 > leaves, 4.0 > roots, 2.5. With the exception of the roots, significant changes in gross cardenolide content occurred for each sample type with time of collection during the growing season, whereas variation within this population was found to be small. Labriformin, a nitrogen-containing cardenolide of low polarity, predominated in the latices. Leaf samples contained labriformin, labriformidin, desglucosyrioside, and other unidentified cardenolides. In addition to most of the same cardenolides as the leaves, the stems also contained uzarigenin. The roots contained desglucosyrioside and several polar cardenolides. The results are compared with those for other cardenolide-containing plants, and discussed in relation to anti-herbivore defense based on plant cardenolide content. Arguments are advanced for a central role of the latex in cardenolide storage and deployment which maximizes the defensive qualities of the cardenolides while preventing toxicity to the plant.

Key Words—*Asclepias eriocarpa*, Asclepiadaceae, milkweed, cardenolides, chemical defense, chemical ecology, labriformin, labriformidin, desglucosyrioside, uzarigenin, variation, season, plant part, defense, root, stem, leaf, latex.

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INTRODUCTION

There is much current interest in plant secondary chemicals and the possible ecological functions they fulfill (Levin, 1976; Cates and Rhoades, 1977; Seigler, 1977; Swain, 1977). Many of these compounds are believed to have evolved in response to plant–herbivore, plant–pathogen, and plant–plant pressures while others may play a role in metabolic regulation or storage within plants. Secondary compounds thought to be antiherbivore defense agents have been divided into two groups. The first characteristically occur in relatively low concentrations (usually less than 2% by dry weight), have pronounced physiological effects, and include alkaloids, cyanogenic glycosides, nonprotein amino acids, cardiac glycosides, and many other toxic substances. They are generally found in ephemeral tissues of herbaceous and woody plants. The compounds in the second group occur in much higher concentrations and include digestibility-reducing agents such as tannins and phenolic resins. These occur primarily in mature tissues and organs of woody perennials and have been found in concentrations up to 60% by dry weight (Feeny, 1975; Rhoades and Cates, 1976).

Cardenolides (cardiac glycosides) are produced by plants belonging to several families of angiosperms. Their biological properties which might be involved in herbivore defense include bitterness, emeticity, cardiotonic activity, Na^+K^+-ATPase inhibition, and cytotoxicity (references in Roeske et al., 1976). Many insect species feed on cardenolide-containing plants, and several sequester and store cardenolides for their own defense against vertebrate predators (Brower, 1970; references in Rothschild and Reichstein, 1976). Indications of toxic effects of cardenolides on insects have been indirectly gained from studies on regulation, sequestration, and detoxification of cardenolides by specialist feeders on plants containing these poisons (Duffey et al., 1978; Rafaeli-Bernstein and Mordue, 1978; Seiber et al., 1980). Other investigations both on adapted and nonadapted insects have compared the responses of isolated tissues or organs to purified cardenolides (Vaughan and Jungreis, 1977; Jungreis and Vaughan, 1977; Rafaeli-Bernstein and Mordue, 1978).

The kinds, amounts, and distribution of cardenolides within plants should be major factors in governing the degree of protection conferred by these chemicals to any given plant and the means for bypassing and/or utilizing such chemicals available to adapted herbivores. By analogy with other secondary chemicals, cardenolide content is expected to be influenced by the plant's genetics, development, biochemistry, and physiology and its interaction with and response to biotic and abiotic environments (Levin, 1971; Jones, 1972; McKey, 1974; McKey, 1979; Brower et al., 1981). Biochemical and physiological sources of variation include site of cardenolide synthesis, means of translocation, stabilization and storage, mechanisms for avoiding

self-toxicity, and the presence and distribution of other phytochemical or physical defenses. Ecological factors which might affect cardenolide concentration and distribution include herbivores, pathogens, and competing plants as well as day length, temperature, moisture, and nutrients. Assuming a plant has evolved chemical defensive capabilities in response to this host of environmental factors, then it could also have evolved the biochemical and physiological capacity to control chemical production and distribution in a manner which optimizes chemical protection throughout life while producing no detrimental effect to the plant in the process.

Several milkweeds of the widely distributed North American genus *Asclepias* (Asclepiadaceae) contain varying amounts of cardenolides (Roeske et al., 1976). Of particular interest are the western *A. eriocarpa* Benth. and *A. labriformis* Jones. Both of these species are extremely toxic to livestock due principally to their cardenolides (Seiber et al., 1978; Benson et al., 1978, 1979). Structures for three of the major cardenolides from these two milkweeds [labriformin (I), labriformidin (II), and desglucosyrioside (III), Figure 1], were recently elucidated (Brown et al., 1979; Cheung et al., 1980). Another, uzarigenin (V), is a structurally simple cardenolide which is widely distributed in *Asclepias* spp. and plants of other families (references in Roeske et al., 1976). *Asclepias* plants are host to a number of different insect herbivores, some feeding preferentially on particular plant parts and some sequestering and storing cardenolide in the process (Brower, 1969; Isman et al., 1977a,b; Price and Willson, 1979; Vaughan, 1979).

In this study we examined the distribution of cardenolides within various organs and latices of *A. eriocarpa* during an entire year. We then compared these findings to other studies of cardenolide variation in *Asclepias* and non-*Asclepias* plants and related them to potential lines of defense these plants may possess by virtue of their cardenolide content.

METHODS AND MATERIALS

Sampling of Plants. The *A. eriocarpa* plants were collected at monthly intervals during 1976 from a natural population growing in an occasionally cultivated wheat field located within a 10-hectare rolling hill site along Rd. 19, Woodland (Yolo County), California. Identification was verified by the staff of the Botany Department Herbarium, University of California, Davis (voucher specimen 71895). In August 1975 the field was divided into three plots separated by at least 115 m. Within these plots, five areas (9×20 m) were marked where plant growth was especially dense (ca. 100 plants) to aid in the location of roots during the winter months. Beginning in January 1976, samples were taken in each of the plots toward the middle of each month.

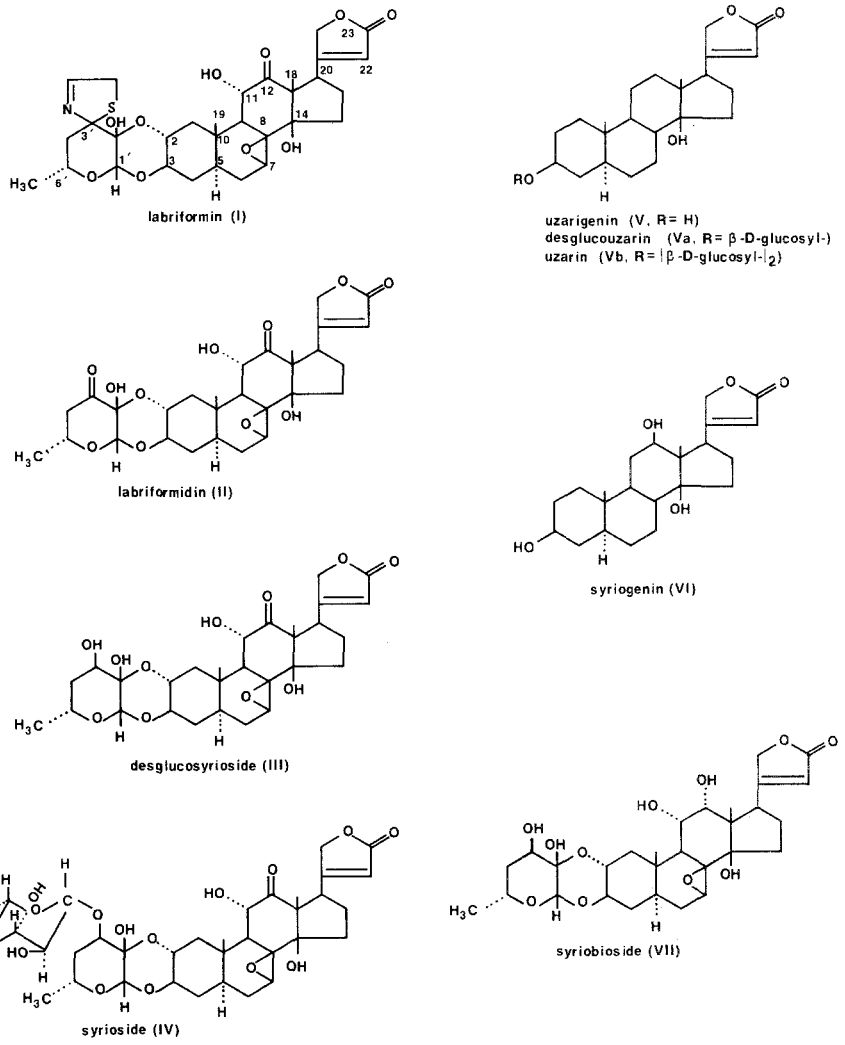

FIG. 1. Structures of some cardenolides occurring in *Asclepias eriocarpa* and/or *A. syriaca*.

Each sample consisted of a composite group of ten plants and each was divided into component parts as follows:

Jan.-Feb.—roots only.
March—roots; shoots (white, 1-2 cm in length).
April—roots; shoots (white and green, up to 16 cm).
May—roots; stems (from root juncture to plant apex); latices (collected

as drops from leaf and stem junctures and from stems when flower buds were removed); lower leaves (from lowest two whorls); middle leaves; upper leaves (from two whorls just below the plant apex); flower buds (unopened); and shoots (12–20 cm in length).

June—same as May, plus flowers, but no shoots.

July–Aug.—same as June, but no flowers or buds were available; unripe seed pods were also taken.

Sept.—same as July–Aug., except lateral shoots (growth off the lower nodes of the stem) were available and taken; mature seed pods were also taken.

Oct.—aerial portions of plants dead due to frost; roots, stems, combined leaves, and empty seed pod shells were collected. No latices were available.

Nov.–Dec.—same as Oct.; leaves and stems were moldy, dry, and deteriorated.

Sample Preparation, Spectroassay, and Statistical Analysis. Collected plants were separated into the different parts in the laboratory and dried in a forced draft oven at 50–60° C for 48–92 hr depending on plant part. Moisture contents were estimated by weighing samples before and after drying. Samples (except latices) were ground in a Wiley mill to pass a 2.0-mm screen (large samples) or a 0.84-mm screen (small samples). They were then stored frozen (−23° C) until air-mailed to Amherst where they were analyzed for total cardenolide content after redrying for 16 hr at 60° C. Latex samples, which had been collected and dried in tared vials, were also redried, weighed, and the latices were dissolved in 95% ethanol and extracted for 40 minutes at 72–78° C.

Plant samples were extracted and total cardenolide content was determined by spectrophotometric assay as milligrams cardenolide (equivalent to digitoxin) per gram dry weight of material as analyzed by the method of Brower et al. (1972) and modified by Brower et al. (1975).

Analyses of variance were carried out using the University of Massachusetts CDC Cyber 75 computer with a University of Michigan Statistical Package for the Social Sciences (Version 7, SPSS, Nie et al., 1977) and by various one-, two-, and three-way ANOVA programs developed by Barthakur (1971) for the Wang 720C electronic calculator system. Since each cardenolide concentration datum was determined on a pooled sample of material from ten plants, the ANOVA input is itself a mean measure and the output therefore does not reflect the whole spectrum of individual plant variability.

Cleanup, Thin-Layer Chromatography, and Densitometry. A lead acetate cleanup developed after that of Rowson (1952) was employed. The residue remaining after evaporation of a 6-ml aliquot of the ethanol extract solution used for spectroassay was dissolved in 1 ml of ethanol, to which was added 2 ml of 10% aq. lead acetate solution. The mixture was vigorously swirled using a mixer which produces a vortex action and then cooled in an ice

bath for 20 min. The supernatant following centrifugation was decanted to a 15-ml test tube containing 1 ml of granular ammonium sulfate and the mixture swirled to precipitate excess lead as sulfate. The residue from centrifugation following lead acetate treatment was rinsed with 2 ml of a 1:2 solution of ethanol–water. The rinse supernatant, separated by centrifugation, was combined in the ammonium sulfate treatment tube, and the remaining residue was discarded. The combined supernatants and ammonium sulfate were swirled and centrifuged. This supernatant was decanted and combined with a rinse of the residue with 2 ml of a 1:2 solution of ethanol–water saturated with ammonium sulfate. The resulting solution was extracted by swirling with 2:1 chloroform–ethanol (2× 2 ml). The organic layers were combined and evaporated to dryness under nitrogen. The residue was dissolved in 2:1 chloroform–ethanol (0.5 ml) and filtered through a Millipore® filter. The filtrate was combined with additional rinses (3× 0.5 ml) of the residue and evaporated to dryness under nitrogen.

Recoveries through the cleanup method were determined by comparing the cardenolide concentration of a subset of samples by spectroassay prior to and after cleanup. The mean molar concentrations of samples, standard deviations, and percent recoveries are given in Table 1.

TLC was carried out on two 20 × 20-cm plates (0.25 mm, Merck Silica

TABLE 1. MEAN CARDENOLIDE CONCENTRATIONS OF *A. eriocarpa* SAMPLES AND RECOVERIES THROUGH LEAD ACETATE CLEANUP[a]

Sample	Concentration ($M \times 10^{-5} \pm SD$)	Recovery (%)
Latices		
Uncleaned	152.2 ± 3.55	67
Cleaned	101.6 ± 1.20	
Roots		
Uncleaned	4.31 ± 0.26	105
Cleaned	4.53 ± 0.16	
Stems		
Uncleaned	23.84 ± 0.60	107
Cleaned	25.64 ± 0.58	
Upper leaves		
Uncleaned	8.71 ± 0.37	78
Cleaned	6.83 ± 0.88	
Middle leaves		
Uncleaned	14.50 ± 0.17	98
Cleaned	14.21 ± 0.08	
Lower leaves		
Uncleaned	7.03 ± 1.17	89
Cleaned	6.25 ± 0.03	

[a] $N = 5$ for all samples except cleaned latices, $N = 4$.

Gel 60 F-254), one developed four times with solvent system I, chloroform–methanol–formamide (90:6:1 by volume), and the other developed twice with solvent system II, ethyl acetate–methanol (97:3 by volume). Development was done in filter paper-lined, preequilibrated chambers. Each plate was spotted with 12 samples corresponding to about 100 μg equivalents of digitoxin and with standards of labriformin, labriformidin, desglucosyrioside, uzarigenin, digitoxin, and digitoxigenin. Visualization was by spraying with a 0.4% solution of 2,2′,4,4′-tetranitrodiphenyl in toluene, then with a 10% solution of KOH in 50% aqueous methanol. Plates were photographed within 1 min after spraying using a ring strobe with Kodachrome 25 film. Color Xerox enlargements were made from the slides for visual comparisons.

Two summary plates were also prepared and developed in each solvent system. These plates contained root, latex, stem, and middle leaf samples for the month of June, and labriformin, labriformidin, desglucosyrioside, and uzarigenin standards. Color prints (20 × 26 cm) from colored slides of the summary plates were submitted to reflectance densitometry to quantitate the relative amounts of individual cardenolides by the method of Seiber et al. (1980). Relative values were obtained from the same samples in both solvent systems. These values were averaged and peak areas of cochromatographing cardenolides (labriformin, labriformidin, and desglucosyrioside) were calculated.

For comparative purposes, three samples of *A. syriaca* (roots, stems, and leaves), grown in Basel by Professor T. Reichstein, were also extracted and assayed for total cardenolide content and TLC profile in the manner described for the *A. eriocarpa* samples.

β-Glucosidase Treatment. The possible occurrence of glucose conjugates such as those reported in *A. syriaca* (Brown et al., 1979) was examined by enzyme treatment. A single sample of *A. eriocarpa* roots was extracted as above and three 6-ml aliquots reduced to dryness under N_2. To one sample, 20 mg β-glucosidase (Sigma Chemical Co.) dissolved in 4 ml sodium acetate–acetic acid buffer (pH 5) was added along with two drops of toluene. To the second sample (reagent blank), only buffer and toluene were added. Samples were capped, stored at room temperature, and shaken several times daily for 12 days after which they were diluted with 4 ml water, saturated with ammonium sulfate, and extracted four times with 2:1 chloroform–ethanol. The organic layer was removed, filtered through anhydrous sodium sulfate, and evaporated. The enzyme-treated, reagent blank, and control samples were cleaned up as described prior to TLC analysis.

RESULTS

Quantitative Cardenolide Variation. The quantitative portion of the study was designed to determine the patterns of variation in gross cardenolide

TABLE 2. CARDENOLIDE CONTENT OF ROOTS, STEMS, UPPER, MIDDLE, LOWER AND DEAD LEAVES, SHOOTS, BUDS AND FLOWERS, PODS, AND LATICES OF *Asclepias eriocarpa* PLANTS FROM A NATURAL POPULATION IN NORTHERN CALIFORNIA[a]

Month	Plot	1 Roots	2 Stems	3 Upper leaves	4 Middle leaves	5 Lower leaves	6 Shoots	7 Dead leaves	8 Buds and flowers	9 Pods	10 Latices
January	1	1.72	*	*	*	*	*	*	*	*	*
	2	3.14 (2.64)	*	*	*	*	*	*	*	*	*
	3	3.06	*	*	*	*	*	*	*	*	*
February	1	1.69	*	*	*	*	*	*	*	*	*
	2	2.44 (2.20)	*	*	*	*	*	*	*	*	*
	3	2.47	*	*	*	*	*	*	*	*	*
March	1	1.51	*	*	*	*	**	*	*	*	*
	2	2.51 (2.14)	*	*	*	*	** (2.95)	*	*	*	*
	3	2.41	*	*	*	*	**	*	*	*	*
April	1	1.74	*	*	*	*	2.34	*	*	*	*
	2	2.44 (2.15)	*	*	*	*	2.57 (2.54)	*	*	*	*
	3	2.26	*	*	*	*	2.71	*	*	*	*
May	1	1.51	7.19	3.43	2.52	2.54	4.06	*	2.49	*	27.1
	2	3.44 (2.80)	7.95 (7.74)	2.65 (3.09)	2.55 (2.76)	2.80 (2.89)	6.24 (5.15)	*	2.23 (2.49)	*	45.2 (43.2)
	3	3.46	8.08	3.20	3.22	3.32	*	*	2.76	*	57.3
June	1	1.63	6.42	3.88	3.25	3.28	*	*	2.20	*	58.0
	2	2.74 (2.19)	8.20 (7.47)	4.24 (4.60)	3.26 (3.72)	3.48 (3.56)	*	*	2.59 (2.63)	*	64.5 (63.3)
	3	2.20	7.78	5.69	4.64	3.91	*	*	3.09	*	67.4
July	1	1.57	5.53	4.42	4.36	4.55	*	*	*	3.23	76.2
	2	2.29 (2.30)	7.14 (6.04)	5.58 (5.26)	5.31 (4.75)	5.02 (4.90)	*	*	*	* (4.59)	60.1 (62.5)
	3	3.03	5.46	5.77	4.58	5.14	*	*	*	5.94	51.2
August	1	1.49	5.66	5.55	3.46	4.08	*	*	*	*	67.7
	2	2.44 (2.28)	5.09 (4.76)	5.89 (5.45)	3.44 (3.47)	3.95 (3.95)	*	*	*	*	68.3 (63.4)
	3	2.92	3.53	4.90	3.52	3.83	*	*	*	3.19	54.2

September	1	2.12	5.26	4.11	3.94	3.76	8.46	*	*	4.30	62.8
	2	4.25 (3.15)	5.53 (4.58)	4.56 (4.10)	3.74 (3.63)	3.88 (3.55)	6.61 (7.54)	*	*	8.36 (6.33)	45.7 (51.7)
	3	3.09	2.96	3.64	3.21	3.02	*	*	*	*	46.6
October	1	1.88	2.74	*	*	*	*	3.09	*	*	*
	2	4.64 (2.95)	2.16 (2.42)	*	*	*	*	2.55 (2.84)	*	*	*
	3	2.34	2.36	*	*	*	*	2.87	*	*	*
November	1	2.02	1.78	*	*	*	*	0.89	*	*	*
	2	3.59 (2.76)	1.42 (1.57)	*	*	*	*	0.92 (0.79)	*	*	*
	3	2.68	1.51	*	*	*	*	0.57	*	*	*
December	1	1.81	1.99	*	*	*	*	1.36	*	*	*
	2	3.19 (2.67)	1.77 (1.78)	*	*	*	*	0.97 (1.06)	*	*	*
	3	3.01	1.57	*	*	*	*	0.84	*	*	*
Values for entire study											
N		36	24	15	15	15	8	9	6	5	15
Mean		2.52	4.54	4.50	3.67	3.77	4.49	1.56	2.56	5.00	56.82
SD		0.77	2.44	1.04	0.78	0.74	2.31	0.99	0.34	2.18	12.33
P time		>0.10	<0.001	<0.01	<0.01	<0.001	<0.05	<0.001	>0.50	>0.50	=0.23
P plot		<0.001	>0.10	>0.50	>0.50	>0.50	>0.75	>0.10	>0.10	+	=0.92

[a]The plant samples were gathered from three separate plots in one field near Woodland, Yolo County, during the January–December 1976 season. Data are cardenolide, equivalent to digitoxin, as mg/g dry weight of plant material. Monthly means are in parentheses; *denotes plant parts which were unavailable on collection dates. *P* values for time and plot are based on 2-way ANOVA tests with one observation per cell. For pods, + denotes a 1-way ANOVA for time only (data insufficient for test of plot effect). The dotted lines circumscribe the complete block of data treated by the 3-way ANOVA test in Table 3. **Represents an average of shoots collected in all three plots; too few were available to run an analysis on each plot separately.

concentration in a single population of *A. eriocarpa* plants with respect to three variables: (1) plant part, (2) time of the year, and (3) plot, i.e., the effect of the local conditions in one large field. The mean gross cardenolide concentrations, expressed as digitoxin equivalents, for samples from each plot over the study period of one year are given in Table 2. The mean cardenolide concentrations for the roots, stems, leaves, and latices are plotted as a function of time in Figure 2.

Statistical analyses were run in three separate sections: The first (A) was done on the latices alone, both because latex is a secretory product rather than an organ and because their cardenolide concentrations were found to be an order of magnitude higher than in all the organs which were studied, including the roots, shoots, stems, leaves, buds and flowers, and pods. The second section (B) presents analyses done on the complete block of data gathered in the three plots for the roots, stems, and lower, middle, and upper leaves for the 5-month period of May through September. The third section (C) presents analysis of all the organs which were gathered in the three plots throughout the entire study period.

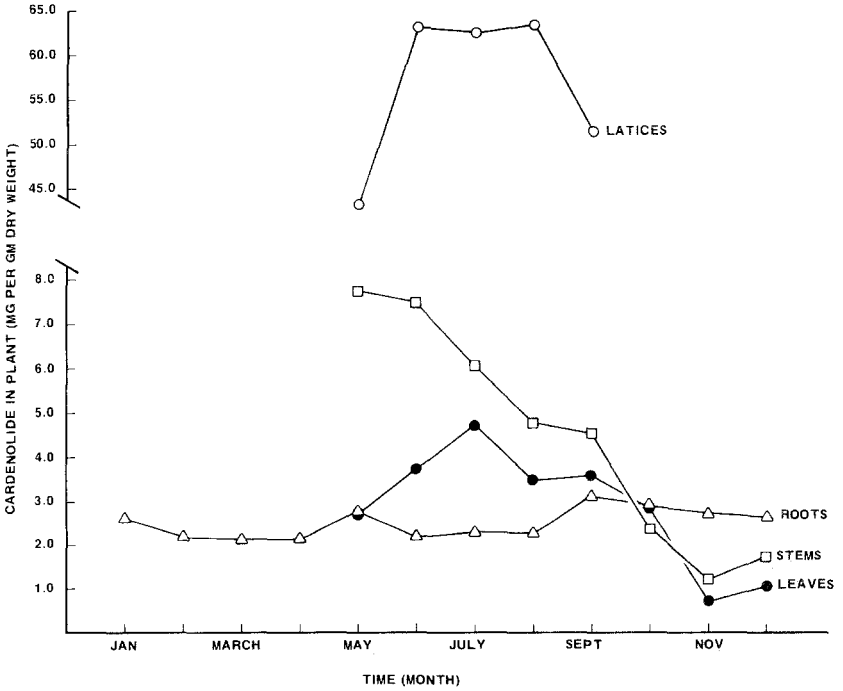

FIG. 2. Cardenolide variation in *Asclepias eriocarpa* during one year based on mean values for plant tissues and latices. The leaf samples are for middle leaves May through September, and dead leaves October through December.

Variability of Cardenolide Concentration of Latices from Three Plots for Five Months (May–September)

A two-way ANOVA with one observation per cell tested for the significance of differences in the cardenolide concentrations in the latices deriving from plot and time (Table 2, column 10). For the three plots, $F_{2,14} = 0.08$, with $P = 0.92$, and for the five months, $F_{4,8} = 1.77$, with $P = 0.23$. Thus neither plot-to-plot differences nor the overall monthly means differed.

Variability in Cardenolide Concentration of Roots, Stems, and Lower, Middle, and Upper Leaves in Three Plots for Five Months (May–September)

These are the most complete data (Table 2, columns 1–5), and the first step in their analyses consisted of a three-way ANOVA with one observation per cell to test the significance of differences in the overall means with respect to the three plots, the five months, and the five different plant parts (Table 3). All three factors differed to a highly significant degree, as did the three interactions (P values all <0.006).

The second step assessed the source of significance for the differences in the three plots. ANOVA tests and Scheffé contrasts (Nie et al., 1977) of the five parts for plot and time showed that plot was a significant effect only for the roots and that this significance was derived solely from a low cardenolide content of the roots in plot 1. The third step in this analysis explored the consistency of the overall time changes by plot. Mean values for all five organs in the three plots showed low May values (3.44–4.26) which then peaked in midsummer (July, 4.09–5.07) but again dropped off as fall approached (September, 3.18–4.39). The only apparent departure from this pattern

TABLE 3. THREE-WAY ANOVA OF CARDENOLIDE CONTENT OF *Asclepias eriocarpa*[a]

Source of variation	Sum of squares	Degrees of freedom	Mean square	F values	P values
A. Time	7.54	4	1.89	8.12	<0.001
B. Plot	3.94	2	1.97	8.49	<0.002
C. Part	104.20	4	26.05	112.20	<0.001
D. Time by plot	7.80	8	0.98	4.20	<0.002
E. Time by part	44.01	16	2.75	11.85	<0.001
F. Plot by part	6.49	8	0.81	3.49	<0.006
G. Residual	7.43	32	0.23	—	—

[a]Three-way ANOVA with one observation per cell testing the significance of differences of overall mean cardenolide concentration values with respect to (A) time (May through September), (B) plot (plots 1–3), and (C) plant part (roots, stems, upper, middle, and lower leaves).

occurred in plot 2 which showed a rise from August to September. However, this rise (6%) was relatively small compared to the one from May to July (31%) and the drop from July to August (18%). The fourth step in this analysis was aimed at describing the three different time trends for the five plant parts which are evident in Table 2 and Figure 2. ANOVA was utilized to test the fit of the data for each organ to linear, quadratic, cubic, or quartic equations. The roots showed no significant changes in cardenolide concentration over the 5-month period (Table 2, column 1). In contrast, the stems in May had a very high concentration which dropped continuously through the summer months (Table 2, column 2; $P < 0.001$). Moreover, these data showed a highly significant fit to a linear equation with a negative slope ($P < 0.001$). In contrast, the lower, middle, and upper leaves began with a low concentration which peaked in midsummer, and then dropped off again in the fall (Table 2, columns 3–5, $P < 0.005$, 0.025, and 0.025, respectively). These three very similar patterns showed highly significant fits to a quadratic function ($P < 0.001$, < 0.008, and < 0.004, respectively). Moreover, the lower and middle leaves also had a quartic component, albeit less significant ($P < 0.11$ and < 0.039, respectively).

A series of Scheffé contrasts (based on the three-way ANOVA, Table 3) was also done to compare the significance of the differences among the overall mean values of the cardenolide concentrations of the five plant parts. Except for the lower vs. the middle leaves ($P \simeq 0.50$), all differed from each other to a highly significant degree ($P < 0.001$).

Variability in Cardenolide Concentrations of All Organs throughout Entire Study Period

Two-way ANOVAS with one observation per cell were run on each of the five plant organs to test for significant differences between plots and over the varying number of months that the collections were made. The results of these tests are summarized as P values in Table 2, from which the following conclusions were made:

Roots, 12 Months. The roots showed remarkably little change throughout the year, but as found in the three-way ANOVA, were strongly influenced by location in the study area, i.e., plot ($P < 0.001$). Thus roots in plot 1 had substantially lower concentrations ($\bar{X} = 1.72$) than those in plots 2 and 3 ($\bar{X} = 3.09$ and 2.74, respectively), and this difference was evident throughout every month of the year (Table 2, column 1).

Stems, May through December. In contrast to the roots, the stems, when young, in May had a relatively high cardenolide concentration which dropped during the summer and on into the fall and winter (Table 2, column 2). This decline was highly significant ($P < 0.001$) and, unlike the roots, the stems showed no significant effect of plot ($P > 0.10$).

Lower, Middle, and Upper Leaves, May through September. This highly significant trend of increasing and then decreasing cardenolide concentration with no significant effect of plot was upheld in the two-way ANOVA tests on the same 5-month period (Table 2, columns 3–5). All P values for time were <0.01, and for plot were >0.50.

Dead Leaves, October through December. Dead leaves showed a highly significant ($P < 0.001$) decline in cardenolide from October through December and also were unaffected by plot ($P > 0.10$, Table 2, column 7).

Shoots; April, May, and September. Although few data could be gathered, the initial spring shoots were relatively low in cardenolide and increased in May (t test, April vs. May, $P < 0.05$). The lateral shoots appearing from the stems in September were not significantly higher than the shoots generated from the roots in May ($0.25 > P > 0.10$), but are significantly higher than the spring shoots in April ($P < 0.01$). (The overall plot effect is again not significant, $P > 0.75$, Table 2, column 6).

Buds and Flowers, and Pods; May through September. These relatively ephemeral organs showed no significant changes over the 2- to 3-month spans that they were present (Table 2, columns 8 and 9). The flowers and buds also showed no influence of plot. (Insufficient data were available to test for the effect of plot on the pods). Since numerous mature pods were collected from plot 1 plants in September, some were divided into their component parts and assayed. The seeds, silk, and pod shells had concentrations of 2.96, 1.89, and 7.36 mg/g, respectively.

In summary, the quantitative analyses indicated the following: (1) latices had cardenolide concentrations of an order of magnitude greater than all the other plant parts studied. Minimum to maximum values for latices were 27.1 to 76.2 mg/g, and for all other living parts were 1.49 (roots in August) to 8.46 (lateral shoots in September). (2) Roots had the lowest mean concentration (2.52) of all the living plant parts and, in addition, exhibited no significant variation throughout the year. (3) Stems in the spring began with a moderately high mean concentration (7.74 in May) which dropped continuously through the growing season and by September was down to 4.58. (4) Mean concentration in spring shoots in April was low (2.54) but doubled by May (5.15), and the lateral shoots in September were nearly 50% higher than the May shoots. The lateral shoots were higher than the leaves and stems for the same month (September) and comparable to stems sampled in May and June. (5) Leaves began with a low concentration (mean for all leaves in May = 2.91), increased to a maximum in midsummer (mean in July = 4.97), and then dropped as fall approached (mean in September = 3.76). (6) The upper leaves were consistently higher in concentration than both the middle and lower leaves (monthly means ranged from 0.5 to 1.5 mg/g higher). (7) Plot-to-plot variation affected the concentration of cardenolide in the roots, but was without a measurable effect on any of the above-ground parts. (8) During the

May to September period for which most data were available, the overall mean values differed significantly from each other as follows: latices (56.82) >> stems (6.12) > upper leaves (4.50) > lower leaves (3.77) ≅ middle leaves (3.67) > roots (2.54). (9) Buds and flowers, present only in May and June, had a mean concentration similar to the roots (2.56). (10) The pods, present from July to September, had a concentration (5.00) which was intermediate between the May–September stems and upper leaves. (11) Finally, as might be expected, the dead stems and leaves continued to decline in cardenolide content as fall progressed, but by December still contained an average of 23% and 37%, respectively, of their mean May values.

Quantitative Moisture Variation

Table 4 contains the moisture content for most samples analyzed and listed in Table 2. Comparing different samples, the roots were consistently lower in moisture than the other tissues and latices. Their mean moisture content for all months was 57% which is 15–25% lower than all other sample means. The seasonal trend evident in most of the other samples was for the moisture to begin high or peak in April and then decline through senescence.

Qualitative Cardenolide Variation

The objectives in this part of the study were similar to those in the quantitative section, that is, to examine samples for qualitative and quantitative variation of individual cardenolides with respect to the three variables—plant part, time of year, and plot. Variation was assessed qualitatively by TLC and quantitatively by densitometry. The principal identified cardenolides of the plants were labriformin (I), labriformidin (II), desglucosyrioside (III), and uzarigenin (V) (Figure 1). These four compounds have been previously isolated from *A. eriocarpa*, purified, and fully characterized (Seiber et al., 1978, and unpublished; Cheung et al., 1980). The presence of other cardenolides (syriogenin, syrioside, and glucosides of uzarigenin) was suggested from TLC comparison with standards in two solvent systems and TLC evaluation of samples treated with the enzyme β-glucosidase.

Subjective comparison of color photocopies from slides of TLC plates showed significant variation in type and amount of individual cardenolides among the plant parts. This variation is evident in the redrawn chromatograms (Figure 3). There was no apparent variation due to plot, and only slight qualitative variation appeared within the sample plant parts over time. Because of this lack of plot and temporal effects, we measured the relative amounts of individual cardenolides by densitometry for samples from one month as representing the principal trends which emerged in the results (Table 5 and Figure 4).

TABLE 4. MEAN MOISTURE CONTENT AS PERCENT OF FRESH WEIGHT IN *A. eriocarpa* WITH CARDENOLIDE CONTENT AS REPORTED IN TABLE 2.

	Roots	Stems	Upper leaves	Middle leaves	Lower leaves	Shoots	Buds and flowers	Pods	Latices
January	59	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
February	59	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
March	58	[a]	[a]	[a]	[a]	70	[a]	[a]	[a]
April	62	[a]	[a]	[a]	[a]	87	[a]	[a]	[a]
May	67	84	80	80	79	83[b]	84	[a]	[a]
June	60	74	73	73	73	[a]	80	[a]	72[b]
July	54	72	73	73	73	[a]	[a]	79[b]	71[b]
August	53	72	70	70	70	[a]	[a]	72[c]	70
September	53	72	69	68	68	64[b]	[a]	65[b]	73
October	50	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
November	55	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
December	55	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
N	36	15	15	15	15	10	6	5	10
Mean ± SD	57 ± 5	75 ± 5	73 ± 5	73 ± 5	73 ± 5	76 ± 12	82 ± 3	72 ± 3	72 ± 8

[a]Denotes plant parts which were unavailable on collection dates or for which moisture content was not determined.
[b]Denotes value determined from two plots.
[c]Denotes value determined from one plot.

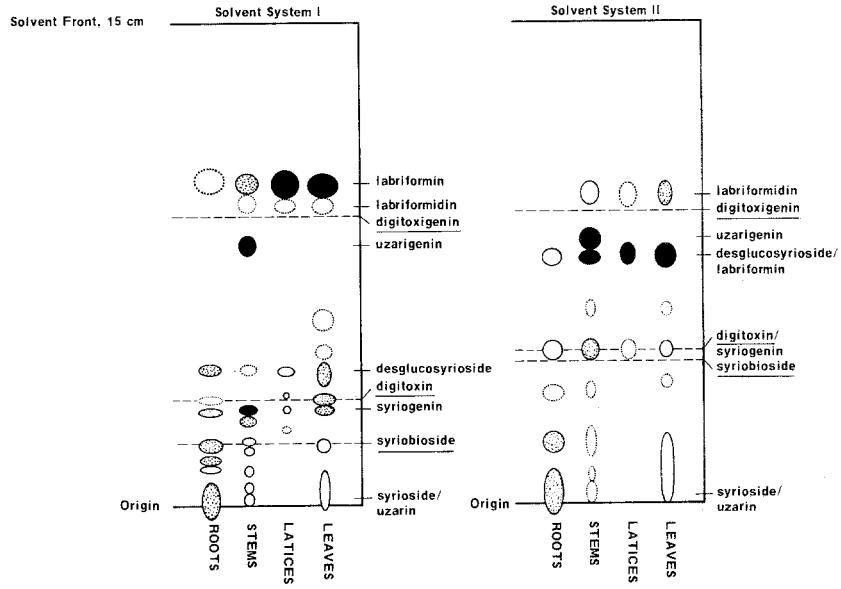

FIG. 3. Redrawn thin-layer chromatograms of cleaned extracts of *Asclepias eriocarpa* roots, stems, latices, and middle leaves collected in June. Chromatograms were developed four times in solvent system I, chloroform–methanol–formamide (90:6:1 by volume), and two times in solvent system II, ethyl acetate–methanol (97:3 by volume). Mobilities of standards are marked. Relative spot intensities correspond to: solid, most intense; stippled, medium; solid outline, light; broken outline, faint.

TLC R_fs indicated that the major proportion (ca. 73%) of cardenolides in the roots was more polar than digitoxin. A spot corresponding to syriogenin, a cardenolide in the R_f range of digitoxin, was observed in all root samples. Some of the very polar components with TLC R_fs near the origin may correspond to cardenolide glucosides, especially uzarin, desglucouzarin, and syrioside. This evidence comes from the increase in uzarigenin and desglucosyrioside upon treatment of root extracts with β-glucosidase and the similarities in R_fs of uzarin, desglucouzarin, and syrioside to cardenolide spots in the root extracts. The roots also contained amounts of desglucosyrioside corresponding to ca. 25% of the total in all samples, and traces of labriformin (ca. 2%) in the May through August samples. Labriformidin was not detected. These observations on the root contents were derived both from Table 5 data and qualitative examination of the TLC plates spotted with root extracts subjected to the lead acetate cleanup.

The stems contained cardenolides generally less polar than those in the roots and one compound, uzarigenin, which was unique to the stems. Uzarigenin occurred in substantial amounts (22%) relative to the other stem

TABLE 5. MEAN RELATIVE PERCENTAGE OF CARDENOLIDES IN *A. eriocarpa* FOR SAMPLES COLLECTED IN JUNE[a]

	Roots	Stems	Latices	Leaves
Labriformin	2	11	49	12
Labriformidin	0	14	16	28
Desglucosyrioside	25	22	10	20
Uzarigenin	0	22	0	0
Other[b]	73	31	25	40

[a]Values from two TLC solvent systems averaged after values for cochromatographing cardenolides determined.
[b]Not identified with certainty; primarily includes compounds with lower TLC R_f than digitoxin.

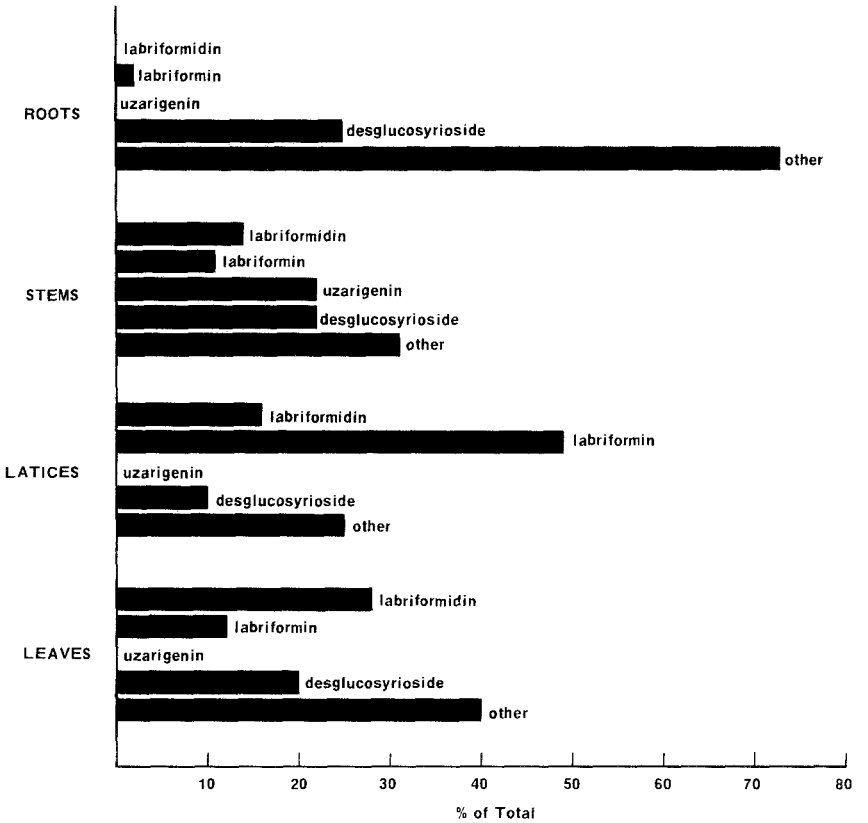

FIG. 4. Variation in four major cardenolides in June *Asclepias eriocarpa* samples of roots, stems, latices, and middle leaves as determined by reflectance densitometry.

cardenolides. Other major stem cardenolides were labriformin (11%), desglucosyrioside (22%), and an unidentified compound, along with smaller amounts of labriformidin (14%) and syriogenin. Samples were quite uniform in their TLC profiles with time from May through September; however, after the first frost in October the proportions of more polar cardenolides were slightly increased.

Latex samples showed a high concentration of less polar cardenolides, labriformin (49% of the total) being the principal component identified. Labriformidin and desglucosyrioside were also present but in substantially lower amounts (16% and 10%, respectively). A few cardenolides more polar than digitoxin were present but at very low concentrations. The latices also contained fewer unidentified cardenolides (25% of the total) than the other plant parts. There appeared to be a slight temporal variation in the labriformidin content of the latices: May samples had a higher content than the June, July, and August samples, which in turn were slightly higher than the September samples.

No qualitative or proportional changes in relative amounts of cardenolide components were observed in the TLC patterns for leaf samples collected from different parts of the stem, and neither time nor plot variation occurred in samples from May through September. Major concentrations of identified cardenolides in the leaves were in the order: labriformidin (28%) > desglucosyrioside (20%) > labriformin (12%). Several cardenolides of greater polarity were also present including syriogenin. The leaves, like the stems, showed a slight change in their TLC pattern after fall frost.

The cardenolides in the first shoot growth to appear in March resembled the roots in that they contained primarily labriformin, desglucosyrioside, and more polar cardenolides. When the shoots first developed both stem and leaf tissue (April), the cardenolide pattern closely resembled that of the stems with labriformin, labriformidin, desglucosyrioside, and uzarigenin being the major cardenolides observed. The lateral shoots—new growth from lower nodes on stems—which were present on some of the September plants had the same four major cardenolides and were particularly concentrated in labriformin and desglucosyrioside.

Buds and flowers (May and June), pods (July through September), and a single sample of pod shells and silk (September) showed TLC patterns similar to leaf samples, while a single sample of seeds contained primarily labriformin as well as small amounts of an unidentified polar cardenolide.

The qualitative analyses thus revealed significant differences among the individual cardenolides in plant parts and latices but no variation among plots and only slight variation in cardenolide proportions of living tissues with time. The latices, which had the highest concentration of cardenolides, contained relatively few in number; these were principally labriformin and lesser

amounts of the structurally related labriformidin and desglucosyrioside. The stems and leaves contained a greater number of cardenolides with a broader TLC R_f range than the latex samples. The stems and leaves also had relatively high concentrations of low-to-medium polarity cardenolides, especially labriformin, labriformidin, and desglucosyrioside. The stems were unique in having a substantial amount of uzarigenin. The cardenolide composition of the stems and leaves (which did not vary among lower, middle, and upper) was fairly constant during the growing season (May through September). The relatively ephemeral plant parts (shoots, buds and flowers, and pods) showed a combination of cardenolides similar to that in the leaves. The roots showed no significant quantitative or qualitative variation during the year. In contrast to the aerial plant parts, the cardenolides of the roots were relatively polar and corresponded in TLC R_f and in behavior with β-glucosidase enzyme to syriogenin, uzarin, desglucosyrioside, and syrioside.

DISCUSSION

Comparison with Other Asclepias *Species*. The comparison of variation in cardenolide content in *A. eriocarpa* to other *Asclepias* species will be limited to species which have been shown to contain some of the same cardenolides as *A. eriocarpa*.

The range in concentration for all living *A. eriocarpa* leaf samples in this study was 2.52–5.89 (mean = 3.98) mg cardenolide/g dried material (Table 2). This range is encompassed by the larger range, 1.02–9.19 mg/g (mean = 4.21) found by Brower et al. (1981) on a larger number (172) of *A. eriocarpa* leaf samples. Qualitative TLC analysis of 85 of these 172 samples collected from several stands in California indicated geographic and temporal factors had only minor effects on the proportions of the various cardenolides, which is consistent with our findings. In addition, our observations with lower, middle, and upper leaves showed that this constancy extends to leaves from all parts of the plant. The TLC profiles of lower, middle, and upper leaves from this study also are within the range of variation (for both type and quantity) observed for the 85 samples analyzed by Brower et al. (1981). We assume, then, that *A. eriocarpa* from our study plots were representative of those over the plant's geographical range in terms of cardenolide content.

A previous survey of *Asclepias* species revealed that the western U.S. species *A. labriformis* and *A. erosa* have cardenolide contents similar to *A. eriocarpa* (Roeske et al., 1976). A small number of samples (pooled aerial parts) of *A. labriformis* had 7.2 mg/g, in the same range as *A. eriocarpa*; TLC profiles for the two species were similar in pattern and proportions (Seiber et al., 1978; Benson et al., 1978).

A. erosa leaf samples collected at several different sites and times during the year had concentrations ranging from 0.79 to 21.02 mg/g (mean = 5.62, $N = 71$) (Brower et al., unpublished). Considering the smaller number of samples in that study ($N = 71$) compared to the similar *A. eriocarpa* study ($N = 172$), *A. erosa* appears to have a greater quantitative cardenolide variability than *A. eriocarpa*. TLC analysis of 24 of the 71 samples revealed cardenolide profiles for *A. erosa* which appeared to be generally indistinguishable from those of *A. eriocarpa*. That is, the variability in TLC profiles among individuals (which varied in collection by season, geographic location, and location on stem) of these two species appears to be at least as great as the variability between the species.

The cardenolide contents of *A. syriaca* growing in its native habitat in North America, and in Europe and Japan where it has become introduced, have been investigated by several different groups (see references in Roeske et al., 1976). Masler et al. (1962) isolated uzarigenin (V), desglucouzarin (Va), syriogenin (VI), syriobioside (VII), and syrioside (IV) from overground parts of *A. syriaca*. A recent examination by Brown and coworkers (1979) of roots from *A. syriaca* plants cultivated by Reichstein indicated the following compounds: syrioside, syriobioside, D-glucosyriobioside, and desglucosyrioside (III). Isolation and further analysis resulted in structural revision for syrioside and syriobioside and a proposed structure for desglucosyrioside. This investigation by Brown and coworkers (1979) indicated that the roots of *A. syriaca* have some similarities to the roots of *A. eriocarpa* in the very polar character of the cardenolides and in the specific cardenolides present.

Quantitative cardenolide analysis of the three samples of *A. syriaca* from Professor T. Reichstein yielded 1.42 mg cardenolide per g dry wt in roots; 1.34, in leaves; and 3.40, in stems. These single samples are slightly lower than the mean for similar *A. eriocarpa* samples (roots, 2.52; leaves for May through September, 3.98; stems for May through September, 6.12; from Table 2). TLC analysis of *A. syriaca* showed a greater number of cardenolides in the roots than stems and leaves. In addition to the compounds reported by Brown et al. (1979), we also found that the roots contained traces of labriformin and labriformidin. Uzarigenin was the major cardenolide component of the leaf and stem samples of *A. syriaca*. Traces of labriformin, desglucosyrioside, and syriogenin were present in the leaves, and traces of desglucosyrioside were present in the stems. Comparison of the TLC profiles for similar parts of *A. eriocarpa* and *A. syriaca* showed a greater variety of cardenolides in every part of *A. eriocarpa*. The most striking differences were that the aerial parts of *A. syriaca* showed only traces of compounds which were major components in the aerial parts of *A. eriocarpa*, *A. erosa*, and *A. labriformis* (i.e., labriformin, labriformidin, and desglucosyrioside) and that the aerial parts of *A. syriaca* lacked the diversity of cardenolides contained in the leaves and stems of *A.*

eriocarpa. Furthermore, syriogenin was a major component of *A. eriocarpa* roots but was absent in roots of *A. syriaca*. Considering their cardenolide commonality, the TLC profiles for the three western US species, *A. eriocarpa*, *A. erosa*, and *A. labriformis*, contrasted sharply with *A. syriaca* in relative cardenolide proportions.

*Comparison with Non-*Asclepias *Species*. Several previous studies on variation in cardenolide content were mainly initiated to evaluate production in species of commercial importance. Evans and Cowley (1972) followed cardenolide production in *Digitalis purpurea* (Scrophulariaceae) for first-year seedlings and for second-year plants. They estimated total cardenolide content and the ratio of digitoxigenin to gitoxigenin after hydrolysis on a fresh weight basis. The seedlings showed a rapid increase in cardenolide content from 0.014 to 0.115 mg/g during the first seven months, corresponding to the period of rapid growth, followed by a decrease to 0.011 mg/g during the subsequent three months when little growth occurred. With *A. eriocarpa*, the leaf samples showed an increase during midseason but the change was not of the magnitude observed for *D. purpurea*. Assessment by plant part for *D. purpurea* showed the following (mg/g): ripe dry fruits (0.480) > upper leaves (0.131) > flowers and immature fruits (0.075 to 0.108) > middle (0.055) and lower (0.051) leaves > stems (0.030) > roots (0.020). Converting our results to a wet weight basis shows that similar plant parts of *A. eriocarpa* contain cardenolide concentrations at least an order of magnitude greater than for *Digitalis*, and in the order: latices (16.2) >> stems (1.46) and pods (1.34) > upper leaves (1.24) > roots (1.05), middle (1.01) and lower (1.04) leaves > buds and flowers (0.47).

Karawya et al. (1973) estimated the total cardenolide content and the amount of oleandrin and adynerin in different tissues of red and white flowering *Nerium oleander* L. (Apocynaceae) during four different seasons. The mean of quantitative determinations (mg cardenolide/g dry weight) were: seeds (5.30) and roots (5.25) > fruits (4.64) and leaves (4.50) > flowers (3.83) > stems (3.18). These concentrations are within the same order of magnitude as for *A. eriocarpa*, but the distribution of concentrations by plant part differed markedly. Furthermore, the proportions of two aforementioned cardenolides varied considerably by plant part, most notably in the seeds and fruits, both of which lacked adynerin. It should be noted that both *A. eriocarpa* and *D. purpurea* are herbaceous plants while *N. oleander* is a woody perennial.

Ecological Implications and Speculations. Our results showed that *A. eriocarpa* latices had a much higher concentration of cardenolides and were enriched in less polar cardenolides when compared with the plant tissues. We believe that these findings, along with more subtle quantitative and qualitative variations among individual plant parts and with time, have implications both for avoidance of autointoxication and for plant defense.

The toxicity of cardenolides toward vertebrates has been well established. Acute effects include cardiac arrhythmias, which may be fatal, and emeticity, which may prevent fatal intoxication (references in Roeske et al., 1976). Most cardenolides are potent inhibitors of Na^+K^+-ATPase, and some of the relatively small number which have been tested are cytotoxic (Kupchan et al., 1967, 1977). Benson and coworkers (1978, 1979) determined that the acute toxicities (LD_{50} mice, intraperitoneal, mg/kg) of labriformin (9.2), labriformidin (3.1), and eriocarpin (≡ desglucosyrioside, Cheung et al., 1980) (6.5) isolated from *A. labriformis* and *A. eriocarpa* were similar to ouabain (6.4) and were only slightly less inhibitory to lamb cardiac Na^+K^+-ATPase than ouabain. These results, along with those from oral dosing of extracts to sheep, implicated cardenolides as the toxic constituents in these two species, both of which have a well-documented involvement in livestock poisoning (Kingsbury, 1964). The bitterness of some cardenolides may contribute in part to the plants' unpalatability (Kingsbury, 1964) and hence may explain why milkweeds growing in heavily grazed pastures are seldom taken by livestock (Brower and Huberth, 1977).

There is less information available on cardenolide toxicity to invertebrates. When uscharidin, calotropin, uzarigenin, and digitoxigenin were administered to *Danaus plexippus* larvae at levels well in excess of those naturally occurring in *Asclepias curassavica*, no mortality occurred and only uzarigenin was noticeably detrimental, producing a dose-dependent melanism in larvae (Seiber et al., 1980). Duffey et al. (1978) observed no toxic effects of digitoxin or ouabain orally administered to or injected into the hemolymph of *Oncopeltus fasciatus*. Both *D. plexippus* and *O. fasciatus* utilize *Asclepias* species as hosts, and both can concentrate and store cardenolides at levels which are demonstrably toxic to vertebrates (see references in Roeske et al., 1976). A different picture emerges for nonadapted insect species. Daily 10-μl injections of 10^{-8} or 10^{-12} M ouabain solutions into hemolymph decreased the survival rate of *Locusta migratoria*, a species not disposed to feeding on cardenolide-containing plants, but did not affect survival of *Zonocerus variegatus*, a cardenolide-adapted species (Rafaeli-Bernstein and Mordue, 1978). Vaughan and Jungreis (1977) found that 1.0 and 5.0×10^{-5} M solutions of ouabain caused 50% inhibition of Na^+K^+-ATPase when dosed in vitro to neuronal tissue isolated from the moths *Hyalaphora cecropia* and *Manduca sexta* in the presence of 7.5 mM of potassium ions. In comparison, tissue from *D. plexippus* was about 300 times less sensitive to ouabain under the same conditions.

The toleration of high diet and/or storage levels of cardenolides in adapted insects is a subject which has received rather intense scientific study. For some insect species, stored cardenolides may be kept isolated from susceptible tissue in specific glands and organs; examples include the

dorsolateral space fluid for *O. fasciatus* (Duffey et al., 1978), poison glands and cast skins for *Poekilocerus bufonius* (von Euw et al., 1967), Malpighian tubules for *Z. variegate* (Rafaeli-Bernstein and Mordue, 1978), pronotal and elytral glands for chrysomelid beetles (Daloze and Pasteels, 1979; Pasteels et al., 1979), and wings and larval/pupal exoskeletons for *D. plexippus* (Brower and Glazier, 1975; Thomashow, 1975). *D. plexippus* may also avoid cardenolide intoxication by maintaining high hemolymph potassium ion concentrations, which may lessen or block the action of these chemicals on the nervous tissue (Vaughan and Jungreis, 1977). Metabolism and rapid elimination represent other possibilities for insects' avoiding the toxic effects of cardenolides, but only circumstantial evidence is available for these routes (Roeske et al., 1976; Seiber et al., 1980).

Whatever unique adaptations exist for explaining the insensitivity of some insect species to cardenolide toxicity, they appear to be connected in most cases with positive defensive advantages for the adapted insect. We hypothesize that a similar situation may hold for those plant species which are adapted to produce and store cardenolide.

The general mechanisms by which cardenolides exhibit toxicity toward cells and certain enzymes in invertebrates and vertebrates suggest a potential for their interference with plants at a cellular level. Studies with two plant species support this hypothesis. MacRobbie (1962) found that 5×10^{-5} M ouabain inhibited potassium ion influx in the cell vacuoles and whole cells of *Nitella translucens* (Characeae). Brown et al. (1965) found that ouabain solutions ranging from 3×10^{-3} to 3×10^{-7} M had both inhibitory and stimulatory effects on different ATPase preparations from *Arachis hypogaea* (Leguminosae). The views of Müller (1976) and Swain (1976), that storage sites are necessary within a plant which accumulates secondary products, particularly those which are cytotoxic, appear to apply to a number of classes of secondary plant chemicals. For example, poppies (Papaveraceae) localize alkaloids in special cells and/or within vacuoles (Neumann, 1976; Matile et al., 1970; Matile, 1976; Fairbairn et al., 1974). Cardiac glycosides in the leaves of *Convallaria majalis* (Liliaceae) are stored primarily in the vacuoles (Löffelhardt et al., 1979). In *Sorghum* leaves (Gramineae) the cyanogenic glycoside dhurrin is isolated in different tissues from the enzymes capable of catalyzing the release of HCN (Kojima et al., 1979). Glucosinolates in the Cruciferae are also stored separately from the enzymes which effect their hydrolysis (Feeny, 1970).

The latex may represent a means for compartmentalizing toxic secondary chemicals, particularly lipophilic substances, so that these substances are stored and transported in the latices independent of the plant's vascular system (McKey, 1979). The laticifers, which may continuously penetrate the meristematic tissue (Esau, 1965), would provide protection to the rapidly

growing and differentiating tissues prior to the development of other storage capacity. Indeed, several examples of latex storage of toxic secondary products have been forthcoming, including *Papaver somniferum* (Fairbairn et al., 1974) and *Chelidonium majus* (Mattle, 1976), both of which contain alkaloids; three *Euphorbia* species, which concentrate toxic diterpenes in the latices (Evans, 1977; Evans and Schmidt, 1976); and other *Euphorbia* species which contain high concentrations of rare, nonprotein amino acids in their latices (Haupt, 1976).

The concentration of cardenolides in the latices of *A. eriocarpa* is an order of magnitude greater than for the other tissues when calculated on either a wet or dry basis. Extending Swain's (1976) hypothesis to include milkweed cardenolides, then the laticifers afford plants such as *A. eriocarpa* with storage sites for cardenolides isolated from the plant's vascular system but permeating throughout most tissues, producing a pressurized secretion of latex rich in toxic metabolites at any rupture. As this secretion collects and dries at the site of injury, the concentration of cardenolides in the immediate area increases. Assuming a defensive function for cardenolides, this might discourage further attack and/or secondary infection by microorganisms. We suggest that the laticifers may provide a means of avoiding autotoxicity via physical isolation in a system which distributes cardenolides throughout the plant making them available for defense. This is in addition to the physical barrier presented by the sticky latex oozing at a point of injury which may trap or deter some potential herbivores.

The concentration ratio of latex–leaf cardenolides for *A. eriocarpa* is approximately 15 and a high ratio is also found in *A. curassavica*, *Calotropis procera*, and *Nerium oleander*. In contrast, species which tend toward low concentrations of leaf cardenolides, such as *A. fascicularis*, *A. cordifolia*, *A. californica*, and *A. speciosa*, tend also to have low concentrations in the latices (Seiber et al., 1981). These observations suggest that the function of the laticifers as storage sites may be relatively more important in plants with a generally high cardenolide content and that at low levels the physical isolation afforded in the laticifers may not be necessary.

TLC of the latices of *A. eriocarpa* revealed that they were relatively high in labriformin. This cardenolide contains a thiazoline ring and as such is a counterpart to uscharin and its hydrogenated analog voruscharin from *Calotropis procera* and *A. curassavica*. The latices of these latter two species bear certain similarities to those of *A. eriocarpa* in terms of cardenolide content. Uscharin was the major cardenolide isolated from the latices of *C. procera*; its presence was noted, but it was not isolated from the leaves and stems (Brüschweiler et al., 1969a). The occurrence of thiazolidine and thiazoline cardenolide derivatives is relatively rare and their propensity to accumulate in the latices is striking. It is also notable that the cardenolide components of the latices are relatively nonpolar (Figure 3). This structure-

related physicochemical characteristic is a factor which may govern their solubility and/or binding in the latex and other lipophilic systems. The presence of nitrogen and sulfur in an additional ring system within labriformin, uscharin, and voruscharin creates the potential for still other interactions for stabilization or binding in the latex. It is notable that some other secondary metabolites localized in latices, specifically alkaloids and amino acids, also contain nitrogen. Assuming a defensive function for cardenolides and a storage and transportative function for laticifers, we then speculate that these compounds (specifically, labriformin, uscharin, voruscharin, labriformidin, and uscharidin) represent a later step in the evolution of the plants' cardenolide defenses and that their presence in the latices is a further adaptation, maximizing their ability for translocation and thus increasing their defensive potential over storage elsewhere. Labriformin can be converted to desglucosyrioside via labriformidin in vitro (Seiber et al., 1978) and in vivo in *D. plexippus* larvae (Tuskes, unpublished); the analogous conversion of uscharin to calactin/calotropin via uscharidin has been reported in vitro (references in Bruschweiler et al., 1969b) and evidence for its occurrence in vivo also exists (Seiber et al., 1980). Thus, latex cardenolides could conceivably be enzymatically modified for deployment by the plant or following ingestion by a herbivore. Aside from observations on the latter with *D. plexippus* (Seiber et al., 1980), there presently exists no experimental basis for assessing the role of such transformations in antiherbivore defense.

Besides the notable differences in concentration of cardenolides in the leaves and stems when compared with the latices, it is difficult to gauge the importance of qualitative differences in cardenolides noted among these tissues because residual latex was unavoidably included in their analysis. That is, our analysis of these parts included both tissue and latex cardenolides but in unknown proportions. An exception lies with uzarigenin which was present in the stems but not in leaves or latices, and thus must be associated with stem tissues. It is notable that only uzarigenin abnormally affected development of *D. plexippus* larvae among four cardenolides tested, producing a dose-related melanism (Seiber et al., 1980). From a chemical defense viewpoint, perhaps the presence of this compound in the stems may be a deterrent to larval feeding on this tissue, consistent with observations that *D. plexippus* larvae feed almost exclusively on the leaves and flowers of *A. eriocarpa* in the wild. A deterrent to feeding on the stem may in fact be to the monarch's advantage in the long run, for the preserved stem could provide vegetative regrowth for sustaining a subsequent generation of the insect. The relatively high concentration of cardenolides, including uzarigenin, in the stems of very young plants might similarly represent a strategic defensive adaptation, in this case toward a variety of herbivores which might otherwise prevent the plant from achieving maturity.

The roots, on a dry weight basis, have the lowest cardenolide content of

all the living tissues. The moisture content is also the lowest, so that when cardenolide content is calculated on a wet weight basis the range from May to September is 0.88–1.48 mg/g, comparable with that of the leaves for the same period (0.55–1.64 mg/g). A low moisture content has been considered a "quantitative" defense by Scriber (1978). Among the *A. eriocarpa* plant tissues examined, the roots were also notable in having a higher ratio of more polar cardenolides, particularly desglucosyrioside (OH at C-3'); preliminary evidence also indicated that the glucose conjugate of this compound at C-3', syrioside, was present along with syriogenin and several unidentified polar cardenolides. In occupying a distinctive habitat from aerial plant parts, roots are exposed to a different set of potential herbivores and pathogens and, if cardenolides serve a defensive function, then the very polar nature of the cardenolides in the roots may be a strategic adaptation. The resistance of onions to the fungus *Colletotrichum circinans* is due to the presence of water-soluble phenols in the dead cells which diffuse and inhibit germination and penetration (see Levin, 1971).

CONCLUSIONS

We have found that significant variations exist in the amounts of total cardenolide and the proportions of individual cardenolides among the plant parts and latices of *A. eriocarpa*. Assuming a defensive function for cardenolides, the temporal variation in the storage patterns may reflect the plants' response to environmental factors, synthesizing and storing cardenolides at growth stages in proportion to the plants' susceptibility to herbivore attack and damage. This suggests the presence of a complex defense system in *A. eriocarpa* (and, presumably, other *Asclepias* species similar in cardenolide content) in which the laticifers play a central role. Relatively high levels of cardenolides, particularly the unusual cysteine-derived conjugate labriformin in *A. eriocarpa*, are stored in the latices; this fluid is expelled when the laticifers are ruptured, thus delivering a relatively large quantity of cardenolide to the site of disruption. Through this process the defensive potential (bitterness, emeticity, cardiac activity) of the cardenolides is enhanced, and the possibility for cardenolide-induced toxicity to the plant is reduced. Very likely, specialized milkweed feeders, such as monarch butterfly larvae and certain lygaeid bugs, have adapted to this defense system and may even utilize the differential content of particular tissues to maximize sequestering of these chemicals for their own defense. Even with well adapted herbivores, such as the monarch, the plant may possess reserves of cardenolide-based defense, such as the presence of uzarigenin in the stem, which when ingested causes a melanic response. The potential role of cardenolides in the

roots for plant defense is less clear, but the propensity toward storage of very polar cardenolides in the roots contrasted sharply with findings for above-ground plant parts and the latices.

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IDENTIFICATION OF A SEX PHEROMONE OF *Heliothis subflexa* (GN.) (LEPIDOPTERA: NOCTUIDAE) AND FIELD TRAPPING STUDIES USING DIFFERENT BLENDS OF COMPONENTS[1]

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Abstract—Eight compounds were isolated from the sex pheromone gland of *Heliothis subflexa* (Gn.) and identified as hexadecanal, (*Z*)-9-hexadecenal, (*Z*)-11-hexadecenal, (*Z*)-7-hexadecen-1-ol acetate, (*Z*)-9-hexadecen-1-ol acetate, (*Z*)-11-hexadecen-1-ol acetate, (*Z*)-9-hexadecen-1-ol, and (*Z*)-11-hexadecen-1-ol. Although the whole blend was found to be an effective male attractant, the deletion of alcohols from the blend increased trap captures considerably. Further, although the binary mixture of (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal caught some male *H. subflexa*, significant increases in captures were noted when the three acetate components were included in the blend.

Key Words—Sex pheromone, *Heliothis subflexa*, Lepidoptera, Noctuidae, reproductive behavior, field trapping, capillary chromatography.

INTRODUCTION

Heliothis subflexa (Gn.) is an innoxious noctuid moth which feeds exclusively on ground cherry (*Physallis* spp.) (Brazzel et al., 1953). Although not a pest, it has recently become the subject of numerous pest control studies due to

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success in laboratory hybridization 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 hybrid and backcross progeny which, when released into a natural *H. virescens* population, should reduce the numbers of *H. virescens* via the induction of male sterility. However, the considerable difficulties in obtaining viable hybrid progeny or even interspecific mating under laboratory conditions (Brazzel et al., 1953; Laster, 1972; Proshold and Lachance, 1974) indicate that the pair have developed highly effective mechanisms of premating reproductive isolation. Due to the broadly sympatric distributions and intersecting reproductive periods of these species (Tingle et al., 1977), barriers to interspecific gene flow are most likely the result of differences in their respective sex pheromone communication systems (Roelofs and Cardé, 1974).

Recent chemical studies on several *Heliothis* species have indicated the existence of a common trend in the types of compounds forming the pheromone blend, with (*Z*)-11-hexadecenal and (*Z*)-9-hexadecenal being components common to all species studied thus far (Klun et al., 1980a,b; Nesbitt et al., 1979, 1980). Hence, it appears that pheromonal mechanisms of isolation between species result from different blends and ratios of components. We report the identification of a highly effective species-specific sex pheromone blend produced by female *H. subflexa*.

METHODS AND MATERIALS

General. H. subflexa used for both the collection of pheromone and bioassay studies were reared from laboratory stocks maintained at either Gainesville, Florida, or Stoneville, Mississippi. After pupation the insects were sexed and allowed to emerge in isolation from members of the opposite sex under test conditions. Newly emerged adults, collected daily, were placed in 30-cm^3 Plexiglas cages and provided with a 10% sucrose solution for nutrient. All laboratory studies were carried out on moths maintained under a 16:8-hr light–dark cycle at a temperature of 20° C and a relative humidity of 60%. Insects used for both the collection of pheromone and bioassay studies were selected on the basis of good physical appearance at between 2 and 5 days of age.

Pheromone extracts were prepared from groups of five actively calling females which had entered into a protracted bout of calling as indicated by ovipositor extension for a minimum of 2 min (Teal et al., 1981). Individual calling females were removed from the holding cage and the ovipositors removed as described by Klun et al. (1980a). Immediately after removal, the ovipositors were placed (5/microvial) in 250 μl of ethyl ether (Mallinckrodt®,

anhydrous reagent grade) and allowed to soak for a period of 2–3 min. The ethyl ether extract was pipetted into another vial which contained 15 μl of isooctane (Fisher, 99 mole %) and the ether allowed to evaporate under a fine nitrogen stream. Extracts were then stored at −60° C until use.

Chemical Analysis. Gas chromatographic (GC) analysis of 3- to 5-μl samples [1–2 female equivalents (FE)] of the ovipositor extracts was done with a Hewlett-Packard model 5710A® GC equipped with a splitless injector system. The output of the flame ionization detector was interfaced to a Nicolett 1180® data system capable of storing 32,000 real time data points. Nitrogen (linear flow velocity of 9.8 cm/sec) was used as a carrier gas. Initial GC studies were performed with a 66-m × 0.25-mm (ID) glass capillary column coated with SP 2340 (Supelco, Belefonte, Pennsylvania) capable of separating most of the geometrical and positional isomers of C_{14} and C_{16} alcohols, aldehydes, and acetates (Heath et al., 1980). Samples were injected at an initial column temperature of 60° C (injector temperature = 250° C) with a 60-sec delay prior to injector purging. The column temperature was programed after 2 min at 32° C/min to a final temperature of 150° C. Extracts were also chromatographed on a 31-m × 0.25-mm (ID) glass column coated with cholesteryl cinnamate (Heath et al., 1979). Splitless injections were made at the mesophase transition temperature (159° C) using decane as the solvent.

The retention times of the compounds eluting during GC analysis of the ovipositor extracts as well as those of the synthetic standards were reduced to equivalent chain length (ECL) units with slight modification (Jamison and Reid, 1969). For the purpose of this study, the acetates of primary saturated alcohols varying in chain length from 12 to 20 carbons were used as the functional retention index (Swoboda, 1962), regardless of the compound's functionality. In subsequent studies, ovipositor extracts were cochromatographed with isomerically pure (+98%) synthetic standards.

Further chemical characterization was accomplished by GC–mass spectrometry with a Finigan model 3200® chemical ionization mass spectrometer equipped with a GC inlet. The combined extract from 50 calling females was injected onto a 2-m × 2-mm (ID) glass column packed with 3% OV-17® on Gas Chrom Q® (100/120 mesh) and the total effluent was introduced directly into the ionization source. Methane was used as both a carrier and reagent gas. Spectra of the natural products were compared with those of candidate synthetic compounds.

All synthetic standards used (Figure 1a), with the exception of (*E*)-7-hexadecenal, (*E*)-9-hexadecenal, (*E*)-11-hexadecenal, (*E*)-9-hexadecen-1-ol, (*E*)-11-hexadecen-1-ol, and (*Z*)-9-hexadecen-1-ol acetate, were obtained from Chemical Samples Co. (Columbus, Ohio), as were the starting materials used in preparation of the above standards. (*E*)-9-Hexadecen-1-ol was obtained from a lithium bronze reduction of 9-hexadecyn-1-ol (Mueller and

Gillick, 1978) and a portion of the product was oxidized to (*E*)-9-hexadecenal with pyridinium chlorochromate (Corey and Suggs, 1975). (*E*)-7-Hexadecenal and (*E*)-11-hexadecenal were similarly prepared by oxidation of their corresponding alcohols. The (*E*)-11-hexadecen-1-ol was obtained by saponification of (*E*)-11-hexadecen-1-ol acetate, while the (*Z*)-9-hexadecen-1-ol acetate was prepared by acetylation of (*Z*)-9-hexadecen-1-ol. Isomeric purity was assessed by GC analysis with the SP2340 column and, when necessary, geometric isomers were separated by high-performance liquid chromatography on a 25 × 2.5-cm (OD) $AgNO_3$ column eluted with toluene (Heath and Sonnet, 1980). All standards were assesssed as being at least 98% pure.

Bioassays and Field Testing. Laboratory-reared males were used to assess the ability of gland extracts to elicit male reproductive behaviors. In these tests, 1-FE samples of the extracts were placed on 1 × 3-cm filter papers and suspended in the upwind end of a 1.5 × 0.5 ×0.5-m Plexiglas wind tunnel (Teal et al., 1981a). Individual males were then released into the center of the downwind end and behaviors monitored over a 5-min period. The data were recorded on audio cassette tape and later transcribed (Teal et al., 1981a). The ability of each extract to induce upwind taxis, landing on the dispenser, and genital segment exposure was assessed.

Field studies were conducted during July and August of 1980 in a fallow field near Gainesville, Florida, containing ground cherry, *Physalis* spp., and supporting a high larval *H. subflexa* population. Cone traps (Hartstack et al., 1979) spaced 10 m apart were set in two lines at 90° to one another in the field. The traps were randomly baited with three calling-age females, a 2-ml polyethylene vial containing 30 mg of the synthetic mixture plus 5 μg of BHT [2,6-bis(1,1-dimethylethyl)-4-methylphenol, as an antioxidant], or a blank vial. Both the females and vials were rebaited every 2 days. In a second series of tests, traps were baited with 3 females, a blank 8.5-cm-diam. filter paper, or filter papers baited at 30-min intervals during the calling period with either 5 FE of the ovipositor extract or an equivalent amount (ca. 75 ng as indicated by GC analysis) of the synthetic blend. All baits were rerandomized daily.

The species specificity of the *H. subflexa* synthetic blend was assessed during field trapping studies in tobacco and corn fields having populations of either *H. virescens* or *H. zea* (Boddie) and in the original test field. All sites had fruiting ground cherry present. Cone traps, spaced 10–12 m apart and located in the vicinity of *Physalis* plants, were baited with 3 female *H. subflexa*, 30 mg of the *H. subflexa* synthetics dispensed from polyethylene vials, 3 female *H. zea* or *H. virescens*, or 30 mg of either the 4-component *H. zea* or the 7-component *H. virescens* synthetic blend reported by Klun et al. (1980a,b) depending upon the field. Tests in the original test field included females and synthetic blends of all three species.

Two series of tests were conducted to assess the effects of groups of

components on trap capture. In the first series the effect of deleting the alcohol, acetate, or aldehyde components from the blend was assessed with rubber septa (A.H. Thomas Co.) (Flint et al., 1979) impregnated with the same concentration of blend components as in the 25-mg complete blend. The second test was designed to assess the effectiveness of the aldehyde, alcohol, and acetate component groups independently, and to assess combinations of the aldehyde and acetate fractions. Pherocon® 1C sticky traps spaced 10 m apart were positioned in two lines in the original field and randomized daily during the test periods. Daily trap captures were recorded and the data transformed to $\log_{10}(n + 1)$ prior to statistical analysis.

RESULTS AND DISCUSSION

Components contained within the ovipositor extracts were tentatively identified by comparison of their ECL values with those of a standard mixture of synthetic compounds. This mixture contained the Δ7, Δ9, and Δ11 isomers of hexadecenal, hexadecen-1-ol, and hexadecen-1-ol acetate. The saturated analogs of the above compounds were also added to the mixture. Although the SP2340 column provided adequate resolution of most of the compounds (Figure 1a), the cholesteryl cinnamate column was also used to further define the assignments of the components. Table I lists the ECL units for the synthetic compounds used in this study on both the SP2340 and cholesteryl cinnamate columns.

Bioassays of 1-FE concentrations of the whole ovipositor extracts gave consistent results, indicating that the complete range of reproductive behaviors assessed was elicited with glandular extracts while only minimal random flight was observed when solvent blanks were presented.

Capillary chromatography of these extracts revealed several major peaks having retention times coinciding with hexadecanal, (*Z*)-9-hexadecenal, (*Z*)-11-hexadecenal, (*Z*)-7-hexadecen-1-ol acetate, (*Z*)-9-hexadecen-1-ol acetate, (*Z*)-11-hexadecen-1-ol acetate, (*Z*)-9-hexadecen-1-ol, and (*Z*)-11-hexadecen-1-ol (Figures 1 and 2). Several other peaks were also variably present but, when present, each composed less than 1% of the total mixture.

Mass spectra obtained from peaks eluting from the OV-17 column when ovipositor extracts were injected had identical fragmentation patterns with the monounsaturated aldehyde, acetate, and alcohol standards. Points of unsaturation were further confirmed by cochromatography of the ovipositor extracts and synthetic compounds on the SP2340 capillary and cholesteryl cinnamate columns. The relative proportion of each of these compounds within the natural blend is indicated in Table 2.

Field tests of the synthetic blend formulated either in polyethylene vials

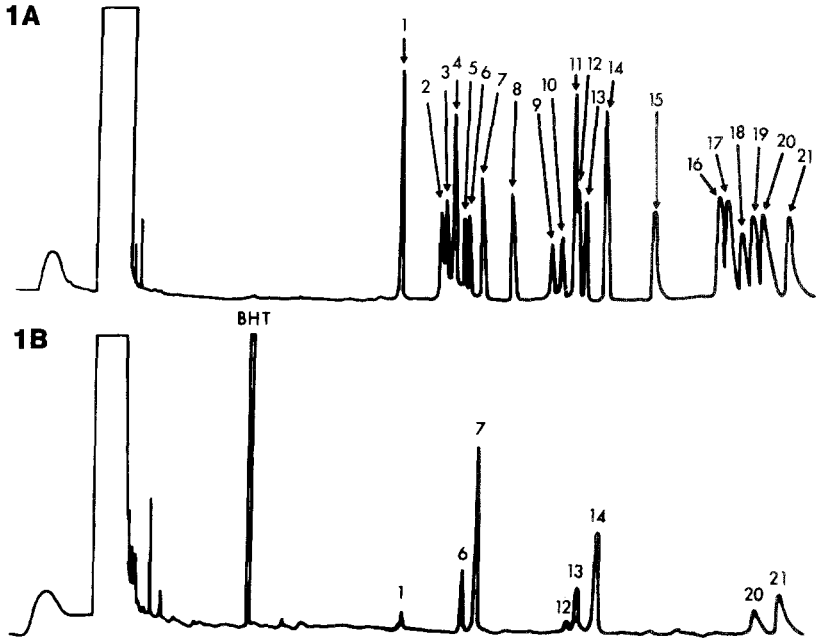

FIG. 1. (A) Chromatogram of standard compounds eluting from the SP2340 column. 1 = Hexadecanal, 2 = (*E*)-7-hexadecenal, 3 = (*E*)-9-hexadecenal, 4 = (*E*)-11-hexadecenal, 5 = (*Z*)-7-hexadecenal, 6 = (*Z*)-9-hexadecenal, 7 = (*Z*)-11-hexadecenal, 8 = hexadecan-1-ol acetate, 9 = (*E*)-7-hexadecen-1-ol acetate, 10 = (*E*)-9-hexadecen-1-ol acetate, 11 = (*E*)-11-hexadecen-1-ol acetate, 12 = (*Z*)-7-hexadecen-1-ol acetate, 13 = (*Z*)-9-hexadecen-1-ol acetate, 14 = (*Z*)-11-hexadecen-1-ol acetate, 15 = hexadecan-1-ol, 16 = (*E*)-7-hexadecen-1-ol, 17 = (*E*)-9-hexadecen-1-ol, 18 = (*E*)-11-hexadecen-1-ol, 19 = (*Z*)-7-hexadecen-1-ol, 20 = (*Z*)-9-hexadecen-1-ol, 21 = (*Z*)-11-hexadecen-1-ol. (B) Chromatogram of components in the ovipositor extracts eluting from the SP2340 column. 1 = Hexadecanal, 6 = (*Z*)-9-hexadecenal, 7 = (*Z*)-11-hexadecenal, 12 = (*Z*)-7-hexadecen-1-ol acetate, 13 = (*Z*)-9-hexadecen-1-ol acetate, 14 = (*Z*)-11-hexadecen-1-ol acetate, 20 = (*Z*)-9-hexadecen-1-ol, 21 = (*Z*)-11-hexadecen-1-ol.

or on filter paper indicated that the synthetics were as effective in capturing males as either females or the crude ovipositor extracts in cone traps (Table 3). Field observations indicated that males generally underwent upwind taxis toward the traps baited with the synthetic blend, extending their genitalia and hovering at 10–15 cm from the dispenser prior to flying up and into the trap. However, close-range courtship behaviors (Teal et al., 1981a) such as landing on the dispenser, abdominal curving, and copulatory attempts were not observed when the synthetic blend was used, which indicates that cues responsible for eliciting courtship behaviors were absent, masked, or inhibited in these tests. Such stimuli may result from several different features absent in

our tests such as the precise blend of components released by calling females and visual and tactile stimuli (Baker and Cardé, 1979a,b). Further, the absence of stimuli responsible for landing and close-range copulatory behaviors are presumed to have little effect upon cone trap efficiency since males commonly move up and out of the pheromone plume when incomplete stimuli are presented (Teal et al., 1981b) and are therefore captured in cone traps.

Neither the synthetic blends nor caged females of the three species captured males of other *Heliothis* species, indicating that the pheromone blends are species specific. However, while both the *H. subflexa* and *H. virescens* synthetic blends caught as effectively as caged females (15 ♂ to *H. subflexa* synthetics/21 ♂ to *H. subflexa* ♀ , and 6 ♂ to *H. virescens* synthetics/10 ♂ to *H. virescens* ♀) the blend described for *H. zea* by Klun et al. (1980a) was considerably less effective than females (3 ♂ to synthetics/56 ♂ to ♀). This may indicate a disparity in the actual blend released during calling and that maintained within the pheromone gland or cuticle overlying it

TABLE 1. EQUIVALENT CHAIN LENGTH UNITS OF GC STANDARDS ON SP2340 AND CHOLESTERYL CINNAMATE CAPILLARY COLUMNS AS CALCULATED USING SATURATED C_{14}–C_{16} ACETATES AS THE FUNCTIONAL RETENTION INDEX

Standard	Equivalent chain length	
	SP2340	Cholesteryl cinnamate
Hexadecanal	1513	1434
(*E*)-7-Hexadecenal	1555	1412
(*Z*)-7-Hexadecenal	1577	1402
(*E*)-9-Hexadecenal	1559	1415
(*Z*)-9-Hexadecenal	1582	1404
(*E*)-11-Hexadecenal	1567	1419
(*Z*)-11-Hexadecenal	1584	1410
Hexadecen-1-ol acetate	1600	1600
(*E*)-7-Hexadecen-1-ol acetate	1628	1578
(*Z*)-7-Hexadecen-1-ol acetate	1645	1566
(*E*)-9-Hexadecen-1-ol acetate	1634	1583
(*Z*)-9-Hexadecen-1-ol acetate	1653	1571
(*E*)-11-Hexadecen-1-ol acetate	1643	1585
(*Z*)-11-Hexadecen-1-ol acetate	1665	1575
Hexadecan-1-ol	1693	1561
(*E*)-7-Hexadecen-1-ol	1734	1521
(*Z*)-7-Hexadecen-1-ol	1752	1508
(*E*)-9-Hexadecen-1-ol	1737	1526
(*Z*)-9-Hexadecen-1-ol	1756	1511
(*E*)-11-Hexadecen-1-ol	1744	1541
(*Z*)-11-Hexadecen-1-ol	1768	1518

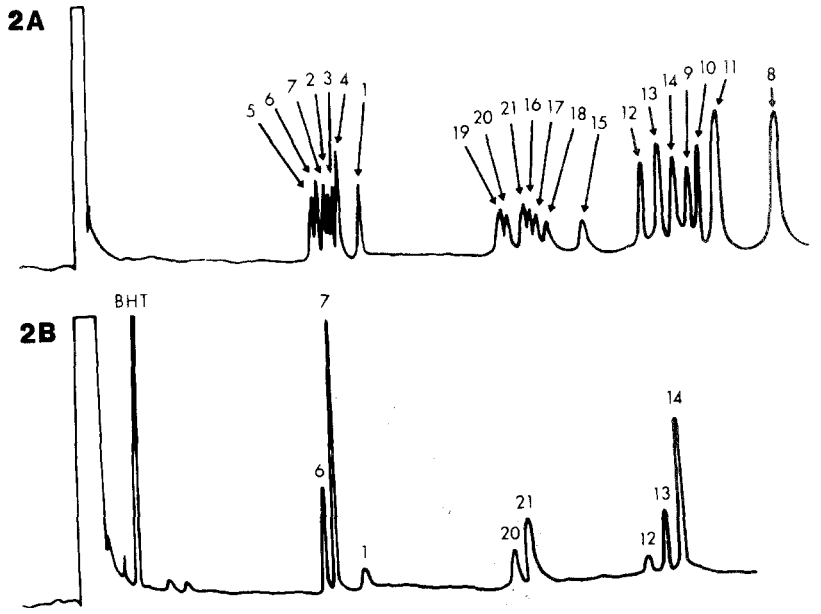

FIG. 2. (A) Chromatogram of standard compounds eluting from the cholesteryl cinnamate column. Compound labels as in Figure 1A. (B) Chromatogram of components in the ovipositor extracts eluting from the cholesteryl cinnamate column. Component labels as in Figure 1B.

TABLE 2. CHEMICAL COMPONENTS ISOLATED FROM *H. subflexa* OVIPOSITOR WASHES

Compound	Mean % composition (15 ♀)	Composition by weight (ng/♀)
Hexadecanal	5.4	0.82
(*Z*)-9-Hexadecenal	19.8	3.01
(*Z*)-11-Hexadecenal	30.0	4.56
(*Z*)-7-Hexadecen-1-ol acetate	1.6	0.25
(*Z*)-9-Hexadecen-1-ol acetate	4.3	0.65
(*Z*)-11-Hexadecen-1-ol acetate	12.3	1.87
(*Z*)-9-Hexadecen-1-ol	14.4	2.19
(*Z*)-11-Hexadecen-1-ol	12.2	1.85

TABLE 3. COMPARISON OF CONE TRAP CAPTURES OF MALE *H. subflexa* USING FEMALES, SYNTHETIC CHEMICALS, AND CRUDE EXTRACTS[a]

Trap bait	Mean trap capture/night[b]
3 females	7.6 a
20 mg synthetics in vials	8.0 a
5 FE synthetics on filter papers	7.3 a
5 FE ovipositor extracts on filter papers	3.0 a
Blank	0.0 b

[a]Means followed by the same letter are not significantly different at a 0.05 level in a Duncan's multiple-range test.
[b]Six replicates.

(cf. Weatherston and Maclean, 1974). Although the obvious differences in the blends of the three species suggest several avenues by which reproductive isolation could be effected, we have not assessed the behavioral effects of differences in the blend ratios or presence or absence of components on any of the three species and are unable to define the basis for chemical isolation at present. Laboratory interspecific communication studies between *H. virescens* and *H. subflexa* (Teal et al., 1981b) indicate that long-distance semiochemical isolation occurs between female *H. virescens* and male *H. subflexa*. However, it is the close-range orientation that is disrupted when the male *H. virescens* are released downwind from calling *H. subflexa* females.

Studies conducted to assess the effects of deleting the alcohol, aldehyde, or acetate components from the blend indicated that the removal of any one of these groups did not completely stop trap capture (Table 4, experiment 1). While the whole synthetic blend was as effective as virgin females in cone trap studies, a significant decrease in captures relative to females was noted when the whole blend was employed in sticky traps. Further, considerably more males were captured in the sticky traps when the alcohols were deleted, suggesting that the alcohols may act as an inhibitor to landing, a prerequisite for both mating (Teal et al., 1981a) and being caught in sticky traps. This is further supported by field observations of moths orienting to cone traps which indicated that, although males did approach dispensers containing the whole blend, none landed or exhibited any close-range copulatory behaviors.

Tests employing baits composed of various blends of components in sticky traps indicated that those containing either the acetates or alcohols alone were ineffective in trapping males, while the binary mixture of (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal was considerably more effective than the whole blend of alcohols, aldehydes, and acetates (Table 4, experiment 2). The acetates alone are ineffective trap baits, but their addition to the binary mixture of monounsaturated aldehydes causes a pronounced increase in the

TABLE 4. COMPARISON OF STICKY TRAP CAPTURES OF *H. subflexa* MALES USING DIFFERENT BLENDS OF COMPONENTS[a]

3♀	16:Al	Z9-16:Al	Z11-16:Al	Z7-16:Ac	Z9-16:Ac	Z11-16:Ac	Z9-16:OH	Z11-16:OH	Mean (males/night)[b]
Experiment 1									
+									2.742 a
	+	+	+	+	+	+			1.912 a
	+	+	+				+	+	0.774 b
	+	+	+	+	+	+	+	+	0.706 b
				+	+	+	+	+	0.045 c
Experiment 2									
		+	+	+	+	+			2.899 (a)
	+	+	+	+	+	+			1.000 (b)
		+	+						0.941 (b)
	+	+	+						0.803 (b,c)
	+	+	+	+	+	+	+	+	0.578 (c,d)
				+	+	+			0.148 (c,d)
							+	+	0.148 (c,d)
									0.000 (d)

[a]Raw data transformed to $\log_{10}(X + 1)$ prior to analysis (16 replicates over 8 nights, experiment 1; 10 replicates over 5 nights, experiment 2). Means followed by the same letter are not significantly different in a Duncan's multiple-range test at a $P = 0.05$ level. 16:Al = Hexadecanal; Z9-16:Al = (Z)-9-hexadecenal; Z11-16:Al = (Z)-11-hexadecenal; Z7-16:Ac = (Z)-7-hexadecen-1-ol acetate; Z9-16:Ac = (Z)-9-hexadecen-1-ol acetate; Z11-16:Ac = (Z)-11-hexadecen-1-ol acetate; Z9-16:OH = (Z)-9-hexadecen-1-ol; Z11-16:OH = (Z)-11-hexadecen-1-ol. Means from the two experiments are not compared with one another. The presence of a compound in a test blend is indicated by a +.

number of males captured, indicating their necessity for effective sexual communication. The decrease in captures recorded on addition of hexadecanal to either the monounsaturated aldehyde or monounsaturated aldehyde + acetate blends tends to suggest a slight inhibitory function. However, insufficient behavioral analysis has been conducted to determine the validity of this hypothesis at present.

Although the blend reported here is highly complex and may contain chemicals having no behavioral significance, a definite biochemical theme based on the use of C_{16} compounds is quite obvious. The use of these compounds is common among heliothids throughout the world, with (*Z*)-11-hexadecenal being the major pheromone component in all cases reported (Klun et al., 1980a,b; Nesbitt et al., 1979, 1980). The distinction appears to be the presence of acetates within the *H. subflexa* blend. However, Rothschild (1978) has recently reported that a mixture of (*Z*)-11-hexadecenal, (*Z*)-9-tetradecenal, and (*Z*)-11-hexadecen-1-ol acetate caught considerably more male *H. punctigera* (Wllgn.) than did the aldehyde blend alone. Hence, while the aldehydes are of obvious major importance to sexual signaling, the acetates may indeed form an integral part of the pheromone blend of many *Heliothis* species.

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CHEMICAL COMMUNICATION IN THE PRIMITIVE ANT *Aneuretus simoni*: The Role of the Sternal and Pygidial Glands

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Abstract—Trail and alarm communication in *Aneuretus simoni* are mediated by the secretions of the sternal and pygidial glands, respectively. The sternal gland is composed of a glandular epithelium and an associated reservoir located in the 7th sternum. This gland produces a relatively long-lived mass recruitment pheromone. The pygidial gland opens between the 6th and 7th tergites and produces a secretion that releases aggressive alarm. The evolution of glandular physiology in the Aneuretinae and Dolichoderinae are discussed in view of these findings.

Key Words—*Aneuretus simoni*, Hymenoptera, Formicidae, chemical communication, sternal gland, pygidial gland, alarm pheromone, trail pheromone.

INTRODUCTION

Aneuretus simoni is the sole living representative of the phylogenetically important formicine subfamily Aneuretinae. *Aneuretus* is wholly confined to limited regions of the tropical rain forests of Sri Lanka (Wilson et al., 1956; Jayasuriya, 1980), thus, although the Aneuretinae occupy a pivotal position in formicid evolution between the primitive *Nothomyrmecia* and the advanced Dolichoderinae and Formicinae (Taylor, 1978), they have remained almost entirely unknown because of their rare, cryptic, and inaccessible nature. In particular, no information on the pheromonal basis of social organization in

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A. simoni is available, although this species does possess exocrine glands that are structurally similar to those found in other ant subfamilies. Miradoli Zatti and Pavan (1957) described a saclike organ located between the 6th and 7th abdominal sternites of *A. simoni*, morphologically equivalent to Pavan's gland (the ventral organ) of the advanced dolichoderine *Iridomyrmex humilis* (Pavan and Ronchetti, 1955), recently termed the sternal gland (Hölldobler and Engel, 1978). The latter authors also described a pygidial gland with a definite reservoir with ducts that open at the intersegmental membrane between the 6th and 7th abdominal terga which is homologous to the dolichoderine anal gland (see also Kugler, 1978). The availability of live *Aneuretus*, made possible by extensive searching and collecting has enabled us to analyze the role of these glands in chemical communication in this rare ant species for the first time.

METHODS AND MATERIALS

Seven queenright colonies and 19 groups of workers, brood, and sexuals were collected in the uninhabited rain forests and dense secondary growth of the Gilimalé region, approximately 20 km northwest of Ratnapura, Sri Lanka. Twig and rotting wood nests were transported to our laboratory at Harvard University, Cambridge, Massachusetts. In the laboratory, colonies quickly moved into pieces of glass tubing 10 cm in length with an inner diameter of 5 mm, fitted with a tight moist cotton plug at one end; the other end was open to allow workers to forage. The artificial nests, which closely approximate the natural twig nests, were placed in 12×17-cm plastic containers, the walls of which were coated with Fluon to prevent escape. Workers of *A. simoni* were fed on flightless *Drosophila*, termites (*Reticulitermes*, *Nasutitermes*), and honey water.

Histological sections were prepared by fixing live specimens in Carnoy's solution and embedding them in JB4. Sagittal sections, 5 μm thick, were cut with a glass knife on a Jung rotary microtome and stained with hematoxylin. Microphotographs were taken with a Zeiss photomicroscope.

RESULTS

Sternal Gland and Recruitment Communication. In the field, workers of *A. simoni* were observed to orient along what appeared to be odor trails. Such trails were established to food sources (rotting fruit) and between entrances of polydomous nests. In the laboratory, scout ants explored the area around the nest and, when liquid food was found, fed to repletion and returned to the nest moving slowly, dragging the gaster along the surface (Figure 1). Inside the

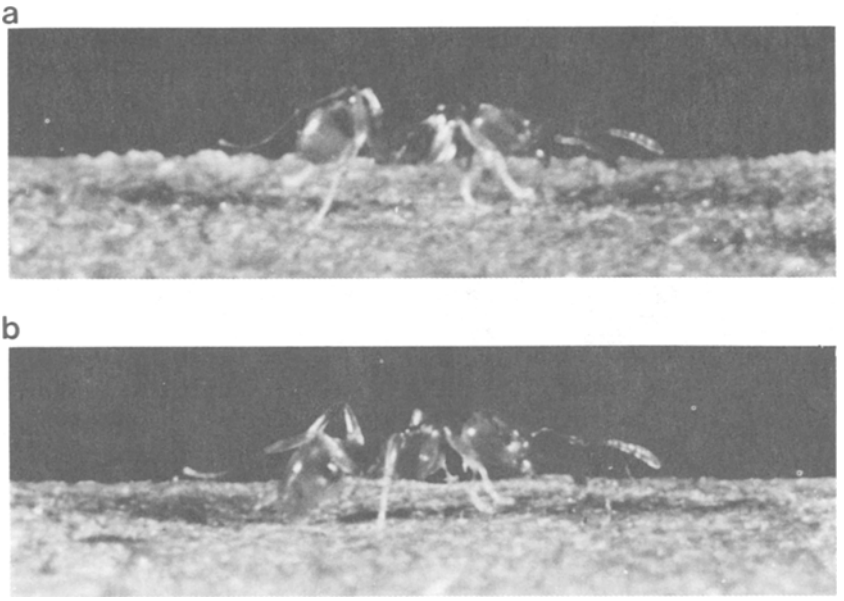

FIG. 1. Minor worker of *Aneuretus simoni* in normal locomotor posture (a) and trail laying (b), dragging the gaster over the substrate.

nest, scouts made vigorous antennal contact with nestmates and performed prominent alerting motor displays. Often trophallaxis ensued or occurred prior to recruitment behaviors. When insect prey too large for workers to retrieve alone were placed in the arena, scouts engaged in similar trail-laying and recruitment behavior, which resulted in the formation of a group of workers that cooperatively retrieved the prey. Trail communication was also employed during nest emigration.

From the description of the ventral organ by Miradoli Zatti and Pavan (1957), the histological investigations of Hölldobler and Engel (1978), and observations of trail-laying behavior, we assumed that the sternal gland played a role in recruitment communication. The sternal gland appears to be composed of groups of cells that make up the glandular epithelium of the 7th abdominal sternite (Figures 2 and 3). Both major and minor worker castes possess this gland. The cells apparently discharge their contents through ducts into the reservoir in which the secretion is stored. Also, through dissection, the glandular anatomy of *A. simoni* was examined, revealing a well-developed Dufour's gland and poison gland associated with the sting. When the contents of the hindgut, Dufour's gland, poison gland, pygidial gland, and sternal gland were extracted in ethanol (1 gland/10 μl solvent) and artificial trails 8

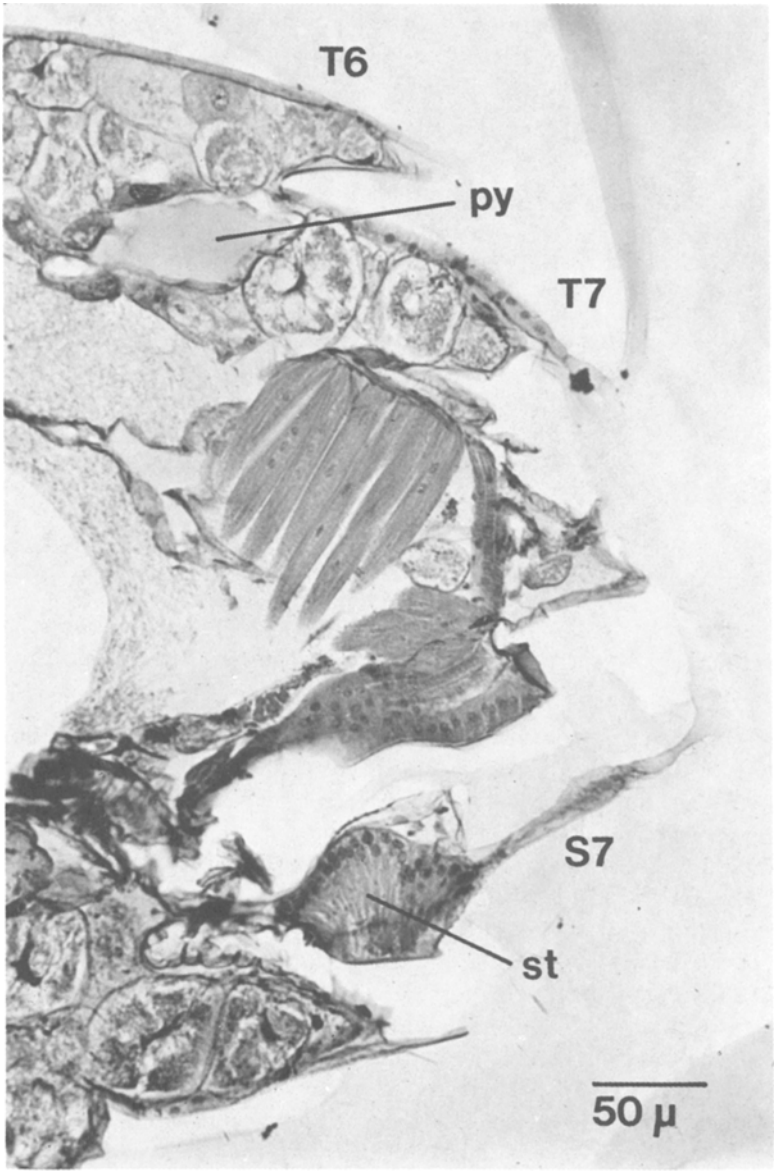

FIG. 2. Overview of a sagittal section through the gaster of *A. simoni* showing the location of the pygidial gland (py) between the 6th and 7th tergites (T6, T7), and the sternal gland (st) on the 7th sternite (S7).

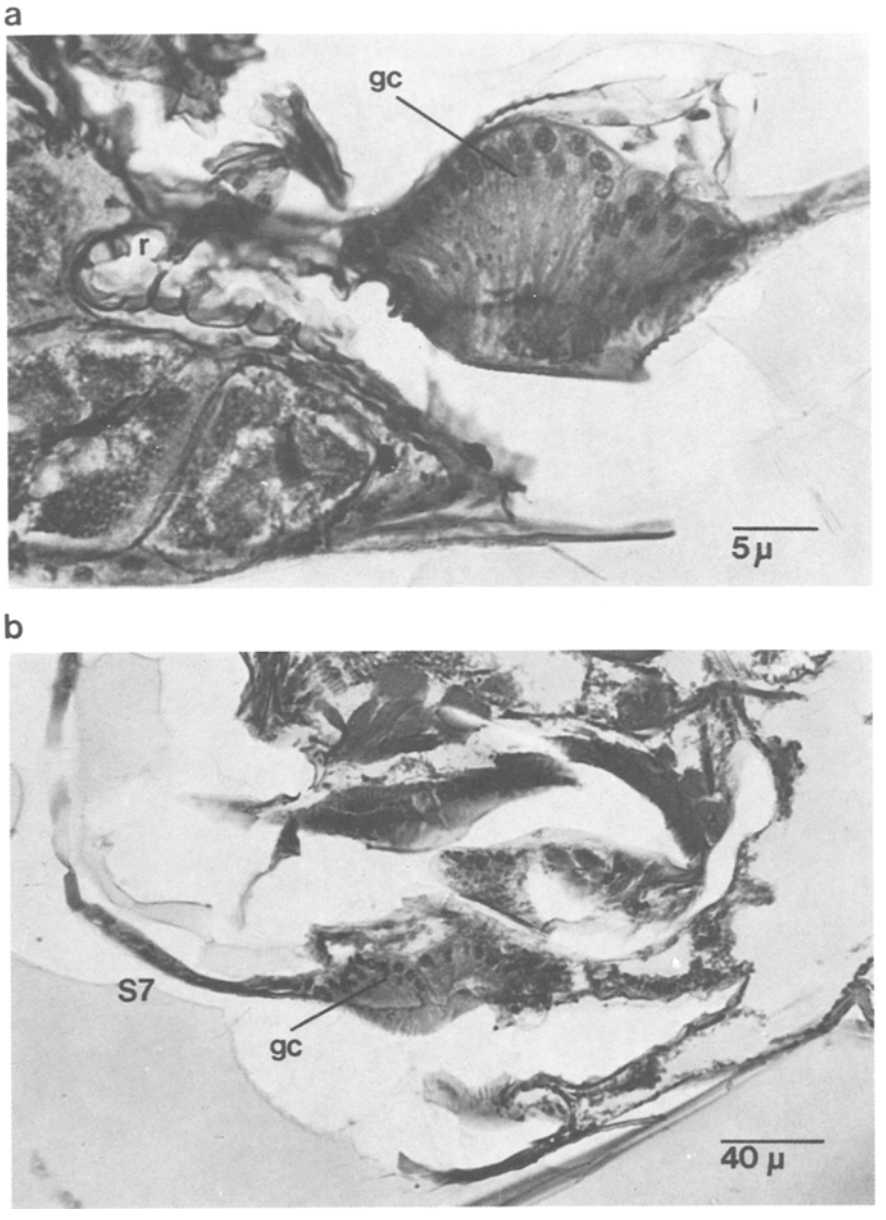

FIG. 3.(a) Sternal gland of a major worker, showing a cluster of glandular cells (GC) and reservoir (R). (b) Sternal gland of a minor worker.

cm in length were drawn out from groups of workers, only sternal gland trails were effective in orienting individuals. In six replicates, a mean of 43.5 ± 5.6 workers followed trails composed of sternal gland extract. Artificial trails composed of Dufour's and poison gland secretion elicited no trail following response, and the response to hindgut material and pygidial gland secretion was insignificant (hindgut: 1.2 ± 1.2 ♀♀ ; pygidial gland: 0.3 ± 0.5 ♀♀). Because field observations indicated that workers oriented along persistent odor trails when traveling between nest entrances or from the nest to food, we next attempted to characterize the nature of the sternal gland trail pheromone by examining the dependence of the recruitment response on the concentration of trail substance and the duration of its activity.

Sternal gland trails alone were capable of inducing recruitment, although motor displays were a prominent feature of recruitment behavior. This suggests that mass recruitment occurs in *A. simoni*. In species of ants exhibiting mass recruitment, colony response is modulated by the concentration of pheromone on the trail (Wilson, 1971). However, when the concentration of sternal gland pheromone was increased by factors of two to three, in three replicates the recruitment response remained the same. The

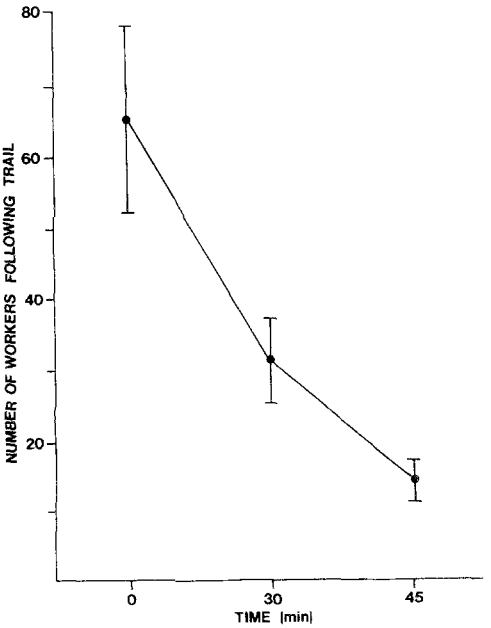

FIG. 4. Duration of the recruitment effect of the sternal gland pheromone. Six replicates were carried out for each series. The mean and standard deviation are given. Additional details in text.

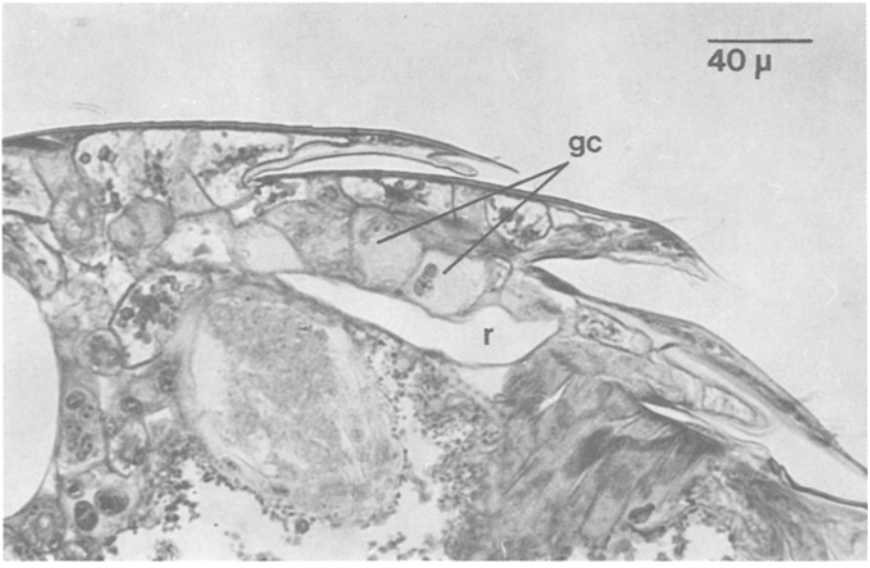

FIG. 5. Pygidial gland of a minor worker, showing gland cells and reservoir. Lettering as in Figure 2.

recruitment effect of the pheromone, although independent of concentration, is quite persistent. Artificial trails composed of one sternal gland in 10 μl ethanol were drawn out on paper and were tested for their recruitment effect when fresh and when aged for 30 and 45 min. The recruitment effect decayed exponentially (Figure 4). In a second series of experiments, trails were tested at 20-min intervals for a period of 4 hr. Even 3-hr-old trails induced a low level of recruitment. It appears as though the sternal gland trails of *A. simoni* retain their orienting effects over long periods of time and serve both as durable odor guides and as alerting signals to recruit workers to new food sources or nest sites.

Pygidial Gland and Alarm Communication. Ant species in the formicoid complex that possess a pygidial gland include *Nothomyrmecia macrops* and the dolichoderines *Iridomyrmex*, *Conomyrma*, and *Liometopum* (Hölldobler and Engel, 1978). The gland, which exits between the 6th and 7th abdominal terga, is well developed in both minor and major workers of *A. simoni* (Figure 5). The pygidial gland of *Aneuretus* is morphologically similar to that of the Dolichoderinae. It appears that large glandular cells empty their contents into channels which transport the secretion to exit pores on the intersegmental membrane. In dolichoderine ants the pygidial ("anal") gland is known to produce chemical alarm signals (see review by Blum and Hermann, 1978).

TABLE 1. RESULTS OF BIOASSAYS OF EXOCRINE ORGANS FOR EFFECTIVENESS IN RELEASING ALARM[a]

Gland	Strength of response	Number of workers responding
1. Mandibular gland (crush and extract)	+	1–4
2. Dufour's gland (crush and extract)	++	4–8
3. Poison gland (crush and extract)	+	1–4
4. Pygidial gland (crush)	++++	>10
5. Pygidial gland (extract)	+++	8–10

[a]Single whole glands of a kind were either crushed on the tip of an applicator stick or extracted in 5 μl 100% ethanol; either gland crushes or extract solutions were offered on applicator sticks ~2 cm into nests. The stick was fixed in place to prevent movement. Responses were graded according to the level of aggression and excitation elicited. +, antennation; ++, antennation and body axis orientation toward stimulus; +++, antennation and opening of mandibles; ++++, rapid movement toward stimulus with open mandibles, lunging, assuming defensive posture with gaster forward beneath body and sting extruded. Seven replicates for each gland crush, three replicates for each gland extract.

Because of the close phylogenetic relationship of the Aneuretinae and the Dolichoderinae and the structural similarity of the pygidial glands, we hypothesized that they were functionally similar. Workers of *A. simoni* show strong alarm behavior in response to mechanical disturbance, temperature stress, or the introduction of alien ants into the nest. Depending on the intensity of the stimulus, mature minor workers aggregate at the nest entrance and show varying degrees of excitation, ranging from antennation to lunging forward with open mandibles, while callow workers retreat with brood to the far end of the nest. Because excitation was rapidly communicated through the nest, and the attraction/aggressive reaction of workers was graded according to their proximity to the stimulus, we postulated that alarm was pheromonally mediated. When crushes of whole glands or extracts of secretions were offered at the nest entrance, varying degrees of alarm were observed. The descriptions of the bioassays and the results are presented in Table 1. Mandibular and poison gland crushes or extracts failed to elicit any noticeable response, and the reaction to Dufour's gland secretion was weak. However, workers constantly showed aggressive alarm in response to pygidial gland secretion, characteristically gaping the mandibles and lunging forward in attack. On occasion the gaster was oriented forward and the sting extruded.

DISCUSSION

The results of the bioassays of the sternal and pygidial glands described above illustrate the importance of pheromones in the social organization of *A.*

simoni. Although *Aneuretus* is a relatively primitive ant species, the extensive use of chemical signals in trail and alarm communication suggests a level of sophistication comparable to that of advanced ant species. Our studies provide behavioral evidence in support of the hypothesized close phylogenetic relationship between the Aneuretinae and the Dolichoderinae (Brown, 1954; Wilson et al., 1956; Taylor, 1978), and illustrate the functional similarity as well as morphological homology of the sternal and pygidial glands in the two groups. Dolichoderine species such as *Iridomyrmex*, *Tapinoma*, and *Monacis* produce species-specific recruitment pheromones in the sternal gland (Wilson and Pavan, 1959). In our pilot studies of the specificity of the sternal gland secretion of *A. simoni* we were able to demonstrate a similar specificity. Workers of *Iridomyrmex* spp. could not interpret the trail substance of *A. simoni*, nor would workers of *A. simoni* follow artificial trails prepared from the sternal glands of these dolichoderines.

Sternal glands are not unique to the Aneuretinae or the Dolichoderinae; they are also found in some ponerine, doryline, myrmicine, and formicine species (Hölldobler and Engel, 1978). However, in these groups they differ structurally, are frequently found in different anatomical positions, and appear to have evolved convergently. Their function is highly variable. The ponerine *Paltothyreus tarsatus* produces a weak trail pheromone in its sternal gland (Hölldobler, personal communication), whereas the secretion of this gland in the African weaver ant *Oecophylla longinoda* plays a role in short-range recruitment (Hölldobler and Wilson, 1977).

Wilson and Pavan (1959) have suggested that the sternal gland arose de novo in the Dolichoderinae with the specific function of mediating worker communication. Our discovery that it is also the source of the trail pheromone in *A. simoni* indicates that this hypothesis must be revised: the sternal gland probably arose de novo in the Aneuretinae and this structure was present initially in the ancestral stock which gave rise to the dolichoderines. If the ancestral aneuretines were ecologically similar to *A. simoni* and nested in unstable nest sites that were frequently disturbed, then the sternal gland may have evolved to organize nest emigrations, the function of recruitment to food being secondarily derived. Although Hölldobler (1981) has postulated a similar sequence for the evolution of the dolichoderine trail pheromone, we believe that the glandular apparatus arose first in the Aneuretinae. *A. simoni* organizes nest emigrations quickly and efficiently and the behavior bears great resemblance to that of dolichoderine species. It is unlikely that the sternal gland would have arisen in the aneuretines de novo for the primary purpose of food recruitment. If colonies of ancestral aneuretines were small (as are colonies of *A. simoni*) and had similar feeding habits (preying on insect larvae in the adjacent leaf litter), neither group retrieval or distant foraging would have been necessary. There would have been little selection for a pheromone mediating recruitment to food.

The structural similarity and probable homology of the pygidial (anal) glands of the Aneuretinae and Dolichoderinae provide an additional link between the two groups. In both subfamilies they are involved in colony defense, but they serve different purposes. Dolichoderine pygidial glands secrete 2-heptanone, cyclopentyl ketones, and other chemicals that alarm nestmates and repel intruders (Blum and Hermann, 1978). Our experiments with *A. simoni* indicate that the pygidial gland secretion in this species releases aggressive alarm but has no repugnatorial effect. The pygidial gland secretion of *Iridomyrmex* does not induce any level of alarm in *A. simoni*, although it has a repellent effect. Unlike dolichoderines, workers of *A. simoni* have a functional, exsertile sting used in intra- and interspecific aggressive confrontations and also in prey paralysis (Jayasuriya and Traniello, unpublished data). The loss of the sting, a change from predatory to scavenging food habits, and the development of a powerful chemical repellent may have occurred concomitantly in the evolution of the Dolichoderinae. Kugler (1978) notes that these steps also could have led to the convergence of pygidial and anal glands in the Formicidae. Blum (1969) has suggested that the pygidial gland arose de novo in the dolichoderines "as a parsimonious glandular system with both a communicative and defensive function." We now suggest that the pygidial gland was part of the glandular anatomy of the ancestral aneuretines, therefore only the repellent function of the secretion evolved in the dolichoderines.

The pygidial gland, like the sternal gland, is not limited to the Aneuretinae and Dolichoderinae; similar organs exist in a wide variety of species in all subfamilies including the Nothomyrmeciinae (Hölldobler and Engel, 1978; Kugler, 1978), a group which Taylor (1978) considers to be ancestral to the Aneuretinae. Although pygidial glands appear to be homologous in the Formicidae, their functions vary considerably. In ponerine species they play a role in sexual attraction (Hölldobler and Haskins, 1977), tandem running (Hölldobler and Traniello, 1980a), and foraging organization (Hölldobler and Traniello, 1980b; Maschwitz and Schonegge, 1977). In the Myrmicinae, Kugler (1979) and Hölldobler et al. (1976) have shown that they play a role in alarm and defense.

The glandular anatomy of *Aneuretus* and the role of the secretions of the pygidial and sternal glands in its social organization support the current phylogenetic standing of the Aneuretinae (Taylor, 1978), originally suggested by Wilson et al. (1956). Future studies of the natural products chemistry of the exocrine organs of *A. simoni* should have additional phyletic implications.

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ENVIRONMENTAL AND BIOTIC FACTORS AFFECTING THE PHENOLIC CONTENT OF DIFFERENT CULTIVARS OF *Sorghum bicolor*

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Abstract—Levels of phenolic acids in healthy plants of *Sorghum bicolor* differ considerably with cultivar and always decrease as the plant matures. Laboratory- and field-grown plants show significant differences in phenolics. Environmental factors, particularly light intensity, influence the concentration of phenolics in sorghum. Attack by insects and pathogenic fungi also increase the phenolic content of the plants to varying degrees dependent on the cultivar and the stage of growth of the plant.

Key Words—Phenolic acids, *Sorghum bicolor*.

INTRODUCTION

Recent studies have shown that the observed resistance of young sorghum to attack by *Locusta migratoria* and other Acridoidea is partly related to the level of available phenolic acids in the plant (Woodhead and Bernays, 1978). These acids act as feeding deterrents to insects, and their potency is dependent on their concentration. The phenolic content varies with plant age and cultivar. In laboratory and field tests, those cultivars with high phenolics suffer little damage from leaf-chewing insects (Woodhead et al., 1980).

The present work represents an attempt to assess the feasibility of the extrapolation of data on sorghum resistance factors from laboratory-grown plants to practical use in the field. Phenolics in ten sorghum cultivars were monitored throughout the plant growth period over two seasons (1978 and 1979) in south India to estimate the influence of environmental factors on phenolics in healthy plants. Plants in the field are also exposed to attack by a

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variety of insects and pathogens, all of which cause damage to the plant in some way. In south India in the main sorghum growing season the most important pests are shootfly (*Atherigona soccata* Rond.) and stemborer (*Chilo partellus*), along with a variety of leaf-chewing insects (see Woodhead et al., 1979), and planthoppers and aphids. Sorghum downy mildew (*Sclerospora sorghi*) and a rustlike disease (probably *Puccinia purpurea*) are common pathogens. Plant phenolics are often affected by both pathogen and insect pests, and their response can sometimes be related to their defensive role in the plant (Levin, 1971). The effects of the predominant sorghum pests on the phenolic acid levels of healthy plants in the field are reported here.

METHODS AND MATERIALS

Plant Material. All grain of *Sorghum bicolor* was from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. Plants were grown in the laboratory in controlled environment rooms as described elsewhere (Woodhead et al., 1980). Conditions were as similar as possible to average field conditions.

Field tests were done at ICRISAT in the Kharif (monsoon) season (July–October, 1978 and 1979). Seeds were hand-sown in plots of seven rows of 2.5 m length for each cultivar, in deep black cotton soil (vertisols). Plots were arranged in a random manner within the field and each was surrounded by a 4-m buffer strip of CSH 1, a susceptible cultivar, intended to reduce losses due to shootfly (*Atherigona soccata*) in the early stages of growth. Ten cultivars were used (see Table 1), selected from 110 cultivars screened for phenolic content early in 1978 (see Woodhead et al., 1979). No herbicide, insecticide, or fertilizer treatments were carried out and plots were not irrigated. Some eggs of *Atherigona* were removed by hand to ensure that sufficient numbers of undamaged plants survived in all cultivars.

Sampling Techniques. Samples of leaf material of uniform area (0.5 cm^2) were taken using a No. 5 cork borer. In each experiment, samples were taken from the same leaf on each plant, usually the one below the whorl leaf. Each leaf was usually sampled only once. Preliminary work indicated that differences in leaf thickness between varieties were negligible except where young plants were sampled (i.e., up to 28 days old) in 1978, when a standard weight (10 mg) of leaf material was used to take into account differences in leaf thickness which were present when seedlings were compared with older plants.

Samples were immediately placed in glass tubes containing distilled water (3.5 ml) and returned to the laboratory for analysis within 1 hr. The method of sampling did not affect the phenolics in other parts of the plant (S. Woodhead, unpublished data, and see also Results section on the effects on phenolics of mechanical damage to plants).

Phenolic Analysis. On return to the laboratory, the tubes were immediately placed in a water bath at 90° C for 20 min. After extraction of phenolics in this way, the solutions were filtered to remove plant material and cooled. Folin phenol reagent (0.5 ml, diluted 1 : 1 with distilled water) was added; after 3 min 20% aqueous Na_2CO_3 solution (1.0 ml) was added, and the solutions were thoroughly mixed. The resulting blue color was recorded using a Corning 252 colorimeter equipped with a red filter (710 nm). Preliminary work had shown that a range of phenolics, mainly simple acids, present in sorghum could be estimated in this way, including dhurrin in the form of its hydrolysis product, *p*-hydroxybenzaldehyde. The assay method was designed to measure only those phenolics that might be expected to be readily available to an insect (see Woodhead and Bernays, 1978). Thus, although there are phenolics present in large amounts in the lignin polymer in older plants, the results reflect only those phenolic acids which are still present as soluble sugar esters (Woodhead and Cooper-Driver, 1979) and any other readily extractable phenolic compounds present (Stafford, 1969). Contribution to the assay from phenolic amino acids was negligible (S. Woodhead, unpublished data). Results are expressed in absorbance units per milligram dry weight of plant material under the assay conditions.

Natural Factors Affecting Sorghum Chemistry. Healthy, apparently undamaged plants were randomly sampled at intervals from about 8 days after emergence up to and, for cultivars CSH 1, SC 173, and SWARNA, including flag leaf formation in the field, and up to 60–70 cm height in the laboratory. In cultivars where tillers were produced, these were also sampled. A minimum of 20 plants were sampled on any one occasion. Sampling was also done on the same plants at different times of day from 0600 hr (sunrise) at 2-hr intervals until 1800 hr (sunset).

Meteorological data was obtained from ICRISAT for the periods of sampling, and the phenolic levels in plants over growth periods with different rainfall and temperature patterns were compared with each other and with laboratory data.

Natural damage due to leaf-feeders (acridids and caterpillars, see Woodhead et al., 1980), shootfly and stemborer was recorded over the growth period of the plants, and phenolic acids in the affected plants were measured. Phenolics in unaffected plants of the same cultivar and the same age were measured on the same occasion: this was done on all occasions where damaged and diseased plants were used.

Natural infestation by sorghum downy mildew and by rust was recorded over the growth period and phenolics measured concurrently.

Induced Predation, Disease, and Injury. Young plants (10 days after germination) were randomly infested with newly hatched larvae of *Chilo partellus* by the method developed by Dr. K.V.S. Reddy at ICRISAT (personal communication). *Chilo* eggs ready to hatch were mixed with

sterilized finger millet seeds and placed in the whorl of the plant by use of a dispenser which delivered 3–27 larvae/plant with an average of 5 larvae/plant. Phenolics were measured 5 days later. There was no visible damage at this stage, but plants were dissected after sampling to monitor actual establishment of *Chilo* larvae.

Conidial suspensions of sorghum downy mildew were injected using a syringe into the base of 14-day-old plants (cultivars CSH 1, IS 1082, TAM 2566, IS 2312), close to the soil, by a method currently in use at ICRISAT (R.M. Williams, personal communication). Phenolic content was measured after 4 days when no symptoms were apparent, and after 20 days when typical chlorosis was observed.

Mechanical damage was inflicted on some plants with a hypodermic needle. A series of pricks (approximately 20) were made along the main veins of the leaf next to the whorl leaf; extensive damage was caused to the whorl leaf by pushing the needle through it, and cork borer holes were made in the leaves. Plants aged 23 or 45 days were used, with 20 plants per treatment. Samples for phenolic analysis were taken adjacent to the site of injury after 0.5 hr and at 2-hr intervals for 6 hr, then after 24 hr, and compared with samples from uninjured, healthy plants.

RESULTS

Age, Cultivar, and Environmental Effects. In previous work (Woodhead and Bernays, 1978), phenolic acid content was inversely related to plant height, which in turn was related to age (days after emergence) in a constant manner, and plants were only used up to 28 days (20–30 cm) since by then they were palatable to the insects under test. In all cultivars, the measured phenolic level decreased in healthy plants with increase in age in field-grown plants. Field results also confirmed that height is inversely related to phenolic content in plants up to about 20–30 cm (Figure 1a). In the field, however, the height of older plants is not always an adequate description of physiological age. On any one sampling occasion, it was possible to have plants of the same emergence date that varied in height from, for example, 25 to 110 cm for cultivar CSH 1. Phenolics are generally much lower in plants over 28 days than in younger, small plants, but a wide range of values can be obtained on any one sampling from plants of the same age, and these are apparently unrelated to plant height (Figure 1b). The same pattern was observed in tillers. Consequently, when the effects of insect predation and disease on plant phenolics were considered, plants over 30 cm were not grouped into height ranges, but all plants sampled at any one time were considered together to give mean values for undamaged plants and for damaged ones.

Comparisons of phenolic potential between cultivars were made on

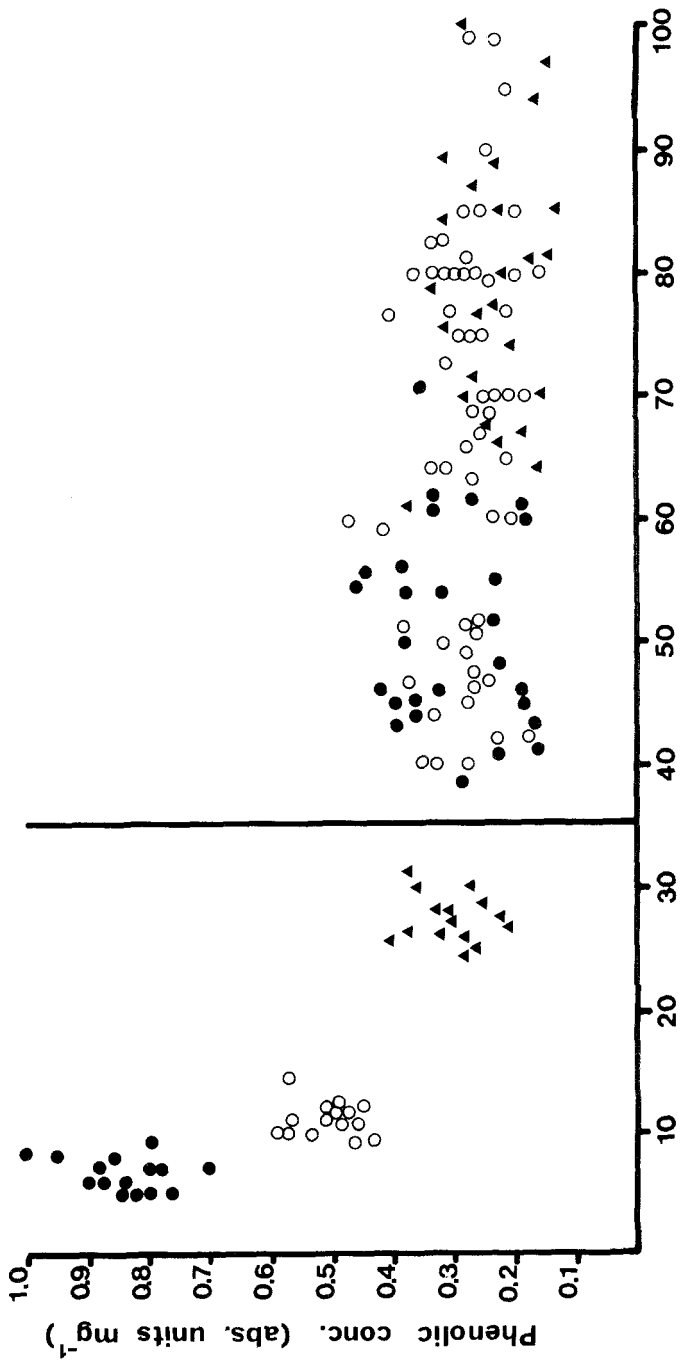

FIG. 1. Changes in phenolic content of sorghum with age and height (cultivar CSH 1, 1979 field season). Each point represents data from one plant. Phenolic concentration is expressed in absorbance units/mg plant dry wt. under the assay conditions (see Methods). (a) Plants aged 8 days (■); 12 days (□); 27 days (▲). (b) Plants aged 32 days (■); 44 days (□); 52 days (▲).

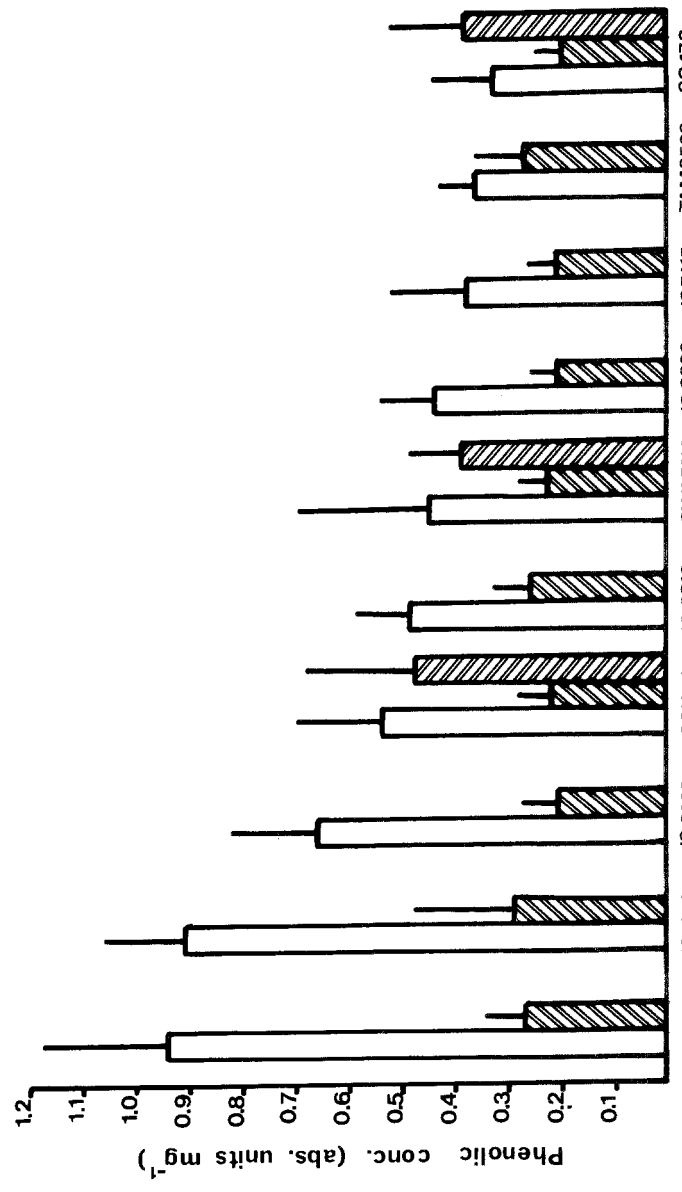

FIG. 2. Differences in phenolic content between sorghum cultivars (1979 field season). Phenolic concentration is expressed as in Figure 1: 23-day-old plants (□); 52-day-old plants (▨); heading plants (▩), mean values ± SD. IS 5642 and IS 1082 at 23 days are significantly different from all other cultivars at this age ($P < 0.005$, t test) but not from each other. At 52 days, cultivars do not differ significantly in phenolic content. Within cultivars, all age differences in phenolics are significant ($P < 0.001$).

young plants up to 28 days. This is the time at which all varieties exhibit the highest phenolic levels and where varietal differences were observed, IS 5642 and IS 1082 having significantly higher levels than the others (Figure 2). Plants were also sampled when they were approaching flag leaf stage: all cultivars had approximately the same low level of phenolics. In cultivars that were sampled when the plants were heading, phenolic content had again increased to about the level found in young plants (Figure 2).

In young plants up to 28 days, heights and leaf number correlated reasonably well between field- and laboratory-grown plants, although field-grown plants were always of a more sturdy appearance (S. Woodhead, unpublished data), e.g., CSH 1: 8-, 15-, and 30-day-old plants were of mean heights 8.0, 16.5, and 30.5 cm, respectively, for laboratory-grown plants and 7.0, 15.0, and 33.0 cm, respectively, for field-grown plants in 1978). However, when the phenolic acid assay, developed on laboratory-grown plants, was applied to field plants of the same cultivars, the values were much higher than for laboratory plants of the same age (Figure 3). This trend was common to all cultivars and was maintained in the older plants also, although the differences were less marked (Figure 3). Young plants of all cultivars also showed statistically significant differences ($P < 0.001$, t test) in phenolic content

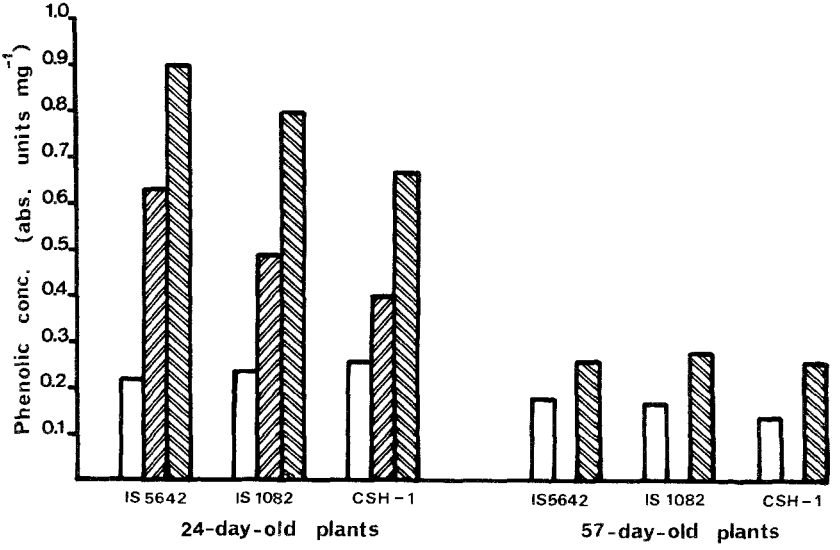

FIG. 3. Phenolic content of cultivars grown under different conditions. Plants were 24 and 57 days old, grown in the laboratory (□), in the field in 1978 (▨) and in the field in 1979 (▧). Phenolic content is expressed as in other figures. Differences in phenolics between two field seasons and between all field plants and laboratory plants are significant at $P < 0.001$ for 24-day-old plants and at $P < 0.005$ for 57-day-old plants.

between plants grown in the field in 1978 and in 1979 (Figure 3), when environmental conditions had differed in light intensity and, to a lesser degree, in rainfall, the 1978 season being much wetter and more cloudy than the 1979 season.

Daily temperature variation at ICRISAT was sometimes as large as 15° C between 0600 hr (sunrise) and midday. The effect of such temperature variation was assessed by sampling the same plants of several cultivars at 2-hr intervals from sunrise to sunset (1800 hr) on days of high radiation. All plants showed significantly higher levels of phenolics between 1000 hr and 1600 hr than at other times. For example, for IS 2312 at 1200 hr, values were increased by 23% ($P < 0.01$, t test) compared with earlier and later readings. In order to avoid this diurnal variation, subsequent sampling was always done before 1000 hr.

Biotic Effects. Plants in the field were monitored for damage from shootfly, stemborer, leaf-chewing insects, mildew, and rust, and the effect of damage on their phenolic content was assessed. Samples were always taken from a visibly undamaged leaf on an affected plant, and phenolics were also measured in healthy plants, i.e., ones with no overt signs of damage or disease, at the same time. Table 1 shows the results for ten cultivars, all sampled on three separate occasions in 1979. Damage by leaf-chewing insects had no effect on the phenolic content of plants of any cultivar. Values measured on plants with obvious signs of damage were not different from those measured on healthy, undamaged plants (Table 1, early September). Plants which were hosts to shootfly or stemborer or were infected with downy mildew or rust all showed higher than normal phenolic content, and in many cases the increase was highly significant (see Table 1). All cultivars reacted strongly to shootfly and stemborer, and most reacted similarly to mildew. Large increases in phenolic synthesis occur, the extent varying with cultivar. CSH 1 and SWARNA, two of the cultivars most susceptible to shootfly attack, showed the largest increases in phenolics in affected plants. IS 1082 and IS 5642, two of the most resistant and also the two cultivars with the highest normal phenolic levels, showed the smallest increases in phenolics in affected plants. Plants with rust gave a variable response: where a cultivar appeared to be resistant to the disease and few plants were affected (e.g., CSH 1, SWARNA, IS 1082, IS 2663, and IS 5642), there was no significant change in phenolics in the plants that were affected, whereas in susceptible cultivars where damage was greater, the response was significant (see Table 1).

Since the original objective was to extrapolate data on sorghum resistance factors to practical use in the field, the obvious differences in phenolics between undamaged laboratory-grown plants and field plants, together with the above observed effects of insect damage and pathogen attack (Table 1) and the well-known hypothesis of increased phenolic synthesis in response to mechanical injury, led to careful examination of field

TABLE 1. EFFECTS OF INSECT PREDATION AND DISEASE ON PHENOLICS IN TEN SORGHUM CULTIVARS ON THREE SAMPLING OCCASIONS IN 1979[a]

Cultivar	IS 5642	IS 1082	IS 2312	IS 2663	TAM 2566	SC 173	IS 7415	IS 8898	CSH 1	SWARNA
August										
Healthy plants	89 ± 23 (48)	86 ± 26 (46)	45 ± 12 (40)	69 ± 16 (33)	32 ± 8 (34)	27 ± 10 (45)	41 ± 14 (31)	42 ± 10 (41)	34 ± 15 (48)	36 ± 24 (44)
Shootfly	125 ± 47*** (17)	100 ± 20*** (9)	62 ± 20*** (27)	94 ± 27*** (41)	59 ± 20*** (44)	54 ± 18*** (33)	76 ± 17*** (34)	75 ± 17*** (35)	81 ± 25*** (40)	85 ± 18*** (45)
Mildew		125 ± 45*** (5)	73 ± 14*** (6)				67 ± 37*** (11)		105 ± 32*** (35)	
Rust					53 ± 18*** (8)			64 ± 9*** (61)		
Early September										
Healthy plants	44 ± 9 (56)	41 ± 19 (85)	41 ± 8 (64)	30 ± 6 (70)	42 ± 20 (78)	28 ± 7 (65)	34 ± 7 (44)	26 ± 7 (54)	29 ± 5 (64)	23 ± 8 (70)
Shootfly	63 ± 8*** (5)	86 ± 25*** (15)	71 ± 23*** (5)	62 ± 19*** (14)	72 ± 22*** (7)					
Stemborer	63 ± 8*** (8)		69 ± 18*** (8)		65 ± 22*** (27)	62 ± 20*** (20)	71 ± 23*** (22)	56 ± 13*** (16)	46 ± 17*** (14)	55 ± 15*** (9)
Leaf feeding	44 ± 7 (14)	39 ± 5 (9)	43 ± 6 (10)	26 ± 10 (12)	42 ± 11 (8)	32 ± 8 (19)	36 ± 5 (15)	30 ± 8 (10)	27 ± 6 (14)	25 ± 6 (16)
Mildew	66 ± 6*** (7)	71 ± 30** (5)	66 ± 13*** (14)		69 ± 30** (7)	48 ± 11*** (10)	60 ± 13*** (11)		53 ± 27*** (5)	62 ± 42* (7)
Rust	52 ± 9* (14)		62 ± 15*** (11)			51 ± 20*** (9)	52 ± 14*** (13)	37 ± 13*** (26)	33 ± 8 (5)	36 ± 12 (8)
Late September										
Healthy plants	27 ± 6 (82)	29 ± 20 (86)	26 ± 7 (42)	21 ± 6 (43)	27 ± 9 (30)	20 ± 5 (32)	21 ± 5 (27)	21 ± 5 (41)	22 ± 5 (45)	23 ± 5 (52)
Stemborer				31 ± 6*** (8)	40 ± 17* (7)	33 ± 12*** (8)	34 ± 14*** (12)	39 ± 2*** (8)	26 ± 4 (5)	
Rust	34 ± 17 (5)	29 ± 7 (7)	29 ± 5 (18)					38 ± 12*** (6)		

[a] Phenolic content is in absorbance units per 100 mg dry wt ± SD under the assay conditions (see Methods). Number of plants sampled is given in parenthesis. Statistical significance between control (healthy) and treatment plants is indicated by ***, $P < 0.001$; **, $P < 0.005$; *, $P < 0.01$ (t test).

sampling techniques. Samples were taken using a cork borer. The type of damage resulting is similar to that of leaf-chewing insects (see Table 1). Browning due to oxidation of phenolics was observed around the site of injury, but the phenolic content of the adjacent area of leaf and of the other leaves of the plant was unaffected.

In addition to the effect on phenolics of the major pests of sorghum, most plants in the field were also infested to some extent by aphids (mainly *Rhopalosiphum maidis*) and planthoppers (mainly *Peregrinus maidis*). Leaf phenolics in laboratory-grown plants were not affected by feeding of aphids and planthoppers (S. Woodhead, unpublished data). In the field, some plants of all cultivars had large numbers of planthopper eggs laid along the main veins, distinguishable by red marks that are characteristic of oxidized phenolics, at the site of oviposition. When leaves of healthy plants were pricked along the veins with a needle to simulate the mechanical damage caused by egg-laying, slightly increased phenolics were detected after 1 hr ($P < 0.025$, t test) but, after 24 hr, phenolics near the damaged area resembled those in untouched leaves. It therefore seems unlikely that the presence of planthoppers or aphids increases the levels of phenolics in sorghum.

Another type of damage not readily visible is that caused by young *Chilo* larvae feeding in the plant whorl. When whorl leaves of plants were damaged, again by use of a needle to simulate injury expected from feeding by young *Chilo*, there was no change in phenolics. However, in experiments where the larvae were introduced into the whorls of plants and left to feed, phenolics increased to different extents in different cultivars: SWARNA, 42% ($P < 0.001$); E303, 25% ($P < 0.01$); CSH 1, 23% ($P < 0.1$); BP53, 13% ($P < 0.4$). Damage caused by the insect is obviously different from purely mechanical damage in this case. Similarly, when plants were injected at the base of the stem with a suspension of downy mildew conidia, there was an initial increase in phenolics (CSH 1, 13%, $P < 0.001$; IS 1082, 25%, $P < 0.01$; IS 2312, 26%, $P < 0.01$; TAM 2566, 11.5%, $P < 0.1$;). When the damage was simulated with an empty syringe, no such increases were seen.

DISCUSSION

Phenolic acid levels in sorghum vary considerably with cultivar (Figure 3) and throughout the growing season (Figures 1 and 2). Seasonal changes in phenolics (tannins in this case) have also been reported in *Quercus robor* (Feeny, 1970) and have been related to resistance in insects. Laboratory- and field-grown sorghum phenolics follow similar patterns, but values for all field-grown plants are much higher than for the corresponding laboratory plants (see Figure 3). Laboratory conditions attempt to simulate the field, but differences in soil moisture and nutrients and in light intensity are likely.

Deficiencies in certain minerals (e.g., N, K, S, B) have been reported to increase phenolics in several species (Newman, 1978), and excesses of N inhibit phenolic accumulation (Margna, 1977). The soil at ICRISAT is low in available N and PO_4^{2-}, and possibly deficiencies may contribute to elevated phenolics as compared with compost-grown plants, where N levels are higher. The difference in phenolics between 1978 and 1979 was as great if not greater than the laboratory–field difference, and since the soil was the same, temperatures were not significantly different, and no correlation could be made with rainfall data, the remaining obvious climatic difference between the two years was light intensity. The monsoon (Kharif) season in 1978 was characterized by typically cloudy skies, but in 1979 there was a much higher sunshine level, and in both years light intensity was probably considerably higher than artificial light. Although no measurements of light intensity could be made at ICRISAT, it seems likely that this factor was largely responsible for the difference in phenolics between the two seasons. Light is known to stimulate the activity of most of the enzymes involved in phenolic biosynthesis in plants (Smith, 1973), and consequently, increased accumulation of phenolics occurs (McClure, 1975). Indeed, phenolics, particularly flavonoids, are considered to protect plants from UV radiation (McClure, 1975).

Plants were often heat and water stressed in 1979, but since sorghum is relatively heat resistant, despite differences of as much as 15° C between maximum and minimum temperatures, heat stress itself should not affect metabolism. Water stress affects the carbon metabolism of the plant (Lawlor, 1979), and so changes in phenolics may result from water deficiency, although this in itself has apparently no effect or a negative one on phenolics (Newman, 1978).

Damage by insects and pathogens causes increases in phenolics in sorghum, the extent of the response being dependent on the cultivar (Table 1). Increased phenolic synthesis by plants in defence against pathogen attack has often been reported (Kosuge, 1969). It is usually accompanied by increases in phenoloxidase activity and subsequent oxidation of phenols to products which are more toxic to the pathogen (Friend, 1979). Increases in phenolics as a result of infection are often correlated with resistance to the disease (e.g., Gans, 1977), but there is some doubt as to whether it is a direct relationship (Frič, 1976). Present data with rust (Table 1) indicate that increased phenolics may be a nonspecific result of infection rather than a specific reaction to the pathogen. Oxidation of phenols at the site of injury in sorghum apparently limits the damage caused by insects which bite a leaf and then leave the plant (see Table 1), but when an insect stays on the plant and feeds, as in the case of stemborer and shootfly, or in pathogenic attack, phenolics are produced in response (Table 1). If the reaction is a defence mechanism, it could operate in several ways: phenolics themselves or their oxidation products affect the digestive enzymes of the insect in an analogous manner to the inhibition of

fungal enzymes (Friend, 1979), or the phenolics may be polymerized to form lignin-like polymers as has been shown in pathogen infection of several Gramineae (Friend, 1979). Another possibility for some insects is that if phenolics are generally distasteful, increased synthesis may have the effect of deterring further feeding. Although a plant under attack may be expected to increase its defences, such increases (or decreases) must be considered in terms of the plant's economy. There are several hypotheses on this subject, recently reviewed by Rhoades (1979).

In the case of sorghum, phenolics not only change significantly when the plant is under attack, but there is a great variation between cultivars, over the growth period of the plant, and as a result of differing growing conditions. The use of phenolics in screening for insect resistance in the field is therefore of limited value unless all plants are free from disease and are not acting as hosts to any insects. Comparisons of laboratory and field data are open to misinterpretation unless conditions are exactly reproduced. It is of course virtually impossible to obtain identical balances of nutrients, moisture, and light intensity in the laboratory and in the field, and it is possible that a plant's metabolism, particularly with respect to secondary compounds, is quite different under the different conditions. Thus, certainly in the case of phenolics, if cultivars of a crop are predicted to be resistant on the basis of studies on laboratory-grown plants, they may behave differently in the field and vice versa. Indeed, in some cases there is evidence that stressed plants are more palatable to insects than healthy ones, presumably as a result of increased nutritional quality (White, 1976). This study has shown that applications of laboratory findings in the field must be made with caution, particularly where so-called antifeedant or defence chemicals are concerned. The nature of their function must make them especially susceptible to variation.

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ANALYSIS OF FIELD-WEATHERED LURES CONTAINING (*E*)-10-DODECEN-1-YL ACETATE, A SEX ATTRACTANT FOR THE PEA MOTH, *Cydia nigricana* (F.)

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Abstract—Natural rubber stoppers treated with 1, 3, or 10 mg of (*E*)-10-dodecen-1-yl acetate, an attractant for male pea moth, *Cydia nigricana* (F.), were exposed in the field during the summer of 1978 and samples analyzed at intervals. The results for all three doses fitted well to first-order loss curves with half-lives of 63.5, 64.7, and 67.3 days, respectively. Thus, lures with an initial dose of 3 mg of the attractant retained approximately 1 mg after 3 months of field exposure; they should therefore maintain a constant level of attractiveness throughout this period because previous field studies showed that moths were equally responsive to fresh lures containing between 1 and 10 mg of the attractant. There was no loss of attractant during the preparation of lures or after 4 months at −15° C and only 13% was lost during 3 months of storage at room temperature.

Key Words—Pea moth, *Cydia nigricana* (F.), Lepidoptera, Olethreutidae, sex attractant, (*E*)-10-dodecen-1-yl acetate, lure, formulation, monitoring.

INTRODUCTION

Pheromones are now used to monitor populations of many economically important Lepidoptera as a guide for growers on their pest management measures. The discovery of two sex attractants for the pea moth, *Cydia nigricana* (F.), (Wall et al., 1976) provided the basis for developing such a monitoring system to produce a more effective procedure for estimating the timing of insecticide application than the previously used egg-counting method (Gould and Legowski, 1964).

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Attractant traps used for monitoring pest populations require lures which are easy to handle, stable in storage, and capable of maintaining a constant level of attractiveness for several weeks when exposed in the field. The lure should be effective from the time traps are placed in the field in mid-May until moths emerge in sufficient numbers to require spray application as indicated by the exceeding of a "threshold catch" (Macaulay, 1977) and, preferably, the rest of the flight season also. In some years the initial period up to this threshold catch for pea moth could be as long as six weeks, as in Britain in 1978 when the flight season continued for a further six weeks after the threshold.

Field studies of lures for pea moth consisting of (*E*)-10-dodecen-1-yl acetate (*E* 10–12: Ac) and (*E*,*E*)-8,10-dodecadien-1-yl acetate sorbed in natural rubber and polyethylene have shown that both compounds are initially attractive (Greenway and Wall, 1981). The latter compound [which has been identified in the female sex pheromone (Greenway and Wall, 1980)], while highly attractive for a few days even at low doses, declined in activity, especially in polyethylene (c.f., Maitlen et al., 1976), so that at present it is unsuitable for incorporation into monitoring lures. However, lures containing *E* 10–12: Ac (1 mg) in either formulation were still attractive after 3 months in the field, but the level of attractiveness of the rubber lures gradually declined after one month as the release rate of *E* 10–12: Ac became slower through depletion of the attractant. Fresh rubber lures with 1, 3, or 10 mg of *E* 10–12: Ac were equally attractive, and so it is probable that an intermediate dose would remain constantly attractive throughout the required period provided that about 1 mg was still retained at the end of the moth's flight season.

In this work, rubber lures with different initial doses of *E* 10–12: Ac were analyzed after various periods of exposure in the field during the summer of 1978 to determine the parameters governing the release rate of *E* 10–12: Ac from the substrate and whether or not a lure providing constant attraction could be produced.

METHODS AND MATERIALS

Natural rubber sleeve stoppers (9 × 18 mm) were obtained from Perkin Elmer Ltd., Beaconsfield, Bucks. (serum stopples, 5000/9602); *E* 10–12: Ac (99.6% pure) was obtained from Farchan Chemical Co., Willoughby, Ohio. Hexane and dichloromethane were redistilled before use. Lures were prepared by applying the desired amount of *E* 10–12: Ac in dichloromethane (100 μl) to the cup of the stoppers and leaving them while the solution was absorbed (15 min). Lures were then stored either in screw-cap glass bottles in the dark at −15° C until required or treated as described below. Each treatment was replicated three times except where stated otherwise.

Analytical Procedure. The amounts of *E*10–12:Ac remaining in lures after field weathering and other treatments were determined as follows: each lure was immersed in hexane (10 ml) containing nonadecane (C19) as internal standard (0.1 mg/ml for 1-mg lures, solution C19A; and 0.5 mg/ml for 3- and 10-mg lures, solution C19B), left for at least two weeks to attain equilibrium, and the solution then stirred vigorously before analysis. These solutions were analyzed on a stainless-steel column (2 m × 3.2 mm OD) packed with 2.5% OV-17 on 80–100 mesh Chromosorb G-AW-DMCS at 158° C in a Perkin-Elmer F11 gas–liquid chromatograph fitted with a flame ionization detector. Each solution was analyzed at least three times and, from the mean ratio of the peak heights of *E*10–12:Ac and C19, the amount of *E*10–12:Ac remaining in the lure was calculated from the appropriate calibration equation. These equations had been obtained by dosing stoppers with known amounts of *E*10–12:Ac to give calibration points for the two internal standard solutions, C19A and C19B. The amounts of *E*10–12:Ac ranged from 0.224 to 1 mg for C19A and from 1 to 10 mg for C19B. Linear regressions were fitted to the resultant data (amount of *E*10–12:Ac vs. ratio of peak heights of *E*10–12:Ac/C19) and, because the intercept was very small for both solutions, it was omitted from the formula for calculating the amounts of *E*10–12:Ac remaining in weathered lures.

The reliability of this analytical method was investigated by analyzing solutions containing freshly dosed 1-, 3-, or 10-mg *E*10–12:Ac lures at intervals up to 13 days, and before and after stirring the solution on days 7 and 13. The *E*10–12:Ac/C19 ratios increased rapidly during the first four days and reached a constant value at day 6 or 7; stirring the solutions on days 7 and 13 had no effect. The eventual *E*10–12:Ac/C19 ratio attained by the lures corresponded to C19 solutions dosed directly with 0.94 ± 0.01 mg. 2.76 ± 0.01 mg, and 9.20 ± 0.03 mg of *E*10–12:Ac, respectively, representing extraction efficiencies of 94.0 ± 1.0%, 92.0 ± 0.3%, and 92.0 ± 0.3%, since the rubber retains more *E*10–12:Ac than nonadecane. Further extraction by soaking lures in fresh portions of hexane (10 ml) for 6 days, increased the *E*10–12:Ac/C19 ratios to recoveries of 98.0 ± 1.0%, 99.1 ± 1.8%, and 97.8 ± 0.1%, respectively, but a third extraction with hexane and dichloromethane (1:1, 6 ml) (Maitlen et al., 1976) failed to extract any more *E*10–12:Ac or C19. The 1-, 3-, or 10-mg *E*10–12:Ac lures that had weathered for 64 days were extracted in the same sequential manner, and the second extraction increased the *E*10–12:Ac/C19 ratios by amounts similar to those with fresh lures, viz. 2.9, 5.7, and 3.1%, respectively. Thus, the method was reliable, and the calibration curves derived from freshly prepared lures could also be applied to weathered lures.

Exposure of Lures in the Field. Lures containing 1, 3, or 10 mg of *E*10–12:Ac were exposed outdoors at Rothamsted on June 17, 1978,

suspended within triangular aluminium traps similar to those used to monitor pea moth (Macaulay, 1977), and sampled after various intervals up to 131 days for analysis of the residual *E* 10–12: Ac by gas–liquid chromatography (GLC). Maitlen et al. (1976) and Flint et al. (1978) have found that losses due to evaporation of pheromone chemicals from rubber stoppers could be expressed by exponential equations, and so both single and double exponential curves [i.e., equations of the forms $y = Ae^{-kt}$, and $y = Ae^{-kt} + Be^{-k't}$ (Phillips, 1971)] were fitted to the results obtained up to day 96 to determine which gave the better fit and thereby obtain the parameters for the rate of loss of *E* 10–12: Ac from the rubber substrate. Daily temperatures were measured at the local meteorological site.

Loss of E10–12: Ac during Dosing of Lures. To investigate whether *E* 10–12: Ac might be lost during the evaporation of dichloromethane immediately after dosing the stopper, glass "cups" of similar dimensions to those of the stoppers were dosed with *E* 10–12: Ac (1 mg) in the usual way. The solvent was allowed to evaporate completely at 24° C (45 min), each cup was then placed in solution C19A (10 ml), and the *E* 10–12: Ac/ C19 ratios of the resultant solutions were compared with those of solutions into which *E* 10–12: Ac (1 mg) had been dosed directly.

Loss of E10–12: Ac from Lures during Storage. To determine the loss of attractant while lures are stored in containers at low or ambient temperatures, lures containing 1 mg (2 replicates), 3 mg (4 replicates), or 10 mg *E* 10–12: Ac were kept in the dark at −15° C for four months and 1-mg lures were exposed in clear glass bottles at a south-facing window for three months and then analyzed.

Distribution of E10–12: Ac within Lures. Freshly prepared and 160-day field-weathered lures were sectioned into four parts (Figure 2) for analysis.

RESULTS AND DISCUSSION

Field Weathering of Lures. Analysis of the residual *E* 10–12: Ac in field-weathered lures showed that simple first-order loss curves (Figure 1) fitted the experimental data well for dose levels of 1, 3, or 10 mg, i.e., the rate of loss of *E* 10–12: Ac at any time, presumably mainly by evaporation, was proportional to the amount remaining in the lure. The more complex double exponential equations only achieved a marginally better fit for the 1-mg lures with a small initial value for the second term which decreased rapidly with time. There was no improvement for 3- or 10-mg lures. Therefore, these losses all followed simple exponential equations, as found by Butler and McDonough (1979) for a range of acetate sex pheromones. Daily mean temperatures did not vary much during the course of the experiment (most were between 12 and 17° C),

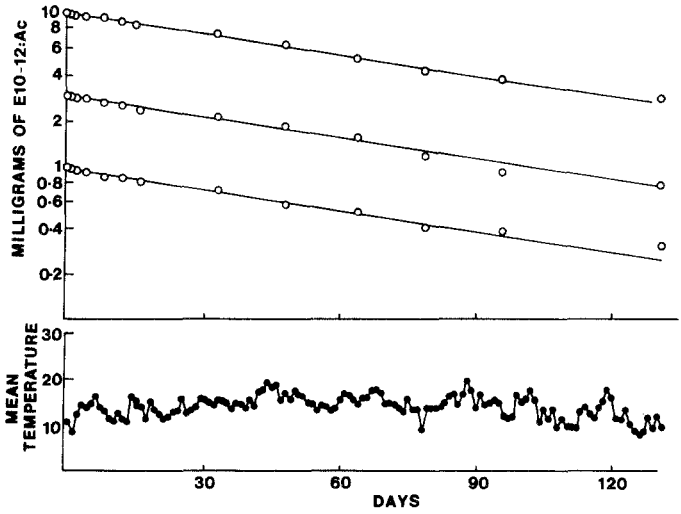

FIG. 1. Loss of (*E*)-10-dodecen-1-yl acetate from rubber stoppers (logarithm of amount remaining vs. time) treated with 1, 3, or 10 mg of the attractant and exposed in the field at Rothamsted. Closed circles denote daily mean temperatures (° C).

and these fluctuations did not markedly affect the overall rate of loss (Figure 1).

There was no pronounced loss of *E*10–12:Ac during the first day of exposure, in contrast to the 58% loss of codling moth pheromone (*E*,*E*)-8,10-dodecadien-1-ol, dosed in hexane onto rubber (Maitlen et al., 1976) and probably due to rapid evaporation from its surface. Presumably, the use of dichloromethane, which reduced the first-day loss of codling pheromone to 8%, and the lower polarity of *E*10–12:Ac aided penetration into the body of the rubber so that rapid initial loss from its surface could not occur.

The first-order curves had rate constants of 0.0109/day, 0.0107/day, and 0.0103/day, respectively, for 1-, 3-, and 10-mg *E*10–12:Ac lures, corresponding to half-lives ($t_{1/2}$) of 63.5, 64.7, and 67.3 days, compared with $t_{1/2}$ of 37.3 and 47.1 days for dodecyl acetate and (*E*,*E*)-8,10-dodecadien-1-yl acetate, respectively, in rubber lures aged in the laboratory at 23° C (Butler and McDonough, 1979). Thus, lures initially dosed with 3 mg of *E*10–12:Ac retained about 1 mg after 3 months in the field and so, from previous field tests (Greenway and Wall, 1981) should maintain a constant level of attractiveness for a further month under British summer conditions. This period should encompass any pea moth flight season in Britain. Even if the summer were hotter than normal so that lures lost attractant more rapidly, they would probably not be depleted below the amount required to maintain constant attractiveness before the end of the flight season which would be correspond-

ingly earlier and shorter. For example, 1976 was an abnormally hot summer in Britain and pea moths emerged in late May (Macaulay, 1977), but few were caught in traps after the first week of July, so that monitoring lures were required for less than two months.

Storage of Lures. In practice, attractant lures are often prepared some time before their use in the field so that storage and transport, probably under less than ideal conditions, are necessary. However, even lures exposed to daylight in clear glass containers for three months retained 87.4 ± 2.6% of the original dose of *E*10–12:Ac, compared with a loss of about 30% after one month in the field (Figure 1), and these lures were as attractive as those stored at −15° C (Greenway and Wall, 1981). Lures kept at −15° C for four months retained 97.2 ± 0.1%, 99.8 ± 0.3%, and 100.0 ± 1.5%, respectively, of their initial doses of 1, 3, and 10 mg. Thus, provided that lures are in closed containers, it appears that they can tolerate a period of poor storage without losing much attractant, and little, if any, loss occurred at deep-freeze temperatures.

Loss of E10–12:Ac during Preparation of Lures. When the dosing procedure was simulated with glass cups, they retained 96.2 ± 1.0% of the original dose of 1 mg of *E*10–12:Ac. This must overestimate any possible loss when preparing the lures because evaporation of solvent from the glass took 45 min compared with 15 min for the evaporation from and absorption into rubber, and glass surfaces do not retain *E*10–12:Ac as does the absorbent rubber. Good recoveries of *E*10–12:Ac from fresh 1-, 3-, or 10-mg lures confirmed the expectation that little if any attractant is lost during the process of preparation.

Distribution of E10–12:Ac within Lures. Figure 2 shows the distribution of attractant within fresh and weathered lures. Initially, most of the *E*10–12:Ac was in the lower wall of the cup of the stopper and none in the tip of the cone. However, during the period of weathering, considerable diffusion within the stopper accompanied evaporative loss. The tip of the cone now contained about one third of the retained *E*10–12:Ac, another third was in the rest of the cone, and the final third was distributed equally between the two sections of the cup wall. Nevertheless, despite the complex shape of the lure and the initial uneven distribution of *E*10–12:Ac within it, the loss curves followed simple exponential functions as discussed above.

CONCLUSIONS

The data now acquired on the rates of evaporation of *E*10–12:Ac from rubber stoppers in the field indicate that an initial dose of 3 mg should provide lures that have a constant level of attractiveness for male pea moths

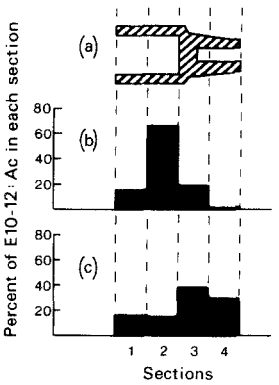

FIG. 2. (a) Cross-section of rubber lure. Distribution of (*E*)-10-dodecen-1-yl acetate within (b) fresh and (c) weathered lures.

throughout a single flight season in Britain as well as allowing for any small losses of attractant which might occur during preparation and storage. Thus, in agricultural practice there should be no need to renew such lures during the flight season and, however early or late the season starts and finishes, growers should not need to adjust the threshold catch, which indicates when treatment with insecticides should be considered (Greenway et al., 1976), in order to compensate for reduced attraction. Subsequent field studies have confirmed that these conclusions are valid in practice (Wall and Greenway, 1981).

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS. XI. Effect of Host Plants on Kairomonal Activity of Frass from *Heliothis zea*[1] Larvae for the Parasitoid *Microplitis croceipes*[2-4]

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Abstract—In laboratory experiments conducted to compare the kairomonal activity for *Microplitis croceipes* (Cresson) of frass from *Heliothis zea* (Boddie) larvae fed on different host plants, *M. croceipes* females responded to extracts of frass from larvae reared on cotton or soybeans but not on corn. The lack of response to frass from larvae reared on corn was shown to be a result of a lack of some appropriate chemical(s) in the corn.

Key Words—*Heliothis zea*, Lepidoptera, Noctuidae, *Microplitis croceipes*, Hymenoptera, Braconidae parasitoids, kairomones.

INTRODUCTION

Lewis and Jones (1971) demonstrated that kairomones found in the frass, salivary secretions, hemolymph, and cuticular washes of *Heliothis zea* (Boddie) larvae elicited a host-seeking response from female *Microplitis croceipes* (Cresson), an important larval parasitoid of *Heliothis* spp. The most active compound was identified as 13-methylhentriacontane, although nu-

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merous other compounds elicited lesser responses from *M. croceipes* females (Jones et al., 1971). Sauls et al. (1979) demonstrated that diet affects the kairomonal activity, for *M. croceipes*, of frass from *H. zea* larvae and that frass from larvae fed on modified pinto bean diet (Burton, 1969) was significantly less active than frass from larvae fed fresh pea cotyledons.

The fact that diet can affect the release or composition of kairomones has been demonstrated for several insect species. For example, Roth et al. (1978) reported that the tachinid parasitoid *Lixophaga diatraeae* (Townsend) was stimulated to larviposit by frass from *Diatraea saccharalis* (F.) larvae that fed on sugarcane but not by frass from larvae fed a soybean–flour–wheat germ diet. Also, Mohyuddin et al. (1981) found that a Pakistan strain of *Apanteles flavipes* (Cameron) responded strongly (with ovipositor probing) to frass from *Chilo partellus* (Swinhoe) larvae fed corn but weakly to frass from larvae fed sugarcane.

We report here results of tests conducted to evaluate differences in the response of *M. croceipes* females to frass from *H. zea* larvae fed different host plants.

METHODS AND MATERIALS

H. zea larvae were reared on cotton squares (Coker 310), soybean pods (Bragg), corn kernels (Silver Queen), or on CSM diet (Burton, 1970). Larvae were reared from fresh eggs obtained from a laboratory culture and held in 1-oz plastic cups at 26° C and 70% relative humidity. The plant material was changed regularly to ensure freshness. Frass was collected daily from 3rd–5th instar larvae.

The *M. croceipes* used in the bioassays were obtained from a laboratory culture and had been reared according to the methods of Lewis and Burton (1970).

Collected frass was lyophilized, weighed, and homogenized with distilled water (1 g/ 100 ml) in a blender. The homogenates were then centrifuged at 750 rpm for 7 min and the supernatant decanted for use in the bioassays. A procedure similar to this was used by Sauls et al. (1979). Although some of the identified kairomones are nonpolar compounds, their concentrations in the supernatant should be relative to their original concentration in the frass.

The bioassay used involved exposing individual 2- to 3-day-old mated *M. croceipes* females to spots treated with 4 μl of an extract on Whatman No. 1 filter paper in the bottom of 150 × 15-mm Petri dishes. Because the parasitoids are negatively geotactic and positively phototactic, we easily guided them to treated sites by holding the dish in a vertical plane with the test side toward the light (Jones et al., 1971). Responses were scored on a three-point scale. When a parasitoid made an intensive examination of a treated

spot with her antennae, exhibited considerable excitement, and occasionally probed with her ovipositor (positive response) on the first pass, a score of 3 was given. If a positive response was elicited on the second pass, a score of 2 was given, etc. When the parasitoid did not respond after three direct passes over a treated spot, a score of 0 was given (Lewis and Jones, 1971). Each replication consisted of the mean score of 10 parasitoids for each of the tested materials.

RESULTS

The initial series of bioassays was conducted to demonstrate the existence of differences in the kairomonal activity of frass from larvae reared on the different host plants. *Microplitis croceipes* females responded significantly ($P > 0.05$) more strongly to extracts of frass from soybean-fed larvae (score: 1.6a)[5] and cotton-fed larvae (1.0b) than to extracts of frass from CSM diet-fed or corn-fed larvae (0.3c and 0.0c, respectively) (data from 10 replications).

It is possible the extract of frass from corn-fed larvae might have contained a chemical(s) that inhibited a response to other chemicals in that extract or contained no chemicals that would elicit a response. Thus we conducted another series of bioassays in which the extracts of frass from larvae reared on soybeans and on corn were compared to spots treated with 4 μl of both extracts. Results (soybean-fed, 0.4a; soybean and corn mixtures, 0.4a; corn-fed 0.0b) (data from 6 replications) showed conclusively that the lack of response to the extract of frass from corn-fed larvae was due to the lack of some appropriate chemical(s) in the frass from corn-fed larvae, not to the presence of any inhibitory chemical(s).

DISCUSSION

The data presented here demonstrate that host plants do influence the kairomonal activity of frass from *H. zea* larvae. It appears that some plants have the necessary chemical constituents while others do not. Whether the chemical(s) is concentrated by the insect as it passes through the digestive system, is chemically altered by the insect, or is mixed with some insect-produced chemical remains to be determined.

Parasitoids are known to exhibit differential responses to various plants (Thorpe and Caudle, 1938; Monteith, 1958, 1967; Read et al., 1970; Taylor and Stern, 1971; Lewis et al., 1972; Mohyuddin et al., 1981); for example, *M. croceipes* females do not generally search in corn or sorghum (Smith et al.

[5] Means followed by different letters are significantly different ($P > 0.05$) according to Duncan's multiple-range test.

1976; Lewis, unpublished data) but do search in cotton and soybeans. Thus, it is not surprising that corn apparently lacks some chemical(s) important to the host-finding behavior of *M. croceipes*. Altieri et al. (1981) have even demonstrated that extracts of certain weeds can be applied to soybeans to increase rates of parasitization by several species of parasitoids.

The data also provide evidence that segments of insect populations escape attack from some entomophages when they are feeding on some particular host plants. This escape is not only due to a breakdown in host-habitat finding, but also in host finding should a parasitoid stray into the habitat. Changes in the semiochemicals released by insects feeding on different host plants certainly complicate the study of those chemicals and may be the reason for some differences in selection pressures within populations.

The importance of considering food plants when studying kairomones or other semiochemicals and when considering a particular entomophagous insect for use in biological control programs becomes obvious in light of these findings. These results also offer some interesting lines of research for plant breeders.

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FIELD AND ELECTROANTENNOGRAM RESPONSES OF THE RED-HEADED PINE SAWFLY, *Neodiprion lecontei* (FITCH), TO OPTICAL ISOMERS OF SAWFLY SEX PHEROMONES

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Abstract—Sawfly sex pheromones, the acetate and propionate esters of 3,7-dimethylpentadecan-2-ol, were field tested for activity toward *Neodiprion lecontei* (Fitch). Only the acetate form of the 2*S*,3*S*,7*S* isomer was active. Field catch decreased with the addition of the 2*S*,3*R*,7(*R*/*S*) acetate isomer sample. Electroantennogram recordings showed a positive correlation between response and degree to which the chirality of each isomer resembled the attractive isomer.

Key Words—Red-headed pine sawfly, *Neodiprion lecontei*, Hymenoptera, Tenthredinidae, electroantennogram, field tests, sex pheromone, 3,7-dimethylpentadecan-2-yl acetate, 3,7-dimethylpentadecan-2-yl propionate, optical isomers, enantiomers, esters.

INTRODUCTION

The red-headed pine sawfly, *Neodiprion lecontei* (Fitch), is one of the most common and destructive of the hard pine feeders in eastern North America. There may be more than one generation per year, especially in Florida where 3–4 generations may occur (Benjamin, 1955; Baker, 1972; Wilkinson, 1980). Economic losses may be considerable unless suppression measures are taken

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(Coppel and Benjamin, 1965). The sex pheromones produced by the adult females are potentially useful for the detection, survey, and suppression of *N. lecontei* and other damaging species of diprionid sawflies.

The sex pheromones of diprionid sawflies appear remarkably similar. Jewett et al. (1976) identified the free alcohol of the pheromone as 3,7-dimethylpentadecan-2-ol (diprionol) in three species from two genera. The acetate of this alcohol is the major component of the sex attractant in *N. lecontei* and *Neodiprion sertifer* (Geoff.), whereas in *Diprion similis* (Hartig) it is the propionate. It was concluded that "while the choice of the acid moiety is the most discriminating factor, a more subtle chemical specificity, based on the difference in alcohol moieties, exists at the level of genus" (Jewett et al., 1976). The presence of three asymmetric centers in the carbon skeleton of the alcohol moeity allows eight ($2n, n = 3$) possible optical isomers. Although a racemic mixture of these isomers was attractive to males in the field, the activity was less than that of the naturally occurring pheromone (Jewett et al., 1978). This was interpreted to mean that the naturally occurring pheromone has a rigid optical requirement and that some of the isomers in the racemic mixture were inhibitory to the attractiveness of the active isomer. It has been established that males of the white pine sawfly, *Neodiprion pinetum* (Norton), respond strongly to only one optical isomer, the acetate form of the 2*S*,3*S*,7*S* isomer (Kraemer et al., 1979). It has also been reported that *N. lecontei* males require an *S* chiral configuration at the 2 and 3 carbons (Matsumura et al., 1978). This paper explores the importance of the seven-carbon optical configuration in *N. lecontei*, the effect of other isomers on the field activity of the active isomer, and the value of electroantennograms (EAG) in predicting field response.

METHODS AND MATERIALS

The optical isomers used in this study are identified by three-letter chiral descriptions which refer to the 2, 3, and 7 carbon positions, respectively. A racemic mixture is indicated by (R/S) at the appropriate carbon position. The ester form is indicated by A for acetate and P for propionate. Thus the acetate of the 2*S*,3*R*,7(R/S) isomer is designated SR(R/S)-A. The isomers were synthesized by two groups of researchers. Code designations are given to the various preparations to allow a comparison with the results of other studies. There are differences in the optical purity of these samples and in some cases contaminants are present. Mori et al. (1978) prepared the four erythro isomers in both acetate and propionate forms: RRR-A (TA-1); RRR-P (TP-1); RRS-A (TA-2); RRS-P (TP-2); SSR-A (TA-3); SSR-P (TP-3); SSS-A (TA-4) and SSS-P (TP-4). These are pure erythro isomers and, except

for TA-4 and TP-4, show only a single peak by GLC analysis (3% SE-30; 1.5 m × 2 mm at 180°, carrier gas N_2, 30 ml/min). The alcohol precursor was present in TA-4 (10%) and TP-4 (5%). These samples were purified by GLC (SE-30 column) and column chromatography (Silica Gel 100; eluted with hexane–EtOAc, 20:1). The alcohol was totally removed by both techniques. The purified samples were designated TA-4 (GLC) and TA-4 (c), respectively.

Tai et al. (1978) prepared all eight isomers in semipure optical forms. Their chiral configurations, purity, code designations, and activity towards *N. lecontei* were reported by Matsumura et al. (1979). Only the RR(R/S)-A isomer (A-1) was used in this study. Tai (unpublished) has since prepared the four threo isomers in an optically more pure form. The isomer samples used here are SR(R/S)-A (CA-1); SR(R/S)-P (CP-1); and RS(R/S)-A (CA-2). These preparations were checked for purity by GLC analysis (SE-30 column), and all showed unknown contaminant peaks (2–5%) just before and after the isomer peak. The CA-1 sample was purified by TLC (Whatman PLK5F, benzene) and column chromatography (Celite-activated charcoal, 4:1; ether). At least 95% of the impurities were removed. This sample is designated CA-1 (TC).

Field bioassays were conducted in two typical slash pine (*Pinus elliotti* Engelm. var. *elliottii*) stands located near Gainesville, Florida. The *N. lecontei* populations were enzootic in this area during the test periods. Isomer samples were prepared in 1.0-ml aliquots of hexane (Skelly B) and poured onto 2.5-cm cotton dental wicks. Each wick was suspended by a hair clip from the roof of a white Pherocon II insect trap (Zoecon Corp., Palo Alto, California). The traps were attached to the trunks of the pine trees at 1.5 m elevation and spaced at least 3 m apart. Each test consisted of two replicates randomly placed in one block distribution. The wicks were not renewed during the test periods, and further randomizations of trap positions were not made. The relative strength of the different treatments can thus be compared over time with minimal positional effect. However, positional effects distort the comparative strength of the different treatments. The test performed in 1980 included a rotation of trap positions after each weekly count.

The EAGs were performed with equipment and techniques described by Jewett et al. (1977). Uniform strips of filter paper impregnated with an isomer sample were placed inside pasteur pipets. A 2-ml pulse of air was passed thru a pipet and into a moisturized air stream (6 liter/min) directed at an antenna. The amount of each isomer impregnated on the filter paper strips was kept constant (10 μg/isomer) and the EAG deflection amplitudes compared. The racemic threo isomer samples SR(R/S) and RS(S/R) were tested on the EAG and in the field at twice the concentration of the optically pure erythro isomers. Thus each isomer in the racemic mixture was tested at the same quantity as the pure erythro isomers.

RESULTS AND DISCUSSION

The first field test (Table 1) established that the chirality of the 7-carbon position is important despite being removed from the functional group, i.e., the acetate ester moiety at the 2 carbon. The 2*S*,3*S*,7*R* isomer (TA-3) failed to catch a single male. It is now clear that both *N. lecontei* and *N. pinetum* respond strongly to the same optical isomer, SSS-A (TA-4). The mechanism of species pheromone specificity of these two sawflies has not been resolved, although in nature they are separated to some degree by host tree preference. The larvae of *N. pinetum* are found almost exclusively on eastern white pine (*Pinus strobus* L.) whereas *N. lecontei* larvae are found on many hard pine species but seldom on eastern white pine unless they are growing close to a preferred species (Baker, 1972). It is also possible that a mixture of isomers is involved in species specificity similar to that found with the geometric isomers of lepidopterous insects (Roelofs and Cardé, 1974). Therefore, TA-4 was bioassayed in combination with the other isomer samples. The large number of isomers, both acetate and propionate forms, precluded the testing of all possible combinations and mixture ratios. Also, the small number of replicates (2) and strong trap positional effect allow only an indication of possible isomer interactions. It was previously found (Matsumura et al., 1979) that the racemic mixture of all eight isomers was less attractive than the semipure SS(R/S)-A isomer. Field tests (Table 2) indicate the threo isomer SR(R/S)-A (CA-1) is probably responsible for this decreased activity. The TA-4/CA-1 mixture (1:2) caught only 10% of the number of males caught by the pure TA-4 trap. The other isomers, RR(R/S)-A (A-I) and RS(R/S)-A (CA-2), did not reduce trap catch. The increased trap catch of TA-4/CA-2 may be due to positional effect as Matsumura et al. (1979) found the threshold dose for SS(R/S)-A was the same as the natural pheromone (1 μg). The second field test (Table 2) again showed that SSS-A (TA-4) was the only active isomer and that the addition of CA-1 reduced trap catch (22% of TA-4). Also tested were the propionate esters of SSS and SR(R/S), TP-4 and CP-1. The propionate of diprionol was reported attractive to several diprionid species (Jewett et al., 1976). *N. lecontei* was, however, neither attracted to these isomers nor inhibited by their combination with TA-4 (Table 2).

The isomer samples used in the above field tests were not checked for purity. After GLC analysis showed several types of contamination, these samples were purified as described earlier and retested (Table 3). The amounts field tested were reduced from previous tests due to the limited supply of these samples. The removal of the alcohol precursor (diprionol) from TA-4 did not appear to affect trap catch. The unpurified TA-4 caught a total of 90 males and the purified TA-4 (GLC) and TA-4 (c) caught 89 and 62, respectively. The variance within treatments was, however, very high in this test. For example,

TABLE 1. NUMBERS OF *N. lecontei* MALES CAUGHT IN PHEROMONE-BAITED STICKY TRAPS (NOON TO NOON) AT AUSTIN CARY FOREST, NEAR GAINESVILLE, FLORIDA; JULY 30–SEPT. 12, 1978

Isomer	Code	Amount (μg)	Trap No.	Date caught							Trap total	Total
				8/4	8/11	8/18	8/25	9/1	9/8	9/12		
SSS-A	TA-4	30	1	4	5	0	1	1	1	3	15	20
			2	1	1	0	1	0	1	1	5	
SSR-A	TA-3	30	1	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	
SSS-A/SR(R/S)-A	TA-4/CA-1	30/60	1	0	0	0	0	0	0	0	0	2
			2	0	2	0	0	0	0	0	2	
SSS-A/RS(R/S)-A	TA-4/CA-2	30/60	1	6	3	1	9	6	6	0	31	48
			2	8	5	1	1	2	0	0	17	
SSS-A/RR(R/S)-A	TA-4/A-I	3/60	1	0	3	0	0	0	0	1	4	17
			2	10	1	1	0	0	1	0	13	
				29	29	3	12	9	9	5	87	87

TABLE 2. NUMBERS OF *N. lecontei* MALES CAUGHT IN PHEROMONE-BAITED STICKY TRAPS (NOON TO NOON) AT UNIV. FLORIDA AGRI. EXP. STN., GAINESVILLE, FLORIDA; AUG. 1–SEPT. 26, 1979

Isomer	Code	Amount (μg)	Trap No.	Date caught 8/8	8/15	8/22	8/29	9/5	9/13	9/19	9/26	Trap total	Total
RRR-A	TA-1	25	1	0	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0	
RRS-A	TA-2	25	1	0	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0	
SSR-A	TA-3	25	1	0	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0	
SSS-A	TA-4	25	1	13	3	4	0	2	5	0	0	27	37
			2	3	3	1	0	1	2	0	0	10	
SR(R/S)-A	CA-1	50	1	0	0	0	0	0	0	0	0	0	1
			2	0	0	1	0	0	0	0	0	1	
RS(R/S)-A	CA-2	50	1	0	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0	
SSS-P	TP-4	25	1	0	0	0	0	0	0	0	0	0	1
			2	1	0	0	0	0	0	0	0	1	
SR(R/S)-P	CP-1	50	1	0	0	0	0	0	0	0	0	0	0
			2	2	0	0	0	1	0	0	0	3	
SSS-A/SR(R/S)-A	TA4/CA-1	25/50	1	3	0	1	1	1	1	0	0	7	8
			1	1	0	0	0	0	0	0	0	1	
SSS-A/SR(R/S)-P	TA-4/CP-1	25/50	2	8	0	2	0	0	1	0	1	12	36
			2	19	1	0	2	0	2	0	0	24	
SSS-A/SSS-P	TA-4/TP-4	25/25	1	7	2	0	0	3	2	0	0	14	36
			2	8	2	1	1	4	4	0	2	22	
Total				65	11	10	4	12	17	0	3	122	122

TABLE 3. NUMBERS OF *N. lecontei* MALES CAUGHT IN PHEROMONE-BAITED STICKY TRAPS (NOON TO NOON) AT UNIV. FLORIDA AGR. EXP. STN., GAINESVILLE, FLORIDA; MAY 8–JUNE 20, 1980

Isomer	Code	Amount (μg)	Trap No.	Date caught 5/18	5/23	5/30	6/6	6/13	6/20	Trap total	Total
SSS-A	TA-4	15	1	27	2	7	6	4	3	49	90
			2	8	6	13	9	3	2	41	
SSS-A	TA-4(GLC)	15	1	9	3	56	5	1	0	74	89
			2	0	0	14	0	0	1	15	
SSS-A	TA-4(c)	15	1	14	1	2	14	9	2	42	62
			2	1	1	9	8	1	0	20	
SSS-A/SR(R/S)-A	TA-4(c)/CA-1(TC)	15/0.75	1	0	0	12	5	0	3	20	35
			2	4	0	2	0	8	1	15	
SSS-A/SR(R/S)-A	TA-4(c)/CA-1(TC)	15/7.5	1	1	0	3	4	1	0	9	21
			2	3	2	6	0	1	0	12	
SSS-A/SR(R/S)-A	TA-4(c)/CA-1(TC)	15/15	1	4	0	2	6	9	0	21	37
			2	10	0	4	1	0	1	16	
Total				81	15	130	58	37	13	334	334

the TA-4 (GLC) treatment caught 15 males in one trap and 74 in the other. It is apparent that an accurate comparison of active treatments requires a larger number of replicates or numerous randomizations of trap positions. Three different mixtures of TA-4 (c) and the purified threo isomer SR(R/S)-A, CA-1(TC), were also tested (Table 3). Although all three treatments caught fewer males than the pure TA-4 (c) [34–60% of TA-4 (c)] there was no strong relationship between trap catch and quantity of CA-1 (c). The traps containing 0.75 μg of CA-1 (c) caught about the same number of males as those containing 15 μg. It is not clear why the previous tests with unpurified CA-1 had relatively lower trap catches than the purified CA-1 (TC). An inhibitory contaminant may have been removed from CA-1. The larger quantities (50 and 60 μg) and higher ratios (1 : 2 vs. 1 : 1) of CA-1 and high trap variance may also be factors. Further field tests would help to resolve the questions of isomer interactions and sample contamination.

These field tests showed that the synthetic isomer SSS-A was attractive over a long period of time. Although the first week generally produced the most catches, the traps remained active for at least 6 weeks. The low catches toward the end of the trapping periods were probably due to the termination of the flight periods. Few adults were found at the end of June and after the middle of September. Although trap catches were generally low, it should be noted that *N. lecontei* was enzootic and low quantities (15–30 μg) of the active isomer were used. Trap catches of *Diprion similis* Hartig in England were relatively lower (Longhurst et al., 1980). The four erythro isomers prepared by Mori (1978, 1979) were field tested at a quantity of 800 μg/isomer and, although both the RRR-P and SSS-P isomer samples caught a significant number of males, the greatest catch in any one trap was 12 males in 11 days. The greatest catch of *N. lecontei* was 56 males in 7 days at 15 μg (Table 3).

The electroantennogram (EAG) was useful in predicting the optical configuration of the active isomer. Here again TA-4 (SSS-4) produced a stronger response than any other isomer, acetate or propionate (Figure 1). Although TP-4 (SSS-P) was about 60% as strong as TA-4 on the EAG it was essentially unattractive in the field. In all cases the acetate isomers were 20–50% more active on the EAG than the corresponding propionate isomers. The amplitude of the EAG response is also related to the degree the optical configuration at the three asymmetric centers corresponds to that of the field active isomer, SSS. Those isomers with an *S* configuration at two of the three positions (TA-3, TP-3) are stronger than those with only one *S* configuration (TA-2, TP-2), and these are in turn stronger than those isomers with no *S* configurations (TA-1, TP-1). Also, an *S* configuration at the functional group, 2-carbon position, was more responsible for EAG amplitude than an *S* configuration at the 3-carbon position. The threo mixtures CA-1 and CP-1, SR(R/S), were twice as strong as the threo mixtures CA-2 and CP-2,

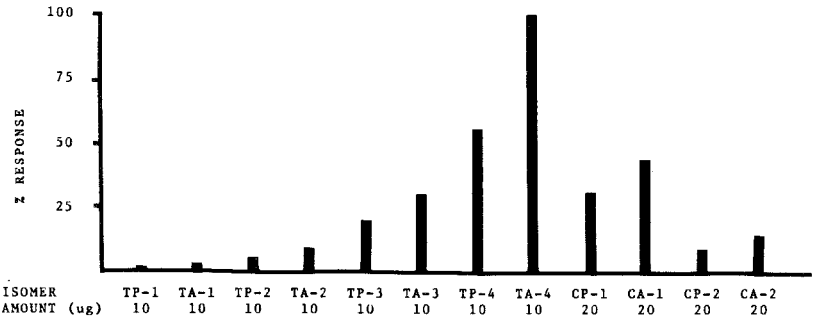

FIG. 1. Relative electroantennogram responses to optical isomers of males of *N. lecontei* where the most active isomer is considered as 100% (2 mV). All responses are the average of 10 males.

RS(R/S), even though they both contained the same number of *S* configurations. The EAG is not a substitute for field assays, but it has been shown to predict the type of acid moiety and optical configuration most attractive in the field.

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SELECTIVE IMPROVEMENT IN RESPONSES TO PREY ODORS BY THE LOBSTER, *Homarus americanus*, FOLLOWING FEEDING EXPERIENCE

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Abstract—Lobsters can detect odors from two natural prey species, the horse mussel *Modiolus modiolus* and the blue mussel *Mytilus edulis*. When lobsters fed exclusively on one of these two prey species for one month, their behavioral response threshold for the prey odor from this species was lowered relative to the threshold for odor from the nonexperienced prey.

Key Words—Lobster, *Homarus americanus*, chemoreception, feeding behavior, behavioral plasticity.

INTRODUCTION

Clawed and spiny lobsters can locate distant prey by means of their sensitive chemoreceptors (McLeese, 1970; Mackie, 1973; Shepheard, 1974; Fuzessery et al., 1978; Reeder and Ache, 1980). Odors from live prey are sufficient to initiate searching behavior by lobsters (Hirtle and Mann, 1978). The American lobster is a predator that can consume a variety of live prey species (Squires, 1970; Weiss, 1970; Miller et al., 1971; Ennis, 1973). Individual lobsters, however, can be selective in their food preferences, as demonstrated by gut analyses (Squires, 1970; Weiss, 1970; Miller et al., 1971; Ennis, 1973; Leavitt et al., 1979) and behavioral observations (Evans and Mann, 1977; personal observations).

For many predators, repeated experience with individuals of a prey species can lead to a preference for that species (Krebs, 1978). Anecdotal information suggests that this may be true for lobsters (Wilson, 1949; Lindberg, 1955). Many features could be learned to form such prey

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preferences (Dawkins, 1971; Krebs, 1973; Curio, 1976). For predators with acute chemical senses, such as lobsters, it may be possible that feeding experience with a single prey species will result in an increased responsiveness to chemicals emanating from that species. Vertebrates capable of such behavioral plasticity of their olfactory responses include fish (yellowfin tuna *Thunnus albacares*) (Atema et al., 1980) and snakes (garter snake *Thamnophis sirtalis*) (Fuchs and Burghardt, 1971; Arnold, 1978). Invertebrates with this capacity include symbionts, herbivores, and predators such as insects (parasitic wasp *Nemeritis canescens*) (Thorpe and Jones, 1937), terrestrial snails (*Achatina fulica*) (Croll and Chase, 1977, 1980), and marine species [oyster drill *Urosalpinx cinerea* (Wood, 1968), polynoid polychaete *Arctonoe pulchra* (Dimock and Davenport, 1971), starfish *Asterias rubens* (Castilla, 1972), and pea crab *Pinnotheres maculatus* (Derby and Atema, 1980)].

We present here data demonstrating that after feeding on a species of prey, lobsters show a selective increase in their responsiveness to odors from this prey species.

METHODS AND MATERIALS

Ten American lobsters of 6–7 cm carapace length were used in this study. Their behavior was observed in two troughs, each 2.50 m long × 0.30 m wide × 0.30 m high. All observations were recorded under red illumination. Sea water from a head tank entered the head of each trough at a rate of 1 liter/min. A 20-ml stimulus was introduced through a funnel that led directly into the sea water inflow and was washed through with 20 ml of filtered sea water in order to remove any residual stimulus from the funnel. A clear Plexiglas shelter was located in the trough 1 m from the funnel tip. Lobsters normally rested in this shelter when not active. A spectrophotometric dye study revealed that an introduced stimulus was diluted 1000-fold when it reached the shelter, 2.0–2.5 min after introduction.

Prey odor was prepared by placing five live undamaged mussels, each of approximately 10 g soft tissue wet weight, in 1 liter of filtered sea water for 10 hr. This water was filtered, diluted in logarithmic steps, and stored in 20-ml aliquots. Prey odors were always used within 36 hr of preparation and were refrigerated when not being used. Stimuli tested included odors of the horse mussel *Modiolus modiolus* and the blue mussel *Mytilus edulis* (two natural prey species for lobsters), as well as a filtered sea water control stimulus.

The effect of feeding experience on the responses of lobsters to prey odors was examined with ten lobsters which for several weeks before the start of the experiment had been maintained on a diet of sea star (*Asterias forbesi*) and cod (*Gadus callarias*). Response thresholds of each lobster to the two mussel prey odors were determined according to the following method. Each lobster

was allowed to acclimate in the trough for 1–2 days before beginning a series of stimulus introductions. At the time of each stimulus introduction, a lobster was observed for 20 min, 10 min before and 10 min after introduction. Responses were analyzed by observing specific movements of the sensory and feeding appendages (chelae, walking legs, maxillipeds, antennules, and antennae), as well as such general movements as walking and shifting. Table I lists these units. The number of occurrences of each behavioral unit for the pre- and postintroduction periods was noted as was the total number of occurrences of all behavioral units for the pre- and postintroduction periods. A response was considered significant when a chi-square test demonstrated a significant ($P < 0.05$) increase in the total number of occurrences of all behavioral units following stimulus introduction. The introductions of filtered sea water and dilutions of each of the two types of prey odors were interspersed, with at least 30 min separating each trial. The response threshold, defined as the lowest dilution to which a lobster responded, was determined for each type of odor. If the threshold of any individual was uncertain upon completion of the first dilution series, dilutions near the threshold were again tested. Upon completion of this series of tests for all ten lobsters, five lobsters were put in each of two 675-liter aquaria (1.25 m long × 0.9 m wide × 0.6 m high). Each aquarium, provided with a surplus of shelters, contained only one species of mussel, either *Mytilus edulis* or *Modiolus modiolus*, at a density of ten mussels per lobster which was maintained throughout the experiment. These two natural prey species of

TABLE I. DESCRIPTION OF LOBSTER BEHAVIORAL UNITS

Behavioral unit	Description
Chela raise	Lift claws high
Dactyl wave	Move legs through water without touching substrate
Dactyl rake	Move legs with tips digging into substrate
Antennule burst	Sudden dramatic increase in flicking rate
Antennule point	Point antennules, often in direction of stimulus
Antennule wipe	Groom antennules, usually with 3rd maxillipeds
Antenna wave	Sweep antenna through water
Antenna point	Place antenna directly in front of body
Antenna wipe	Groom antenna, usually with 3rd maxillipeds
Maxilliped wave	Slow back-and-forth movement of 3rd maxillipeds without touching one another
Maxilliped wipe	Rub 3rd maxillipeds against each other
Maxilliped rake	Touch substrate with 3rd maxillipeds
Walk toward funnel	Move toward site of stimulus introduction
Walk away from funnel	Move away from introduction site
Shift	Change body position without walking

lobsters were chosen since they are phylogenetically related and are similar in terms of size, shape, ease of capture by lobsters, and probably also food value. After 28–30 days in these aquaria, thresholds for the two prey odors were again determined as previously described. Any changes in thresholds for the two prey odors following feeding experience were analyzed with a Wilcoxon matched-pairs signed-ranks test; since other species that had been tested for similar effects are known to show an increased responsiveness to experienced odors (see Introduction), the expectation of such an effect in this experiment permitted usage of a one-tailed test.

RESULTS

Four weeks of feeding experience with a single prey species resulted in both increased sensitivity to odors of that species (mean absolute threshold change = 0.5 log units) and decreased sensitivity to odors from nonexperienced prey (mean absolute threshold change = −1.4 log units). The effect is more readily demonstrable by comparing the relative change in response to experienced odors versus that to nonexperienced odors for each individual (Table 2). This analysis shows that eight lobsters became more sensitive to the experienced odor, one showed no change, and one became less sensitive.

TABLE 2. CHANGES IN THRESHOLD RESPONSES TO PREY ODORS FOLLOWING FEEDING EXPERIENCE[a]

Lobster	Prey species	Change in threshold (log units)[b]		Relative change in threshold (log units)[c]
		Modiolus odor	*Mytilus* odor	
1	*Modiolus*	2.0	−5.0	7.0
2	*Modiolus*	6.2	1.2	5.0
3	*Modiolus*	−1.8	−3.8	2.0
4	*Modiolus*	1.2	−0.8	2.0
5	*Modiolus*	0.0	−1.0	1.0
6	*Mytilus*	−1.9	1.1	3.0
7	*Mytilus*	−6.0	−4.0	2.0
8	*Mytilus*	−0.9	−0.6	0.3
9	*Mytilus*	−0.9	−0.9	0.0
10	*Mytilus*	5.2	2.2	−3.0

[a] Wilcoxon matched-pairs signed-ranks test (one-tailed) demonstrates an increase ($P < 0.05$) in responsiveness to the experienced odor relative to the nonexperienced odor.

[b] Represents the change of response threshold to each prey odor after 30 days of feeding on only the prey species listed; a positive value signifies an increase in responsiveness.

[c] Represents the difference between "change in threshold" for the experienced prey odor and the nonexperienced prey odor; a positive value signifies that the behavioral response to the experienced prey odor became more sensitive relative to the response to the nonexperienced odor.

DISCUSSION

These results demonstrate that feeding experience results in a significant improvement in the response of lobsters to odors from experienced prey, i.e., exposure of lobsters to a single species of mussel improves the responsiveness of the lobsters to odors of that species relative to the responsiveness of the lobsters to odors from another mussel species. For some individuals, these changes are very large, e.g., after lobster 1 (Table 2) fed on *Modiolus* for one month, its threshold to *Modiolus* odor improved by 100-fold while its threshold to *Mytilus* odor became 100,000-fold less sensitive. In only one animal (lobster 10) was the change in the direction of the nonexperienced prey. These effects are striking in light of the fact that these two complex mixtures are probably quite similar (however, their exact compositions remain unknown). These results contradict the opinion of Mackie and Shelton (1972), who concluded that responsiveness of lobsters (*Homarus gammarus*) to tissue extracts of squid (*Loligo vulgaris*) does not change as a result of exclusive feeding on a squid diet. This discrepancy may be due to the fact that their experiments were not specifically designed to examine this hypothesis or possibly that feeding experience with only certain types of foods may result in chemosensory threshold changes.

Although several symbionts, predators, and herbivores that possess sensitive chemical senses can show improved responsiveness to odors from their hosts, prey, or plant foods following experience with them (see Introduction), the basis for such changes has rarely been examined. Croll and Chase (1977, 1980) showed that such plasticity of olfactory orientation in the land snail *Achatina fulica* occurred following exposure to food odors only after ingestion, demonstrating that this behavioral change was not due to sensitization but rather to an associative learning phenomenon. The neuronal basis of associative aversion learning involving changes in chemoreceptive behavior of a number of invertebrate species is presently being studied (Carew et al., 1980; Chang and Gelperin, 1980; Mpitsos et al., 1980). Future studies such as these may shed light on the basis of chemoreceptive response plasticity.

Animals which have demonstrated an increased responsiveness to experienced prey may have formed search images. However, there are many features of a recently experienced prey species that can be learned by a predator which result in the formation of a species-specific preference. These include learning to search in a particular area or type of habitat, learning to capture and handle prey more efficiently, developing a change in acceptability of prey, or learning to detect the prey. Only the last case, where detection of a prey species improves as a result of encounters with that species, can truly be called search image formation (Tinbergen, 1960; Dawkins, 1971; Krebs, 1973; Curio, 1976; Pietrewicz and Kamil, 1979; Atema and Derby, 1981). Search

images have most frequently been attributed to visual predators, and the best behavioral experimental evidence for the existence of search images involves birds feeding on cryptic prey (Pietrewicz and Kamil, 1979); however, it is possible that search images may operate through sensory modalities other than vision, e.g., the chemical senses (Atema, 1977, 1980; Atema and Derby, 1981). The distinction between changes in prey acceptability and changes in prey detection capabilities of a predator is often difficult to differentiate. This distinction, however, is crucial to the definition of search images (Dawkins, 1971; Krebs, 1973). Behavioral threshold determinations are generally good methods for demonstrating a change in detection ability (Blough, 1961), and procedures similar to those used here have been used by others to determine the ability of lobsters to detect various chemical stimuli (McLeese, 1970; Mackie, 1973; Hirtle and Mann, 1978). If the experiments described here do actually measure detection thresholds, then they provide behavioral support for the existence of chemical search images.

The existence of a relationship between feeding experience and formation of prey preferences by lobsters is suggested by anecdotal information of Wilson (1949) and Lindberg (1955). Wilson described "an apparent example of learning in *Panulirus vulgaris*": several lobsters that had never eaten hermit crabs (*Eupagurus bernhardus*) finally did so when no other food was available; subsequently, hermit crabs became one of the most preferred prey of these lobsters, even when other prey species were provided. Lindberg stated that in trapping *Panulirus interruptus*, "the effectiveness of a bait is sometimes dependent on the locality being fished. Crushed mussels are a good bait in areas where extensive mussel beds are normally present, but not elsewhere." The effect of feeding experience on chemoreceptive behavior of lobsters described by us could be one cause of such selective predation by lobsters, especially in areas where the same prey species is available over an extended period of time. This chemoreceptive plasticity, by enabling lobsters to more readily initiate searching in response to chemical signals released by nearby potential prey, could lead to more efficient location and subsequent ingestion of their prey.

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SEX PHEROMONE OF THE TOMATO LOOPER, *Plusia chalcites* (ESP)[1,2]

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Abstract—*Z*-7-Dodecenyl acetate (A) and *Z*-9-tetradecenyl acetate (B), in a 5:1 ratio, have been identified as sex pheromone components of female *Plusia chalcites*. In addition, small amounts of dodecyl acetate, tetradecyl acetate, hexadecyl acetate, and *Z*-9-dodecenyl acetate have been found in the abdominal tip extracts. This is the first known Plusiinae species which utilizes a two-component primary sex pheromone system. The most effective lure found in field experiments was a mixture of 1 mg A and 0.2 mg B absorbed on rubber septa.

Key Words—*Plusia chalcites*, Lepidoptera, Noctuidae, tomato looper, sex pheromone identification, glass WCOT capillary chromatography, gas chromatography–mass spectrometry, *Z*-7-dodencenyl acetate, *Z*-9-tetradecenyl acetate.

INTRODUCTION

The tomato looper, *Plusia chalcites* (Esp). is a polyphagous pest in tropical and subtropical regions of Asia, Africa, Europe, and Australia (Rivnay, 1962). In Israel and Egypt it is an important pest of solanaceous plants (Yathom and Rivnay, 1968; Rashid et al., 1971; Harakly, 1974). The damage caused by the tomato looper in Israel became significant in recent years with the expansion of the areas for growing tomatoes for canning and processing; hence, the need for a monitoring system to aid in population estimates and timing of control. Such a monitoring system is particularly important for this pest, the population of which may suddenly reach high levels as a result of adult migration (Yathom and Rivnay, 1968). Because of the lack of electric

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power in the fields and problems of vandalism, it is impractical to use light traps for monitoring. An alternative method is to use traps baited with chemical attractant, such as sex pheromone.

The composition of the sex pheromone of a number of Plusiinae species has been investigated. These included *Trichoplusia ni* (Hübner) (Berger, 1966), *Pseudoplusia includens* (Walker) (Tumlinson et al., 1972) and *Autographa californica* (Speyer) (Kaae et al., 1973; Steck et al., 1979). In all of them *Z*-7-dodecenyl acetate (*Z*7-12:OAc) was identified. This paper reports the identification of two components of the female sex pheromone of *Plusia chalcites*.

METHODS AND MATERIALS

Chemicals. *Z*7-12:OAc was purchased from Farchan Chemical Co. and *Z*-9-tetradecenyl acetate (*Z*9-14:OAc) was obtained from Tropical Products Institute Laboratories, London, England; both compounds were 98–99% pure; they contained 1–2% of the corresponding *E* isomer. The rest of the compounds used were from our collection (purity 97–99%).

Gas Chromatography (GC) and Combined Mass Spectroscopy (GS-MS). A Varian 3700 model, equipped with a FID detector and a splitless injector system, was used for all capillary GC analyses. The following WCOT capillary columns were used: A = SP 2100 (glass, 60 m × 0.25 mm ID): B = OV-17 (stainless steel, 25 m × 0.5 mm ID), C = DEGS (glass, 25 m × 0.5 mm ID) and D = PCP-sil (glass, 30 m × 0.25 mm ID). Conditions of chromatography for all columns were: injector and detector 250°; initial oven temperature relatively low, purge 1 min after injection, programing 3 min after injection, and then isothermal. Column A, He 1 ml/min, oven 100° programing at 30°/min to 180°. Column B, He 2 ml/min, oven 120° programing at 20°/min to 180°. Column C, He 3 ml/min, oven 60° programing at 7°/min to 150°. Column D, He 2 ml/min, oven 60° programing at 5°/min to 150°. The GC-MS analysis was carried out on a Du Pont 21-490B mass spectrometer coupled with a Varian 2700 chromatograph equipped with a FID detector. A 1.8 m × 4 mm ID glass column packed with 3% OV-17 on Gaschrom Q 80–100 mesh was used. A capillary splitter divided the effluent between the FID detector and the mass spectrometer. Conditions of chromatography were: He inlet pressure of 20 psi, oven at 125°, after 10 min programing at 2°/min to 180°. The pheromone components were eluted before the maximum temperature was reached. Authentic synthetic reference samples were analyzed under the same conditions. All mass spectra were recorded at an electron energy of 70 eV. Spectra were taken at the center of a peak.

Microreactions. Hydrolysis with 10% KOH/MeOH and acetylation with

neat acetyl chloride were carried out in ½-dram vials sealed with Teflon-lined screw caps as described by Kochansky et al. (1978). Crude extract of abdominal tips from about 10 moths were used.

Virgin Females and Extract Preparation. Insects were reared on an artificial medium (Shorey and Hale, 1965). Pupae were sexed and placed in a room with a dark–light regime of 10:14 hr. The abdominal tips of 4- to 6-day old females were clipped, during their calling period, 1–3 hr after the beginning of the dark period and extracted with redistilled hexane. The extracts of about 100 females were filtered through a small glass wool plug and concentrated on a Rotavapor to a small volume (ca. 0.25 ml). A few drops of heptane were added, and the residual hexane was removed with a slow stream of nitrogen. The heptane solutions were analyzed directly on capillary GC and by GC-MS.

Field Tests. For field experiments dry funnel traps were used. The traps were made of a protective wooden roof fixed 5–7 cm above a plastic funnel of 20 cm diameter, to which a 2-liter plastic container was attached. A screen cage, containing two virgin females 4–6 days old or the synthetic pheromone on a rubber septum, was hung from the center of the roof. Male moths attracted to the pheromone fell through the funnel into the container. Traps were located in a tomato field 25 m apart and about 75 cm above the plants. The catches were recorded ever 1–2 days, and the baits were rotated by one position at that time.

RESULTS

Identification of Pheromone Components. Crude female abdominal tip extracts were analyzed on a packed OV-17 column. Three peaks were present which eluted below 180°. The retention times of these peaks corresponded approximately to those of 12-carbon, 14-carbon, and 16-carbon acetates, respectively. A GC-MS analysis showed that peak *A* was a dodecenyl acetate with the highest mass at *m/e* 166, corresponding to M^+-60 (loss of acetic acid), and a diagnostic peak at *m/e* 61 ($CH_3COOH_2^+$). Peak B had the highest mass at *m/e* 194, M^+-60) and *m/e* 61 ($CH_3COOH_2^+$). Peck C was a hexadecyl acetate (16:OAc) with the highest mass at *m/e* 224 (M^+-60) and *m/e* 61 ($CH_3COOH_2^+$). The mass spectra of these three peaks were identical to the mass spectra of *Z*7–12:OAc, *Z*9–14:OAc, and normal 16:OAc, respectively. The mass spectra of positional isomers of long-chain unsaturated acetates such as these are similar but, if run at the same conditions, different in the intensities of various mass peaks, allowing comparison of natural pheromone components with synthetic samples (Klun and Junk, 1977). In order to confirm the identity of the three compounds, a careful analysis on four WCOT capillary columns was performed. The following columns, in order of increasing

polarity, were used: SP 2100, OV-17, DEGS, and PCP-sil. The analysis was carried out by comparison of retention times and coinjection with authentic reference samples. Relatively clean chromatograms were obtained on all four columns containing the main three peaks, A, B, and C, and a number of additional small peaks. A representative chromatogram is shown in Figure 1. A series of positional and geometric isomers of dodecenyl acetates and tetradecenyl acetates, including *Z*5–12:OAc, *Z*9–12:OAc, *Z*7–14:OAc and *Z*11–14:OAc, were analyzed for comparison. The nonpolar columns SP 2100 and OV-17 were preferable for the positional isomer separation, whereas the polar columns DEGS and PCP-sil were preferable for the geometric isomer separation. The SP 2100 column gave the best separation of all the positional isomers whereas the PCP-sil column gave a baseline separation between the *E*- and *Z*-7-dodecenyl acetates and the *E*- and *Z*-9-tetradecenyl acetates.

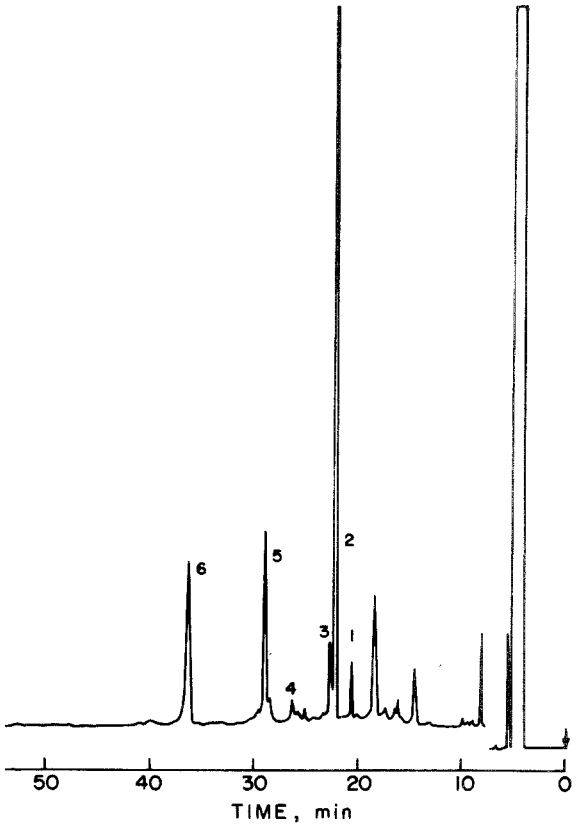

FIG. 1. DEGS high-resolution WCOT column chromatogram of crude extract from *P. chalcites*: (1) dodecyl acetate; (2) *Z*-7-dodecenyl acetate; (3) *Z*-9-dodecenyl acetate; (4) tetradecyl acetate; (5) *Z*-9-tetradecenyl acetate; (6) hexadecyl acetate.

Analysis on the capillary columns showed unambiguously that peaks A, B, and C were *Z*7–12:OAc, *Z*9–14:OAc, and 16:OAc, respectively. No *E*7–12:OAc or *E*9–14:OAc was detected in the tip extracts. Further confirmation of the three main compounds A, B, and C was obtained from the hydrolysis and acetylation of a sample of crude extract from *P. chalcites*. Hydrolysis with KOH/MeOH and analysis on the DEGS capillary column showed the three new peaks formed had identical retention times to those of *Z*-7-dodecenyl alcohol, *Z*-9-tetradecenyl alcohol, and hexadecyl alcohol. Acetylation of the hydrolyzed sample with acetyl chloride regenerated the acetate peaks A, B, and C.

Some of the small peaks (Figure 1) were present in all extracts, and three of them could be identified by comparison of retention times and coinjection on all four capillary columns. All three comprised only about 3% of the identified components. The compounds were dodecyl acetate (12:OAc), *Z*-9-dodecenyl acetate (*Z*9–12:OAc), and tetradecyl acetate (14:OAc). A list of retention times of all identified components is presented in Table 1. An estimated ratio of these components in the abdominal tip extracts of *P. chalcites* is summarized in Table 2. The amount of *Z*7–12:OAc and *Z*9–14:OAc per female obtained was 300–400 ng and 60–80 ng, respectively.

Field Tests. Different combinations of the identified components on rubber septa were tested in a tomato field. The major component identified in the extracts, *Z*7–12:OAc, attracted no moths into the traps. The same was true for the second component *Z*9–14:OAc. However, mixtures of the two compounds at ratios of 1:1, 5:1, and 10:1 were attractive to males in the field. The differences in catches were statistically nonsignificant (Table 3). Addition of the other components identified in the abdominal tip extracts had no influence on attractancy. A comparison of the traps baited with the synthetic pheromone (ratio of 5:1) and traps baited with two virgin females showed that the synthetic pheromone was more efficient and gave consistently higher catches (Table 3).

TABLE 1. RETENTION TIMES (MIN) OF COMPONENTS FROM ABDOMINAL TIP EXTRACT OF *Plusia chalcites* ON DIFFERENT CAPILLARY COLUMNS

	Retention time (min)					
Column	12:OAc	*Z*7–12:OAc	*Z*9–12:OAc	14:OAc	*Z*9–14:OAc	16:OAc
SP 2100	26.1	25.2	25.8	45.8	42.0	83.4
OV 17	15.7	16.0	16.3	26.9	27.8	51.8
DEGS	20.6	22.4	22.7	26.6	29.1	36.5
PCP-Sil	22.1	24.0	24.3	27.0	29.9	35.4
		*E*7–12:OAc			*E*9–14:OAc	
PCP-Sil		23.4			28.8	

TABLE 2. ESTIMATED COMPOSITION OF ABDOMINAL TIP EXTRACT OF *Plusia chalcites*

Compound	%
Dodecyl acetate	1.5
Z-7-Dodecenyl acetate	67.0
Z-9-Dodecenyl acetate	0.5
Tetradecyl acetate	1.0
Z-9-Tetradecenyl acetate	14.0
Hexadecyl acetate	16.0

TABLE 3. TRAP CATCHES OF MALE *Plusia chalcites* WITH SYNTHETIC CHEMICALS DISPENSED FROM RUBBER SEPTA

Test No.	Bait[a]	No. traps	No. nights	Total no. of males[b]
1.	1.0 mg A	4	20	0b
	1.0 mg B	4	20	0b
	0.5 mg A + 0.5 mg B	4	20	96a
	0.83 mg A + 0.17 mg B	4	20	109a
	0.91 mg A + 0.09 mg B	4	20	89a
2.	1.0 mg A + 0.2 mg B	3	17	84a
	1.0 mg A + 0.2 mg B + 0.3 mg C	3	17	82a
	1.0 mg A + 0.2 mg B + 0.3 mg C + 0.2 mg D + 0.02 mg E + 0.02 mg F	3	17	89a
3.	1.0 mg A + 0.2 mg B	6	6	133a
	1.0 mg A + 0.2 mg C	6	6	0b
4.	1.0 mg A + 0.2 mg B	6	11	209a
	1.0 mg A + 0.2 mg B + 0.1 mg D	6	11	170a
	1.0 mg A + 0.2 mg B + 0.1 mg D + 0.2 mg C	6	11	188a
5.	1.0 mg A + 0.2 mg B	8[c]	6	185a
	2 virgin females (3–6 days old)	8[c]	6	36b

[a]A = Z-7-dodecenyl acetate; B = Z-9-tetradecenyl acetate; C = hexadecyl acetate; D = Z-9-dodecenyl acetate; E = dodecyl acetate; F = tetradecyl acetate. For purity of all compound see Materials and Methods.

[b]Numbers in each test followed by the same letter are not significantly different at the 5% level (Duncan's new multiple-range test).

[c]There were only 6 traps per treatment on the first two nights.

DISCUSSION

Two components of the primary sex pheromone system of *P. chalcites* have been identified as *Z*7–12:OAc and *Z*9–12:OAc. These two compounds were found in the abdominal tip extracts in an approximate ratio of 5:1, respectively. Only a combination of the two compounds was an efficient lure for males in the field (Table 3). Thus, *P. chalcites* appears to be the first Plusiinae species for which a primary sex pheromone comprising two compounds has been found.

*Z*7–12:OAc has been identified as the sex pheromone in a number of Plusiinae species. Additional Plusiinae have been found to respond to *Z*7–12:OAc in bioassays and field screening tests (Berger and Canerday, 1968; Roelofs and Comeau, 1970, 1971). From previous work on this subfamily it is clear that *Z*7–12:OAc is the main (Berger, 1966; Tumlinson et al., 1972; Steck et al., 1979; Bjostad et al., 1980), but not the sole, component of the sex pheromone of all these species. The most typical example is the case of *A. californica*, in which *Z*7–12:OAc has been found in the abdominal tips of virgin females, but this compound gave conflicting results in field tests. Kaae et al. (1973) reported attractancy by *Z*7–12:OAc alone, whereas Butler et al. (1977) and Steck et al. (1979) found negligible catches by this compound unless a synergist was added. One group used *Z*-7-dodecenyl formate (Butler et al., 1977), whereas the second group used *Z*-7-dodecenyl alcohol as synergist, although the two compounds were not detected in the female (Steck et al., 1979). Recently, 12:OAc has been identified as a minor second component of the sex pheromone of *Trichoplusia ni*; it appears to function as a short-range pheromone component (Bjostad et al., 1980).

The second component of the *P. chalcites* sex pheromone, *Z*9–14:OAc, has not been detected on other Plusiinae studied so far nor shown to be a stimulant to the males. This compound is, however, quite common as a sex pheromone component in other lepidopterous species (Tamaki, 1977). *Agrotis ipsilon* (Hufnagel) (Hill et al., 1979), like *P. chalcites*, utilizes a combination of *Z*7–12:OAc and *Z*9–14:OAc.

The exact role of the other components identified (Table 2) remains to be elucidated. Among these, the saturated acetates 12:OAc and 14:OAc could be precursors for the biosynthesis of *Z*7–12:OAc and *Z*9–14:OAc. Such a proposition was made by Nesbitt et al. (1979) for hexadecanal and *Z*-11-hexadecenal which is the main pheromone component of *Heliothis armigera*.

Traps baited with a mixture of 1 mg of *Z*7–12:OAc and 0.2 mg of *Z*9–14:OAc (at ratio of 5:1) impregnated on rubber septa were more efficient than traps baited with two virgin females. This ratio, which is similar to that found in the extracts, was used in most experiments, although two different ratios of 10:1 and 1:1 were also tested and were found to catch quite

efficiently. Only *P. chalcites* were caught by this mixture, although adult *Trichoplusia ni* and *Plusia gamma* were present in the test plot. The use of the synthetic pheromone for *P. chalcites* makes it convenient to establish a monitoring system specifically for this pest.

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LARVAL OSMETERIAL SECRETIONS OF THE SWALLOWTAILS (*Papilio*)

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Abstract—The larval osmeterial secretions of six *Papilio* species examined displayed a remarkable qualitative change at the fourth larval ecdysis. The secretions of 4th (penultimate) instar larvae of *P. machaon*, *P. memnon*, *P. helenus*, *P. bianor*, and *P. maackii* principally comprised mono- and/or sesquiterpenoids. The compounds identified from these species included α-pinene, sabinene, β-myrcene, limonene, β-phellandrene, (*Z*)-β-ocimene, (*E*)-β-ocimene, β-elemene, β-caryophyllene, (*E*)-β-farnesene, β-selinene, (*E,E*)-α-farnesene, germacrene-A, germacrene-B, caryophyllene oxide, methyl 3-hydroxy-*n*-butyrate, and acetic acid. In contrast, the secretion of 4th larval instar of *P. xuthus*, although containing similar terpenic compounds, was accompanied by large amounts of aliphatic acids and their esters: isobutyric acid, 2-methylbutyric acid, methyl isobutyrate and methyl 2-methylbutyrate. On the other hand, the osmeterial secretions of 5th (last) instar larvae varied little in quality among the six species, and the identified compounds consisted of isobutyric acid, 2-methylbutyric acid, methyl isobutyrate, ethyl isobutyrate, methyl 2-methylbutyrate, ethyl 2-methylbutyrate, and isovaleric acid, the last of which was specific to *P. bianor* and *P. maackii*. The chemical alteration of osmeterial exudate synchronized at the final larval ecdysis with the larval morphological change (particularly in body coloration) that appears to be of defensive significance against predators.

Key Words—Osmeterial secretion, *Papilio* larvae, Lepidoptera, monoterpene, sesquiterpene, aliphatic acid, ester.

INTRODUCTION

Papilionid larvae are known to possess, on the mid-dorsal surface of the larvae, between the head and the prothoracic segment, a peculiar, eversible, epidermal gland (osmeterium) that secretes a characteristic odoriferous liquid

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regarded as a defensive allomone (Eisner and Meinwald, 1965). The gland, which is ordinarily invaginated invisibly beneath the integument, is extruded by blood pressure when the animal is irritated by abrupt contact, vibration, etc.

The chemical properties of osmeterial secretions and the ultrastructure of osmeterium have been the major subject of investigations undertaken during the last decade. Isobutyric and 2-methylbutyric acids have been identified as major components of the osmeterial secretions of some *Papilio*, *Graphium*, and *Baronia* larvae (Eisner and Meinwald, 1965; Crossley and Waterhouse, 1969; Eisner et al., 1970; López and Quesnel, 1970; Oshima et al., 1975). On the other hand, Eisner et al. (1971) have reported on two sesquiterpenes, β-selinene and selin-11-en-4a-ol, from the osmeterial secretion of *Battus polydamas*, and recently, a monoterpene hydrocarbon, myrcene, has been detected from the secretions of two *Luehdorfia* species (Suzuki et al., 1979).

So far a few papers have insufficiently documented the chemical disparities between the osmeterial secretions of the last and the preceding larval instars of certain *Papilio* species (Seligman and Doy, 1972; Burger et al., 1978).

In previous papers, the author has given detailed reports on the chemical nature of osmeterial secretions of *Papilio protenor* (Honda, 1980a) and several species in the genera *Luehdorfia*, *Graphium*, and *Atrophaneura* (Honda, 1980b). It has been demonstrated that the secretion of *P. protenor* is of heterogeneous type in which the chemical composition of the secretion of the last instar larvae remarkably differs in quality from those of the younger larvae and that the secretions of the genera *Luehdorfia*, *Graphium*, and *Atrophaneura* belong to a homogeneous group where no qualitative alteration of secretion components occurs at the final larval ecdysis. The osmeterial secretion of 5th instar (last) *P. protenor* larvae mainly comprised isobutyric and 2-methylbutyric acids together with their methyl and ethyl esters, while those of the younger larvae were found to consist predominantly of a variety of mono- and sesquiterpenes such as α-pinene, β-myrcene, limonene, β-phellandrene, (*Z*)- and (*E*)-β-ocimenes, β-elemene, β-caryophyllene, germacrene-B, and caryophyllene oxide. The secretions of the last and the penultimate larval instars of *Luehdorfia* species were characterized by monoterpene hydrocarbons composed principally of α-pinene, β-myrcene, limonene, and terpinolene, and those of *Atrophaneura alcinous*, by sesquiterpenes (δ-cadinene, etc.). In contrast, the above two acids and their esters constituted a chemical character of the secretions of *Graphium* species.

As the chemical constitution of osmeterial secretions of *Papilio* species are not fully elucidated, further research is required for a more comprehensive interpretation of defensive systems of papilionid larvae. In the present work, a comparative investigation is conducted in detail of volatile components in the osmeterial secretions of 4th and 5th instar larvae of six *Papilio* species.

METHODS AND MATERIALS

Animals. Larvae of *Papilio machaon hippocrates*, *P. memnon thunbergii*, *P. helenus nicconicolens*, *P. bianor dehaanii*, *P. maackii tutanus*, and *P. xuthus*, derived from respective adult females collected in Kanagawa, Kagoshima, or Fukushima prefectures, were reared at 25° C and under a 16:8 light–dark photoperiod throughout the larval development. The larvae of *P. machaon* were fed with an umbelliferous plant, *Cryptotaenia canadensis*, and those of *P. memnon*, with *Citrus unshiu*; *Fagara ailantoides* was utilized as a diet for the other species.

Collection of Secretions. The secretion was "milked" separately from 4th and 5th instar larvae of individual species on the third or fourth day after larval ecdysis. Each sample for chemical analysis (dichloromethane extract of osmeterial secretion) was prepared from 30–40 larvae by a method similar to that of Eisner and Meinwald (1965). The amount of dichloromethane (Tokyo Kasei Co. Ltd., Spectro Grade) used for extraction was 1.5–2 ml/sample.

Analysis of Components. The secretion components were examined by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Identification of the components was tentatively based on mass spectral data and confirmed by comparison of GC retention data and fragmentation patterns of mass spectra with authentic compounds. GC identity was corroborated by either cochromatography or measurement of Kovats' indices. GC analyses were carried out on a Hitachi 073 gas chromatograph equipped with a flame ionization detector, using two types of glass capillary columns (0.26 mm ID × 40 m, WCOT) coated with FFAP (column A) or OV-101 (column B). The flow rate of carrier gas (nitrogen) was 1 ml/min, and the split ratio was regulated at 1/70. The injection temperature was 200° C. The oven temperature was programed from 50° C at 5° C/min or 2° C/min for general analyses, or maintained isothermal for the determination of Kovats' indices. Mass spectra were measured by means of EI mode (IV: 70 eV) and also by CI mode (reactant gas: isobutane; IV: 200 eV), when necessary, with a JEOL JMS-D 300 mass spectrometer interfaced to a JEOL JGC-20 KP gas chromatograph, employing the same capillary columns under analogous GC conditions described above. The percentage composition of each component was calculated by estimating its peak area/total peak area on gas chromatograms. As 2-methylbutyric acid and isovaleric acid (secretion components coexisting in the secretion of some species) were not separable from each other on column A, for purposes of quantification they were converted to the corresponding *n*-butyl esters with ethereal diazobutane prior to GC injection, according to the procedure reported in a previous work (Honda, 1980b). The peak separation between *n*-butyl esters of the two acids was successfully attained by running on column B.

RESULTS

All *Papilio* larvae examined are provided with a pair of forked osmeteria, colored yellow to orange (4th instar) or yellow to reddish purple (5th instar), secreting a colorless liquid with a faint odor (4th instar) or an offensive intense odor (5th instar).

P. machaon, P. memnon, P. helenus, P. bianor, and P. maackii.

4th Instar Larvae. GC analysis of the osmeterial secretion obtained from 4th instar larvae of *P. machaon* showed the presence of more than 45 components on column A, and those of *P. memnon*, *P. helenus*, *P. bianor*, and *P. maackii* were found to comprise about 23, 10, 26, and 28 components, respectively (Figure 1). Preliminary investigation suggested that in every species the secretion consists mainly of terpenic compounds (the majority of them being hydrocarbons).

Peaks a, c, d, and f, which were detected in common from four species on GC-MS, gave molecular ions (M^+) equally at m/z 136 and exhibited mass fragmentation patterns characteristic of monoterpene hydrocarbons. This mass spectral information (Table 1) combined with GC retention data established the identity of peaks a, c, d, and f as α-pinene, β-myrcene, limonene, and (*Z*)-β-ocimene, respectively. Peaks b, e, and g, indicating M^+ at m/z 136, were also regarded as monoterpene hydrocarbons and determined to be sabinene, β-phellandrene, and (*E*)-β-ocimene, respectively.

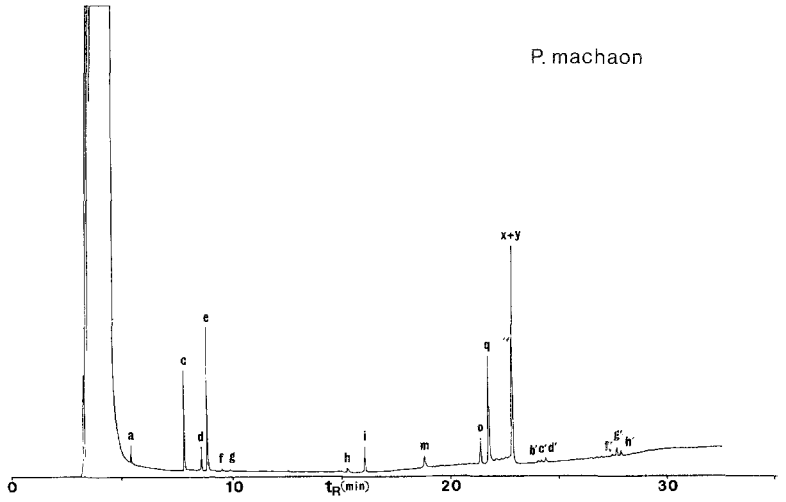
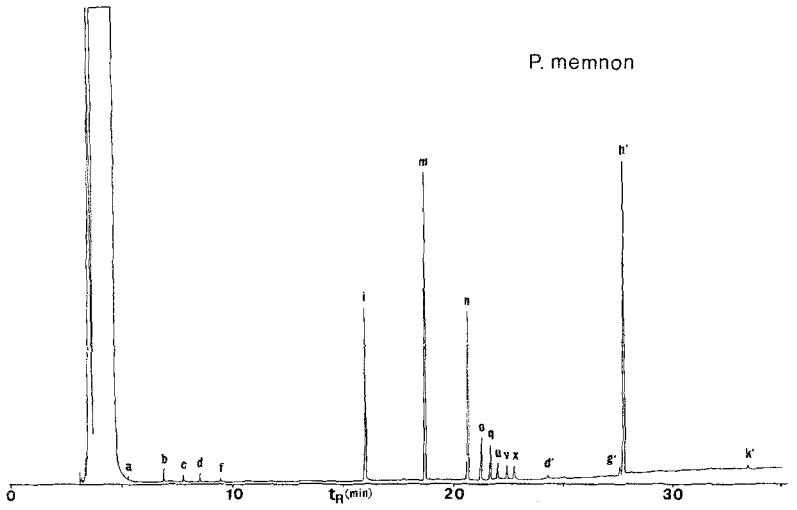


FIG. 1. Gas chromatograms of the osmeterial secretions of 4th instar *Papilio* larvae on a FFAP glass capillary column; 50–180° C at 5° C/min.



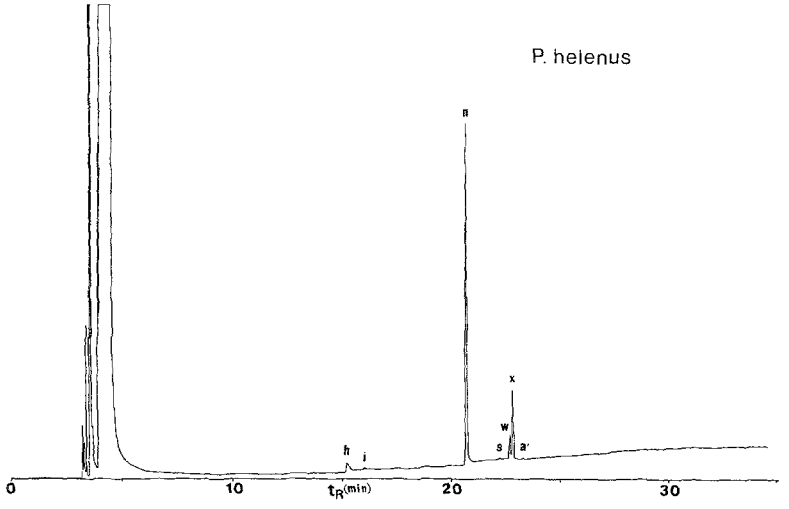
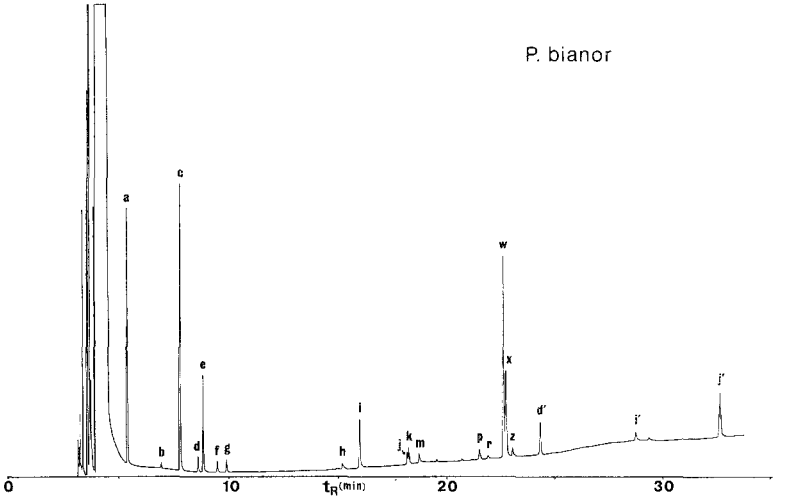


FIG. 1. CONTINUED.



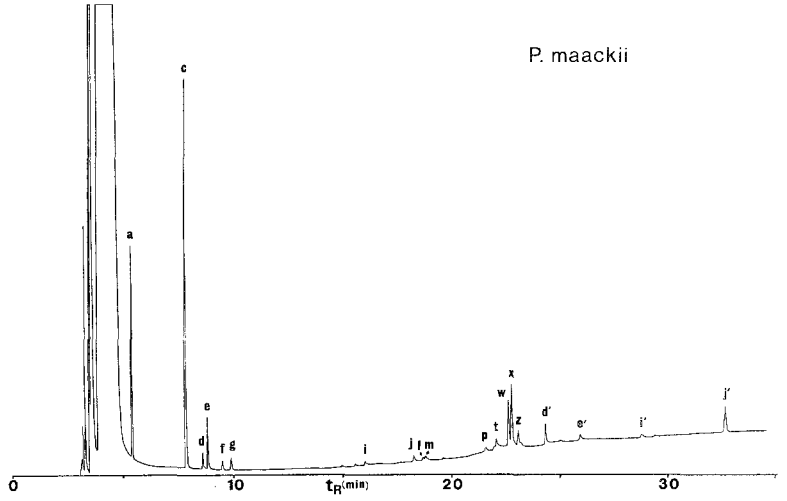


FIG. 1. CONTINUED.

On the other hand, peak h was deemed to be a carboxylic acid, since it was extractable with dilute aqueous potassium bicarbonate solution. Its molecular formula was assignable to $C_2H_4O_2$ from the molecular weight of 60. Compound h was thus identified as acetic acid.

The mass spectrum of peak i, which was found in every secretion, lacked the molecular ion, although it showed several fragment ions with substantial intensities. Another GC-MS measurement with CI mode gave a prominent quasimolecular ion (QM^+) at m/z 119, thereby indicating a molecular weight of 118. This compound was soluble in water and readily hydrolyzed with aqueous sodium hydroxide solution. These spectral and chemical properties were quite analogous to those of the compound previously identified as methyl 3-hydroxy-*n*-butyrate from the osmeterial secretion of young larvae of

TABLE 1. MASS SPECTROMETRIC IDENTIFICATION OF COMPONENTS IN OSMETERIAL SECRETIONS

No.	Component	Mass spectral data m/z (intensity, %)
1	Acetic acid	M^+: 60 (51), 45 (83), 43 (100)
2	Isobutyric acid	M^+: 88 (5), 73 (25), 45 (8), 43 (100), 41 (40)
3	2-Methylbutyric acid	QM^+: 103[a], 87 (23), 74 (100), 57 (75), 45 (10), 41 (60)
4	Isovaleric acid	60[b]
5	Methyl isobutyrate	M^+: 102 (12), 87 (21), 71 (44), 59 (32), 43 (100), 41 (21)
6	Ethyl isobutyrate	M^+: 116 (7), 89 (7), 88 (10), 73 (7), 71 (39), 45 (10), 43 (100), 41 (19)
7	Methyl 3-hydroxy-*n*-butyrate	QM^+: 119[a], 103 (11), 87 (12), 74 (39), 71 (23), 61 (12), 59 (13), 45 (61), 43 (100)
8	Methyl 2-methylbutyrate	M^+: 116 (1), 101 (18), 88 (68), 85 (23), 69 (7), 59 (22), 57 (100), 41 (47)
9	Ethyl 2-methylbutyrate	M^+: 130 (1), 115 (6), 102 (41), 87 (9), 85 (29), 74 (18), 57 (100), 45 (6), 41 (34)
10	α-Pinene	M^+: 136 (8), 121 (12), 107 (6), 105 (10), 93 (100), 79 (25), 77 (30), 67 (10), 53 (12), 43 (11), 41 (30)
11	Sabinene	M^+: 136 (13), 121 (4), 93 (100), 91 (29), 79 (22), 77 (30), 69 (9), 65 (7), 53 (6), 41 (21)
12	Limonene	M^+: 136 (11), 121 (13), 107 (12), 93 (50), 79 (22), 77 (13), 68 (100), 67 (50), 53 (20), 41 (23)
13	β-Phellandrene	M^+: 136 (15), 121 (4), 93 (100), 91 (22), 79 (16), 77 (27), 65 (5), 53 (4), 43 (10), 41 (13)
14	β-Myrcene	M^+: 136 (3), 121 (3), 107 (2), 93 (81), 79 (10), 77 (11), 69 (70), 67 (8), 53 (10), 41 (100)
15	(Z)-β-Ocimene	M^+: 136 (3), 121 (20), 105 (18), 93 (100), 91 (33), 79 (34), 77 (30), 67 (11), 65 (6), 55 (12), 53 (14), 43 (14), 41 (32)

TABLE 1. CONTINUED

No.	Component	Mass spectral data m/z (intensity, %)
16	(*E*)-β-Ocimene	M^+: 136 (8), 121 (17), 105 (15), 93 (100), 91 (31), 80 (42), 79 (37), 77 (29), 67 (13), 65 (7), 55 (14), 53 (17), 43 (18), 41 (36)
17	(*E*)-β-Farnesene	M^+: 204 (5), 161 (8), 133 (19), 120 (13), 107 (7), 93 (56), 79 (22), 69 (100), 55 (19), 41 (86)
18	(*E*,*E*)-α-Farnesene	M^+: 204 (2), 161 (3), 135 (7), 123 (21), 119 (26), 107 (40), 93 (95), 79 (43), 69 (62), 55 (61), 41 (100)
19	β-Elemene	M^+: 204 (3), 189 (14), 161 (18), 147 (28), 133 (18), 121 (34), 107 (49), 93 (75), 81 (100), 68 (79), 55 (46), 41 (69)
20	β-Caryophyllene	M^+: 204 (9), 189 (13), 175 (8), 161 (25), 148 (22), 147 (22), 133 (65), 120 (32), 107 (38), 105 (35), 93 (88), 91 (47), 79 (55), 69 (91), 55 (42), 41 (100)
21	β-Selinene	M^+: 204 (53), 189 (41), 175 (22), 161 (52), 147 (45), 133 (50), 121 (66), 107 (92), 105 (97), 93 (100), 81 (84), 79 (79), 67 (64), 55 (55), 41 (79)
22	Germacrene-A[c]	M^+: 204 (16), 189 (27), 161 (28), 147 (32), 133 (25), 119 (30), 107 (62), 93 (77), 81 (68), 68 (100), 53 (57), 41 (85)
23	Germacrene-B	M^+: 204 (17), 189 (13), 161 (26), 147 (15), 133 (23), 121 (100), 107 (50), 105 (43), 93 (72), 81 (46), 67 (47), 55 (39), 53 (41), 41 (74)
24	Caryophyllene oxide	M^+: 220 (3), 205 (4), 202 (7), 187 (9), 177 (7), 161 (14), 149 (11), 135 (14), 131 (14), 121 (26), 107 (38), 93 (55), 79 (63), 69 (57), 55 (48), 43 (78), 41 (100)

[a] $(M + 1)^+$ ion recorded with CI mode (reactant gas: isobutane).
[b] In the secretions of *P. bianor* and *P. maackii*, the mass spectrum of 2-methylbutyric acid contained this ion of considerable intensity. Identification of isovaleric acid was carried out on the basis of its *n*-butyl ester, mass spectral data of which are given in the text.
[c] Measured at 110° C (ionizing temperature). Other compounds were measured at a temperature between 150 and 200° C.

Papilio protenor (Honda, 1980a). Actually, the mass spectral and GC retention data of compound i exactly coincided with those of authentic methyl 3-hydroxy-*n*-butyrate.

In contrast are peaks j and k whose mass spectra set forth the following M^+ and base peak: j; M^+, 204; B, 91; k: M^+, 204; B, 147 and represented fragmentation patterns suggestive of sesquiterpene hydrocarbons whose molecular formula can be assigned to $C_{15}H_{24}$. However, an attempt to elucidate their chemical structures was unsuccessful.

Peaks l and m, both exhibiting M^+ at m/z 204, also appeared to be

sesquiterpene hydrocarbons and were readily identified as β-elemene and β-caryophyllene, respectively.

Peak n, a major constituent in the secretion of *P. helenus*, was assumed to be either of the two possible geometrical isomers of β-farnesene upon comparison with its published mass spectra (Stenhagen et al., 1974). GC-MS data of the compound were consistent with those of authentic (*E*)-β-farnesene prepared by dehydration of (*E*)-nerolidol in the presence of hexamethylphosphortriamide (HMPA) as a catalyst.

Peak p was anticipated to be β-selinene by making reference to its mass spectrum reported by Yukawa and Ito (1973). The identity was established by comparison with an authentic standard isolated from celery seed oil.

Peak q (M^+: 204; B: 161), a sesquiterpene hydrocarbon, although exhibiting a mass spectral fragmentation closely resembling that of aromadendrene or alloaromadendrene, remained unidentified owing to the failure to conform with their retention indices.

Peaks o (M^+: 204; B: 105), r (M^+: 204; B: 161), s (M^+: 204; B: 41), t (M^+: 204; B: 161), u (M^+: 204; B: 161) and v (M^+: 204; B: 105) are also highly likely to be sesquiterpene hydrocarbons whose molecular formulae can be represented equally by $C_{15}H_{24}$, but their structures are yet unknown.

The mass spectrum of peak w, giving M^+ at *m/z* 204, was very similar to that of a major volatile component of quince fruit, already determined to be (*E,E*)-α-farnesene (Shimizu and Yoshihara, 1977). GC-MS data of (*E,E*)-α-farnesene prepared by dehydration of (*E*)-nerolidol catalyzed with HMPA were in good agreement with those of the peak w.

Peak x, which was detected in common from the secretion of every species, exhibited the mass spectrum shown in Figure 2 when recorded at 110° C (ionizing temperature), while another measurement at an elevated temperature (200° C) resulted in a different fragmentation: essentially that of β-elemene. The higher the temperature, the more salient the ion of *m/z* 81 and the weaker the intensity of the molecular ion. This compound was so labile as to readily isomerize to β-elemene on heating at 120° C for 30 min (probably due to Cope rearrangement) and to yield a mixture of β-selinene and another hydrocarbon on contact with silicic acid for 12 hr at room temperature. These chemical properties appear to be compatible with those of germacrene-A reported by Weinheimer et al. (1970) and Nishino et al. (1977). In addition, by treatment with aqueous $AgNO_3$ solution, the compound easily afforded a silver nitrate adduct as a white gruel-like substance, which, when decomposed with aqueous NaCl solution, regenerated the original compound with no appreciable isomerization. The complex formation proceeded rapidly and, thus, selectively for this compound as long as the reaction was continued for a short time, despite the presence of other unsaturated hydrocarbons; on prolonged reaction, some other compounds seemed to undergo complex formation with $AgNO_3$. These results revealed that compound x is identical

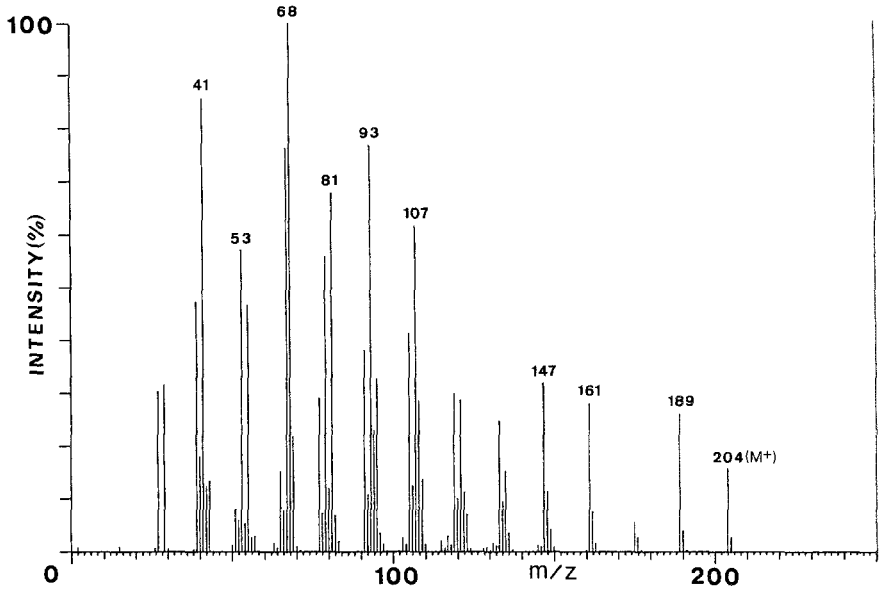


FIG. 2. Mass spectrum of component X (germacrene-A) at 70 eV and 110° C.

with the one tentatively assigned to germacrene-A in *Papilio protenor* (Honda, 1980a). The mass spectrum shown in Figure 2 was found to conform well with that of germacrene-A present in the cornicle exudate of the spotted alfalfa aphid, *Therioaphis maculata* (Nishino et al., 1977). This information, coupled with GC retention data, substantiated the identity of component x as germacrene-A.

In *Papilio machaon*, the mass spectrum of peak x (germacrene-A) was accompanied by an ion of m/z 122 with considerable intensity, thereby suggesting the superimposition of another component. After removal of germacrene-A by treatment with aqueous $AgNO_3$ solution, a small, previously concealed peak (y), on GC/MS, showed M^+ at m/z 204 and the base peak at m/z 122. This compound was also supposed to be a sesquiterpene hydrocarbon with the molecular formula of $C_{15}H_{24}$.

Structural elucidation of peaks z, a′, b′, and c′ was not achieved, although their mass spectra, z (M^+: 204; B: 41), a′ (M^+: 204; B: 93), b′ (M^+: 202; B: 119), and c′ (M^+: 202; B: 159), were strongly expected to be sesquiterpene hydrocarbons. For the components with a molecular weight of 202, the formulae are reasonably assigned to $C_{15}H_{22}$.

Peak d′, the mass spectrum of which had a close resemblance to that of γ-elemene, is likely to be germacrene-B, as it was partially converted to γ-elemene at a higher injection temperature (280° C) and gave a silver nitrate adduct. Such a nature seems to conform to that of germacrene-B (Brown et al.,

1967; Allen and Rogers, 1971). Actually, GC-MS data of the compound d' were in accord with those of germacrene-B contained in the peel oil of *Citrus junos* (Nishimura et al., 1969; Shinoda et al., 1970).

Peaks e', f', g', h', i', and j', in contrast, were all considered to be oxygenated sesquiterpenes from their mass spectra. Among them, peak h', showing M^+ at m/z 220, was determined to be caryophyllene oxide by comparison with the authentic sample.

Peak g' (M^+: 220; B: 41) appeared to be closely related to caryophyllene oxide (probably an isomer), taking into account the similarities between their mass spectra. Furthermore, this compound proved to be identical with the second principal oxygenated sesquiterpene previously detected from the osmeterial secretion of young *Papilio protenor* larvae (Honda, 1980a), but this compound was not pursued further.

The other peaks, e' (M^+: 220; B: 81), f' (M^+: 220; B: 41), and i' (M^+: 220; B: 97), to which molecular formulae of $C_{15}H_{24}O$ seem relevant, remained unidentified.

On the other hand, peaks j' and k' gave M^+ equally at m/z 222, and base peaks at m/z 43 and m/z 204, respectively, and were assigned to oxygenated sesquiterpenes with the same molecular formula of $C_{15}H_{26}O$.

Table 2 summarizes these results, and mass spectral information on the identified components is given in Table 1.

5th Instar Larvae. As shown in Figure 3A, C, and E, the gas chromatogram of the secretion of every species recorded on column A displayed two major peaks (f and i), which can be regarded as carboxylic acids from their solubility in dilute aqueous alkaline solution.

Peak f, indicating M^+ at m/z 88, was thus identified as isobutyric acid.

The mass spectrum of peak i, although devoid of a molecular ion, showed a salient QM^+ at m/z 103 when measured with CI mode. The chemical nature described above and molecular weight (102), together with GC retention index, indicated the compound to be 2-methylbutyric acid. However, in *P. bianor* (Figure 3C) the mass spectrum of the peak eluting at the same position as that of 2-methylbutyric acid contained an ion at m/z 60, significantly higher in proportion than that expected for 2-methylbutyric acid alone. This suggests that this peak may be superimposed upon a much smaller quantity of isovaleric acid whose mass spectrum shows the most abundant ion at m/z 60. Indeed, these two branched pentanoic acid isomers were found not to be separated each other under this GC condition but could be separated when their *n*-butyl ester derivatives were subjected to gas chromatography on column B under the same condition as noted in Figure 3D. A small peak of *n*-butyl isovalerate [M^+ at m/z 158 ($<1\%$), and m/z 116 (4%), 103 (50), 87 (17), 85 (100), 60 (29), 57 (97), 56 (77), 43 (28) and 41 (63)] was clearly discerned just behind a large peak of *n*-butyl 2-methylbutyrate upon GC analysis on column B of the diazobutane-treated secretion from 5th instar *P. bianor* larvae.

TABLE 2. VOLATILE COMPONENTS IN OSMETERIAL SECRETIONS OF 4TH INSTAR *Papilio* LARVAE

Peak code in Figure 1	Component[a]	Composition (%)				
		P. machaon	*P. memnon*	*P. helenus*	*P. bianor*	*P. maackii*
a	α-Pinene	1.6	0.2		15.0	17.8
b	Sabinene		0.8		0.3	
c	β-Myrcene	9.5	0.4		21.8	43.9
d	Limonene	2.3	0.6		1.1	1.3
e	β-Phellandrene	15.3			7.5	5.2
f	(*Z*)-β-Ocimene	0.2	0.3		0.8	0.7
g	(*E*)-β-Ocimene	0.2			0.9	1.0
h	Acetic acid	0.7		3.9	0.8	
i	Methyl 3-hydroxy-*n*-butyrate	2.5	13.8	0.3	4.5	0.5
j	$C_{15}H_{24}$				2.3[b]	1.1
k	$C_{15}H_{24}$					
l	β-Elemene					0.5
m	β-Caryophyllene	2.5	29.7		0.9	0.6
n	(*E*)-β-Farnesene		13.5	80.2		
o	$C_{15}H_{24}$	3.7	3.5			
p	β-Selinene				1.0	0.7
q	$C_{15}H_{24}$	14.4	2.3			
r	$C_{15}H_{24}$				0.4	
s	$C_{15}H_{24}$			0.3		
t	$C_{15}H_{24}$					1.4
u	$C_{15}H_{24}$		1.1			
v	$C_{15}H_{24}$		0.9			

w	(*E*,*E*)-α-Farnesene			3.6	23.0	5.8
x	Germacrene-A	32.1[c]	0.8	11.3	7.6	8.0
y	$C_{15}H_{24}$					
z	$C_{15}H_{24}$				0.6	1.3
a′	$C_{15}H_{24}$			0.2		
b′	$C_{15}H_{22}$	0.3				
c′	$C_{15}H_{22}$	0.3				
d′	Germacrene-B	0.6	0.3		3.1	2.8
e′	$C_{15}H_{24}O$					0.8
f′	$C_{15}H_{24}O$	0.2				
g′	$C_{15}H_{24}O$	1.4	0.8			
h′	Caryophyllene oxide	0.8	30.1			
i′	$C_{15}H_{24}O$				0.9	0.8
j′	$C_{15}H_{26}O$				6.0	4.5
k′	$C_{15}H_{26}O$		0.4			

[a]Components less than 0.2% of the total are not listed.
[b]Combined percentage of components j and k.
[c]Combined percentage of components x and y (the former being major).

Consequently, peak j was identified as isovaleric acid. In a similar manner, a small amount of isovaleric acid was also detected from the secretion of *P. maackii*.

Peak e, giving M^+ at m/z 60, was also considered to be an aliphatic acid and was identified as acetic acid.

On the other hand, peaks g, h, and k, present only in the secretion of *P. memnon* (Figure 3E), represented mass spectra characteristic of sesquiterpenic compounds and were determined to be β-caryophyllene, (*E*)-β-farnesene, and caryophyllene oxide, respectively.

Since the chromatograms on column A (Figure 3A, C, and E, for instance) of secretions of 5th larval instar indicated the presence of additional compounds overlapped upon the solvent, the "acid-free" fraction of the secretion was again gas-chromatographed on column B (Figure 3B, D, and F). Two major peaks were detected along with two minor ones from most species.

Peak a, one of the main components, showed M^+ at m/z 102 and was identified as methyl isobutyrate.

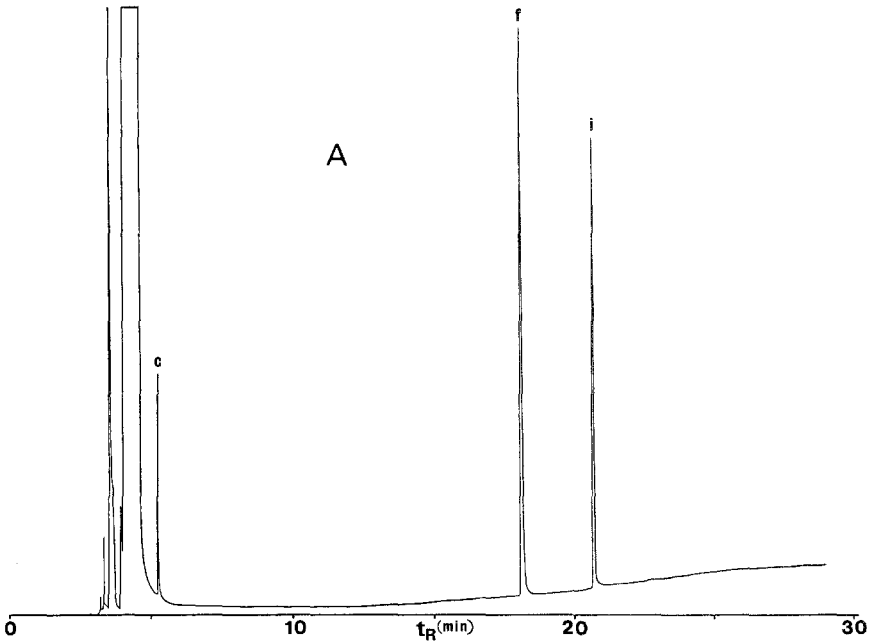


FIG. 3. Gas chromatograms of the osmeterial secretions of 5th instar larvae of *Papilio helenus* (A, B), *P. bianor* (C, D) and *P. memnon* (E, F). A, C, E: Intact extract on a FFAP glass capillary column; 50–180° C at 5° C/min. B, D, F: Acid-free fraction on a OV-101 glass capillary column; 50–120° C at 2° C/min.

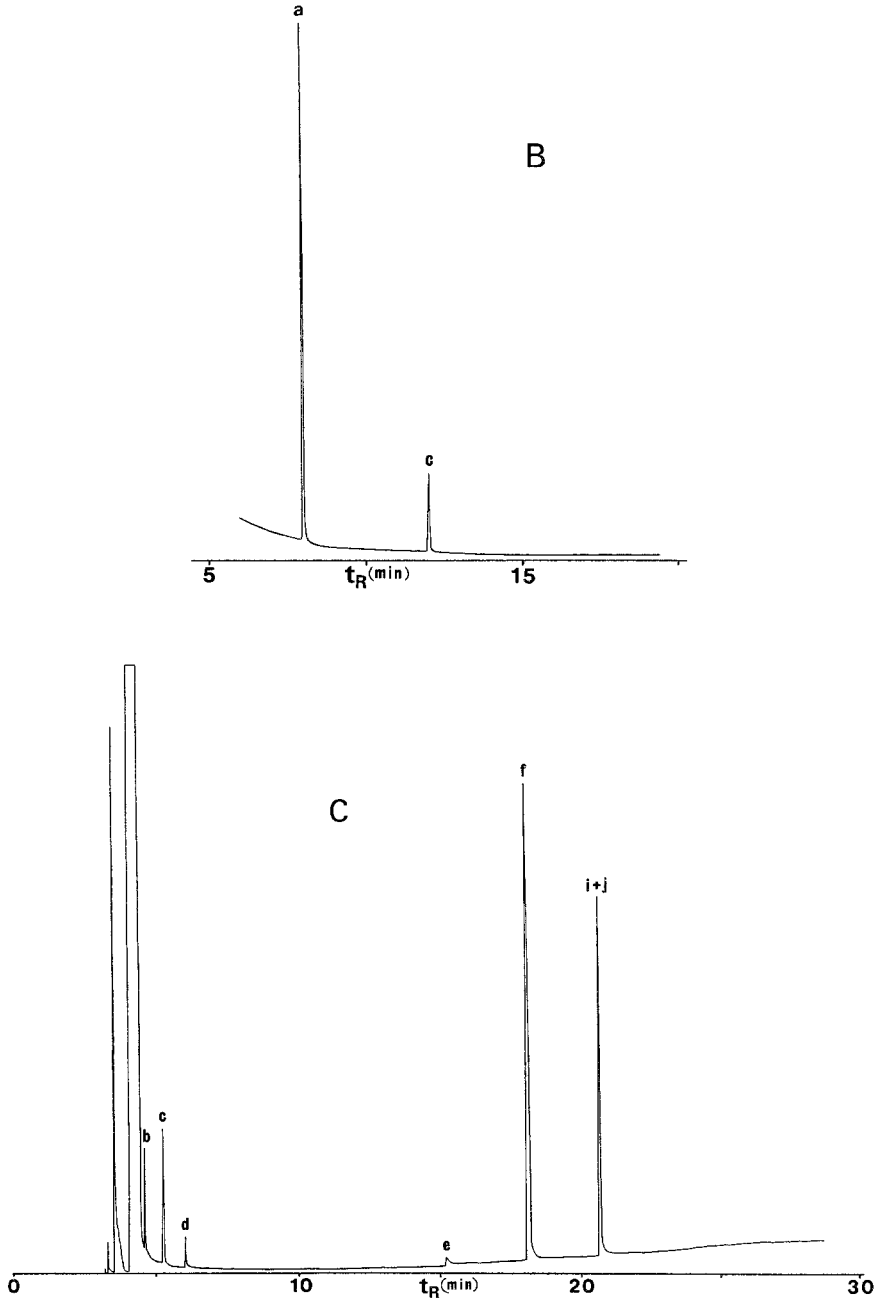


FIG. 3. CONTINUED.

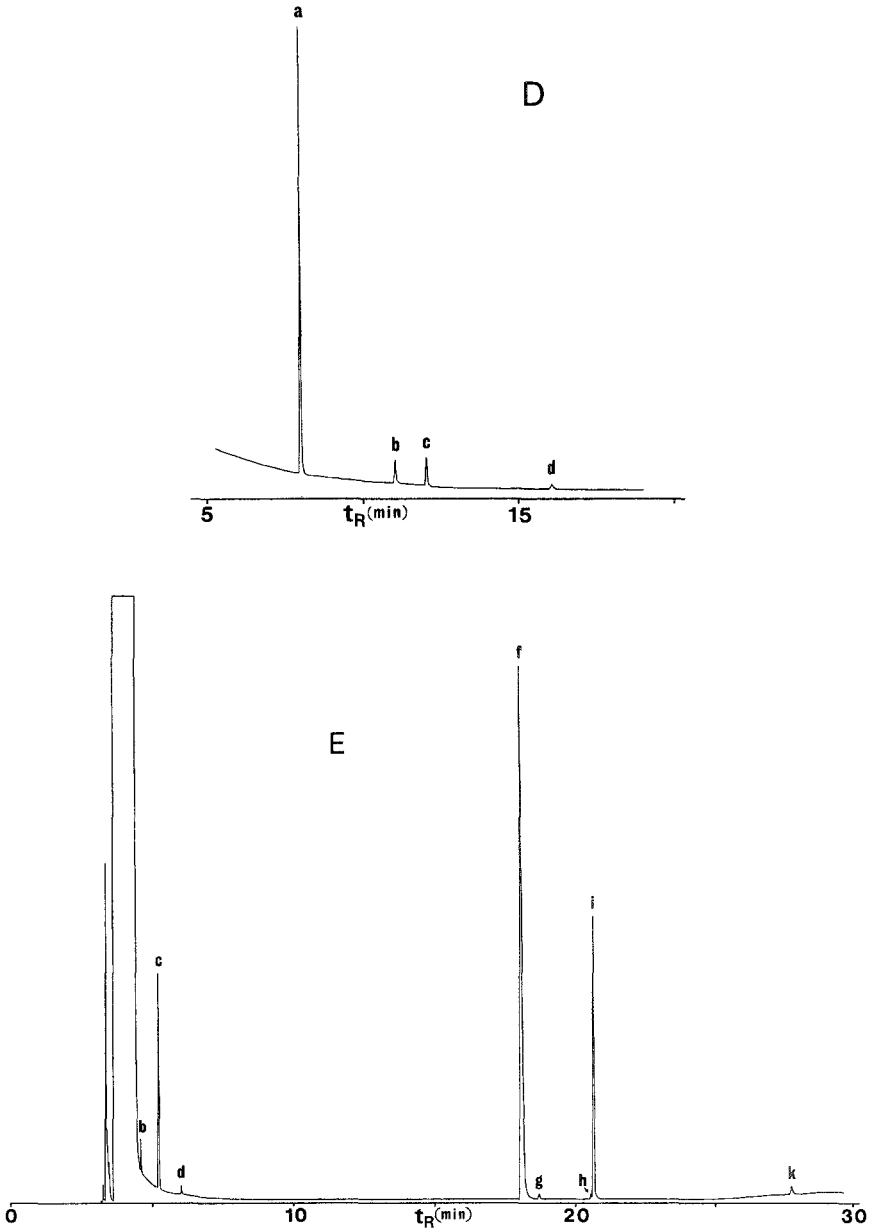


FIG. 3. CONTINUED.

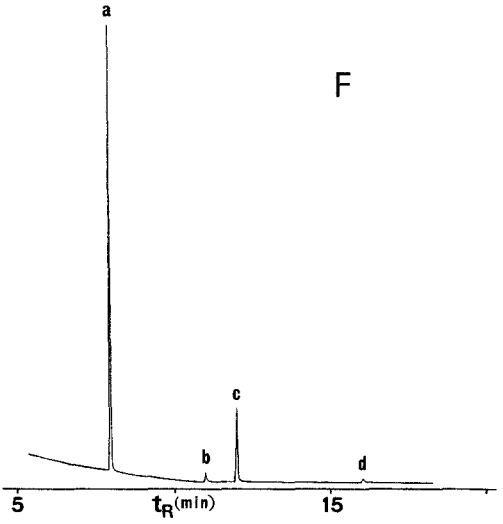


FIG. 3. CONTINUED.

The other peaks, b–d, were all assumed to be homologous esters from their mass spectra. As the molecular weights of compounds c and d determined by EI mode measurement seemed unreliable due to the lower intensities of their molecular ions, further confirmation was definitely provided by their CI spectra (QM^+: 117 and 131). Components b–d were thus concluded to be ethyl isobutyrate, methyl 2-methylbutyrate, and ethyl 2-methylbutyrate, respectively.

Table 3 summarizes the results on the secretion of 5th larval instar.

Papilio xuthus

4th Instar Larva. The osmeterial secretion of 4th larval instar of this species gives out an odor very different from those of other *Papilio* species. The odor was suggestive of the existence of acidic components and/or volatile esters with low molecular weights. The secretion was found to consist of more than 16 components (Figure 4; the solvent peak was superimposed on peak 1, which, however, was detectable by another chromatography on column B).

GC-MS analysis revealed that peak 9 was not a terpenic compound but isobutyric acid and that peaks 1, 2, and 11 were methyl isobutyrate, methyl 2-methylbutyrate, and 2-methylbutyric acid, respectively. Most of the other peaks, however, were supposed to be mono- or sesquiterpene hydrocarbons, and peaks 3–8, 10, 12, and 16 were identified as α-pinene, β-myrcene, limonene, β-phellandrene, (*Z*)-β-ocimene, (*E*)-β-ocimene, (*E*)-β-farnesene, β-selinene, and germacrene-A, respectively.

TABLE 3. VOLATILE COMPONENTS IN OSMETERIAL SECRETIONS OF 5TH INSTAR *Papilio* LARVAE

Peak code in Figure 3	Component	Composition (%)				
		P. machaon	*P. memnon*	*P. helenus*	*P. bianor*	*P. maackii*
a	Methyl isobutyrate	62.3	39.4	31.8	42.3	34.6
b	Ethyl isobutyrate	0.2	1.0		2.5	0.6
c	Methyl 2-methylbutyrate	9.1	8.1	6.0	3.4	4.2
d	Ethyl 2-methylbutyrate	0.2	0.3		0.9	0.3
e	Acetic acid	<0.1			0.6	
f	Isobutyric acid	20.4	35.5	39.6	32.2	43.1
g	β-Caryophyllene		0.2			
h	2-Methylbutyric acid	7.7	15.0	22.6	16.6	16.8
i	Isovaleric acid				1.5	0.5
j	(*E*)-β-Farnesene		0.2			
k	Caryophyllene oxide		0.3			

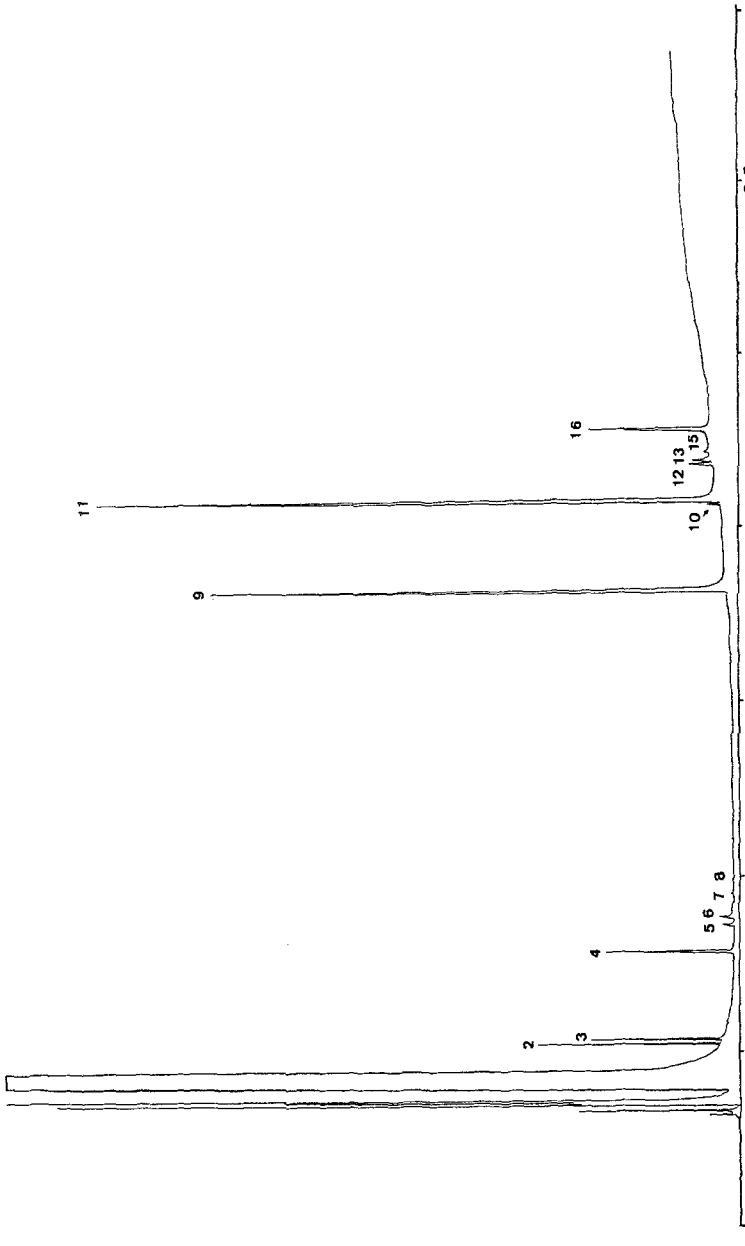


FIG. 4. Gas chromatogram of the osmeterial secretion of 4th instar *Papilio xuthus* larvae on a FFAP glass capillary column; 50–180° C at 5° C/min (peak 1 being superimposed upon the solvent).

Peak 13, exhibiting M^+ at m/z 204 (18%) and fragment ions at m/z 189 (69%), 175 (22), 161 (21), 147 (24), 133 (57), 121 (28), 107 (64), 93 (83), 81 (65), 67 (43), 55 (64), and 41 (100), was assumed to be a sesquiterpene hydrocarbon closely related to β-selinene, taking into account the likeness between their mass spectra and GC retention times. Upon comparison with a mass spectrum reported by Moshonas and Lund (1970), peak 13 was tentatively assigned to α-selinene.

The chemical structure of peak 15, a sesquiterpene hydrocarbon giving M^+ at m/z 204 and the base peak at m/z 41, was not elucidated.

5th Instar Larva. The secretion was found to be composed simply of four components by GC-MS analyses both on columns A and B. They were concluded to be methyl isobutyrate, methyl 2-methylbutyrate, isobutyric acid, and 2-methylbutyric acid, and no ethyl esters of these acids occurred in the secretion of this species. The results on *P. xuthus* are given in Table 4, and the chemical structures of all the compounds identified are collectively shown in Figure 5.

TABLE 4. VOLATILE COMPONENTS IN LARVAL OSMETERIAL SECRETIONS OF *Papilio xuthus*

Peak No. in Figure 4	Component	Composition (%) 4th instar	Composition (%) 5th instar
1	Methyl isobutyrate	15.3	12.7
2	Methyl 2-methylbutyrate	5.0	3.9
3	α-Pinene	3.5	
4	β-Myrcene	4.5	
5	Limonene	0.4	
6	β-Phellandrene	0.5	
7	(*Z*)-β-Ocimene	<0.1	
8	(*E*)-β-Ocimene	<0.1	
9	Isobutyric acid	34.7	48.7
10	(*E*)-β-Farnesene	0.6	
11	2-Methylbutyric acid	28.0	34.7
12	β-Selinene	1.1	
13	α-Selinene[a]	1.0[b]	
14	Unidentified		
15	$C_{15}H_{24}$	0.2	
16	Germacrene-A	5.1	

[a]Tentative identification.
[b]Combined percentage of components 13 and 14.

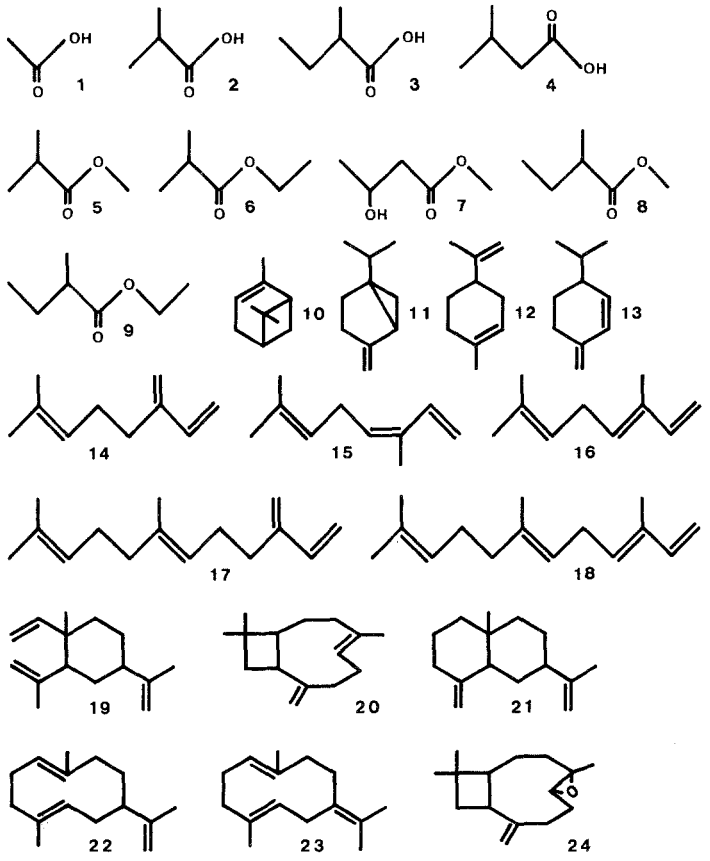


FIG. 5. Chemical structures of the compounds identified from the osmeterial secretions of *Papilio* larvae. (The number given to each compound corresponds to that of Table 1).

DISCUSSION

In all the *Papilio* species examined, the osmeterial secretions displayed remarkable qualitative alteration at the fourth (last) larval ecdysis, which is consistent with results previously reported for *Papilio protenor* (Honda, 1980a).

The secretions of 4th (penultimate) instar larvae of most species were composed principally of both mono- and sesquiterpenoids (hydrocarbons being dominant). *P. helenus* larvae, however, were devoid of monoterpenes, and the presence of relatively larger quantities of (*E*)-β-farnesene and acetic acid seemed to characterize the secretion of this species. The relative content

of monoterpene hydrocarbons was small also in *P. memnon*, whereas the amounts of methyl 3-hydroxy-*n*-butyrate and β-caryophyllene and its oxide were larger in this species. In contrast, a few monoterpene hydrocarbons such as α-pinene and β-myrcene were abundant in the secretions of *P. bianor* and *P. maackii*. The overall patterns of their chemical constitutions, although showing some variations between them, were substantially similar. This probably suggests a close phylogenetic relationship between these two species. The secretion of *P. machaon* consisted of only a few major and many minor components. β-Phellandrene and germacrene-A, present in comparatively larger amounts, can be regarded as characterizing this species. On the other hand, the secretion of *P. xuthus* is quite peculiar and seems to be of an exceptional type. In addition to a variety of mono- and sesquiterpenes, it contained considerable quantities of isobutyric acid, 2-methylbutyric acid, and their methyl esters, which have never been encountered in any of the secretions of the penultimate larval instar of other *Papilio* species examined. The occurrence of these aliphatic acids and their esters in young papilionid larvae has hitherto been reported for two *Graphium* species whose osmeterial secretions do not vary in quality at the fourth (last) larval ecdysis (Honda, 1980b). Moreover, the conspicuous disparity found in the secretion components of two comparatively affinitive swallowtails. *P. xuthus* and *P. machaon* is of great interest.

The osmeterial secretions of 5th instar larvae were all of analogous composition and fundamentally comprised isobutyric and 2-methylbutyric acids and their methyl and ethyl esters, although ethyl esters were not present in detectable amounts in the secretions of *P. helenus* and *P. xuthus*. It is also interesting that isovaleric acid, an isomer of 2-methylbutyric acid, was detected from *P. bianor* and *P. maackii*.

To summarize these results, it is to be noted that the secretion of the penultimate larval instar manifests more definite specific features than that of the last larval instar. Therefore, earlier instars would be more valuable for chemotaxonomic and phylogenetic considerations of the Papilionidae.

Of various constituents of osmeterial secretion, acetic acid, isovaleric acid, (*E*)-β-farnesene, and (*E,E*)-α-farnesene have not so far been known to occur in any of the larval osmeterial secretions of the Papilionidae, although Crossley and Waterhouse (1969) have already surmised the existence of isovaleric acid in the osmeterial secretion of *Papilio demoleus sthenelus*. β-Selinene is known to be one of components of the osmeterial secretion of *Battus polydamas* (Eisner et al., 1971). In Tables 2–4, identified components other than those described above have been found from the secretion of *Papilio protenor* or *Luehdorfia puziloi inexpecta* (Honda, 1980a,b). (With respect to the compound tentatively identified as germacrene-A in *P. protenor*, the present study ascertained the identity.)

Sufficient information is not yet available on the ecological function(s)

of these exocrine substances, except isobutyric and 2-methylbutyric acids which have already been demonstrated to serve as defensive allomones against ants (Eisner and Meinwald, 1965). However, several monoterpene hydrocarbons, such as α-pinene, β-myrcene, limonene, and β-phellandrene, are known to be present in the cephalic defensive secretions of certain termites (Moore, 1964, 1968), and the defensive effectiveness of α-pinene as an irritant or a repellent discharged by a sawfly, *Neodiprion sertifer*, and a termite, *Nasutitermes exitiosus*, has been proved by Eisner et al. (1974, 1976). α-Farnesene is a constituent of Dufour's gland exudate of the ant, *Aphaenogaster longiceps* (Cavill et al., 1967). It is of interest that some aphids secrete from their cornicles (*E*)-β-farnesene which acts as an alarm pheromone (Bowers et al., 1972; Edwards et al., 1973; Wientjens et al., 1973) and that germacrene-A is utilized by another aphid, *Therioaphis maculata* as an alarm substance (Bowers et al., 1977; Nishino et al., 1977). More recently, Pickett and Griffiths (1980) have reported the presence of (*E,E*)-α-farnesene in cornicle droplets from *Myzus persicae* and α-pinene from *Megoura viciae*, and they have substantiated that α-pinene exhibits alarm activity for the latter species. Acetic and isovaleric acids, which are also contained in the pygidial gland secretion of several carabid beetles, are regarded as having a defensive role in these insects (e.g., Moore and Wallbank, 1968; Kanehisa dn Murase, 1977). Further investigations are required on the effectiveness of these secretion components, especially terpenic compounds, in defense against predators such as wasps, ants, spiders, birds, and so on that are probably important in regulation of population density.

Although nothing is known about the origin of components of osmeterial secretions in the species examined, coexistence of aliphatic acids and terpenoids in a single glandular exudate of 4th larval instar of *P. xuthus* may propound an interesting problem on the biosynthetic mechanism and enzymatic system involved in the production of these compounds, because aliphatic carboxylic acids and terpenoids are generally known to originate from different precursors and biosynthetic pathways. For instance, in *Papilio aegeus* isobutyric acid has been reported to arise from L-valine, and 2-methylbutyric acid, from isoleucine, probably by oxidative deamination yielding α-ketoacids and subsequent decarboxylation (Seligman and Doy, 1973). Citral and citronellal in the ant, *Acanthomyops claviger*, are derived from acetate or mevalonate presumably via isopentenyl pyrophosphate, successive condensation of which can eventually give a large variety of terpenoids (Happ and Meinwald, 1965). It is noteworthy that every *Papilio* species examined secretes germacrene-A which is proposed as a possible biogenetic progenitor of many mono- and bicyclic sesquiterpenes (Kulkarni et al., 1964; Morikawa and Hirose, 1969).

All *Papilio* larvae investigated as well as *P. protenor* (Honda, 1980a) without exception convert their body coloration from dark brown and white

(the penultimate and preceding instars) to green (last instar) at the final larval ecdysis, which concurs with the qualitative change of osmeterial secretion mentioned above. The 5th instar (last) larva, provided with eyespots, resembles a small snake, whereas the younger larvae look like amorphous bird droppings. It seems essential to take this mimetic transformation in conjunction with the chemical alteration of osmeterial secretion for a thorough explication of the defensive system in *Papilio* larvae.

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POLYPHENOLS IN BROWN ALGAE *Fucus vesiculosus* AND *Ascophyllum nodosum*: Chemical Defenses Against the Marine Herbivorous Snail, *Littorina littorea*

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Abstract—Polyphenols from two brown algae, *Fucus vesiculosus* (L.) and *Ascophyllum nodosum* (L.) Le Jolis, inhibited feeding by the herbivorous snail, *Littorina littorea*. The active compounds were characterized as phloroglucinol polymers with a wide molecular weight range (mol wt <30,000 to >300,000) by spectroscopic, ultrafiltration, thin-layer chromatographic, and chemical degradation data. As little as 1% (dry wt) polyphenol in food reduced feeding by more than 50%, and polyphenolic extracts inhibited feeding entirely when present in concentrations of 2–5% (dry wt). Commercially available phloroglucinol dihydrate and gallotannin, which are known herbivore feeding deterrents in terrestrial plants, inhibited *L. littorea* feeding when added to food media in concentrations similar to those above. We conclude that polyphenols in *F. vesiculosus* and *A. nodosum* are functionally similar to terrestrial plant polyphenols (tannins) in providing chemical defenses against herbivores. This research is the first demonstration that chemical compounds defend these two dominant, perennial marine algae from the major herbivore found in their community.

Key Words—Chemical defense, feeding deterrents, polyphenols, phloroglucinol polymers, *Fucus*, *Ascophyllum*, *Littorina*.

INTRODUCTION

Fucus vesiculosus (Linnaeus) and *Ascophyllum nodosum* (Linnaeus) Le Jolis are perennial brown algae which dominate the high and middle rocky

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intertidal zones of New England. The predominant herbivore on macroscopic algae in this intertidal community is the periwinkle snail, *Littorina littorea*. Although in abundant supply, neither of these fucoid algae is utilized for food by this herbivore. The only exceptions to this observation appear to be certain instances of littorine grazing on germling stages or on mature fucoids only in winter when all other food species are unavailable (Menge, 1975). Lubchenco (1978) hypothesized that physical or chemical changes occur as fucoids grow and that these changes render *F. vesiculosus* and *A. nodosum* less attractive as food for *L. littorea*.

Our studies began by testing the hypothesis that some algae are not eaten because of their toughness. A feeding preference bioassay was devised where algal homogenates were solidified with agar–seawater and presented to *L. littorea* (Geiselman, 1978, 1980). Snails consumed media prepared with normally preferred algal species such as *Ulva lactuca*, *Enteromorpha intestinalis*, *Porphyra umbilicalis*, and *Ceramium rubrum* and did not eat agar controls (without algal homogenates added). Thus, agar itself was not an attractant. Media prepared with *F. vesiculosus*, *A. nodosum*, and *Chondrus crispus* (normally nonpreferred algal species) were avoided. These results indicated that factors other than toughness or size act as a feeding deterrent and suggested that a chemical factor was involved.

Chemical investigation of the brown algae *F. vesiculosus* and *A. nodosum* began by comparing the effect of compounds extracted from each algal species with methanol and methylene chloride on grazing by littorines. Methanol extracts of *F. vesiculosus* and *A. nodosum* inhibited feeding by *L. littorea* when added to preferred algal homogenates in agar.

The purpose of this investigation was to isolate and identify the compounds in these extracts which act as chemical defenses and to determine their effective concentrations (ED_{50} = dose required to inhibit feeding by 50%) (see Russell et al., 1978) against *L. littorea*.

METHODS AND RESULTS

Algal Collection, Extraction, and *Bioassay. F. vesiculosus* and *A. nodosum* were collected along the coast of Vineyard Sound, Massachusetts. After epiphytes and animals were removed from their surfaces, the algae were extracted either fresh or after drying. Experiments with methanol extracts from dried fucoids showed similar levels of antiherbivore activity as extracts from fresh plants. Hence, analyses were initially carried out on dried plants and, as a precaution against structural changes on drying, were thereafter performed on fresh plants.

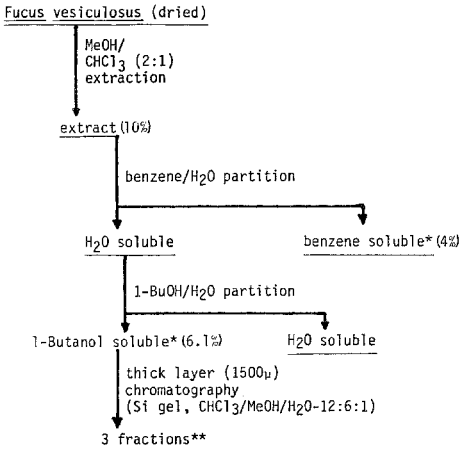
Food media for bioassays were prepared by homogenizing *Ulva* and *Enteromorpha* spp. (1:1 wet wt) in seawater with a Brinkman Polytron,

adding agar (1.8–2.0% w/v), and heating the mixture to 80--90°C to dissolve the agar. The extracted compounds were added to this mixture in a small volume of ethanol–water (depending on solubility), and the media were then poured into Petri plates (11 cm diam) to solidify. An equal volume of ethanol–water was added to the control food media. Extracts were incorporated into the food media in concentrations equivalent to those in the fresh plant unless otherwise noted (e.g., in dose-response experiments). The experimental and control media were offered in pairs in divided Petri plates placed in running seawater (18°C). Rather than being confined to individual plates in screened containers (Geiselman, 1978), the snails were allowed to roam the tank, feeding freely on the plates and media. This change in procedure was initiated because snails tended to climb the walls of the containers when enclosed, causing time delays in the feeding experiments. A grid was used to determine the area (%) of experimental and control media consumed. Results from each experimental medium were expressed as % of the control medium consumed.

Activity of Crude Extracts of Fucus vesiculosus *and* Ascophyllum nodosum. In earlier experiments (Geiselman, 1978, 1980), methanol extracts from fresh *F. vesiculosus* and *A. nodosum* were partitioned between methylene chloride and methanol–water (3:1), and each fraction was bioassayed with *L. littorea*. Compounds active against the herbivores were concentrated in the methanol–water fractions. Thin-layer chromatography (TLC) of these active fractions on silica gel and polyamide revealed spots which absorbed in the UV and which stained reddish brown with Lindt reagent (vanillin HCl). This suggested that the active fractions contain phenolic or tannic compounds, perhaps similar to those identified in brown algae by Glombitza (1977) and Ragan and Craigie (1976) or to those found in terrestrial plants which are proven effective antiherbivore compounds (Feeny, 1970; Dement and Mooney, 1974).

To further investigate the chemical nature of the active compounds, large quantities of dried *F. vesiculosus* (1515 g dry wt) were extracted according to the scheme depicted in Figure 1. Bioassay of the benzene and *n*-butanol partitions indicated that the *n*-butanol partition totally inhibited feeding by *L. littorea* whereas the benzene partition only partially inhibited feeding (Figure 1). The *n*-butanol fraction contained characteristic [^{1}H]-NMR absorptions at δ5.9–6.2 for polyphenols, and showed a positive reaction with vanillin.

The 1-butanol partition of *F. vesiculosus* was next separated into three fractions by preparative thick layer (1500 μm) silica gel chromatography ($CHCl_3$–MeOH–H_2O, 12:6:1). The most polar section (R_f = 0.0–0.3) contained most of the mass of the extract (65%), exhibited the absorptions at δ5.9–6.2 in the [^{1}H]-NMR, and showed a positive reaction with vanillin. The less polar (top, middle) layers reacted much less strongly with vanillin. The bioassay of the three fractions showed that feeding deterrent activity was isolated in the most polar fraction (Figure 1).



	Addition to media	% media consumed (relative to control) mean ± S.E., n = 3
*I.	Benzene partition	23.3 ± 8.8
	1-Butanol partition	0 ± 0
**II.	Fraction 1 (R_f 0.6-0.8)	100
	Fraction 2 (R_f 0.3-0.6)	100
	Fraction 3 (R_f 0.0-0.3)	0

FIG. 1. First extraction scheme for *Fucus vesiculosus* (dried) and results from bioassays.

Activity of Polyphenolic Fractions from F. vesiculosus *and* A. nodosum. The results above showed that feeding deterrent activity against *L. littorea* was isolated in polar fractions of 1-butanol partitions of *F. vesiculosus* MeOH-$CHCl_3$ extracts. Because thick-layer chromatographic separations did not readily yield large enough quantities for several bioassays, the 1-butanol partition was separated into three polyphenolic fractions by trituration and gel and adsorption column chromatography.

Low-molecular-weight polyphenols (Fraction 1) were isolated for bioassay from the 1-butanol partition by column chromatography of the EtOAC-acetone (1:1) soluble material on silica gel (Davison, 60–200 mesh) using a gradient of 2-propanol in EtOAC or acetone in hexane. [R_f = 0.5–0.55: silica gel 60 F_{254} using MeOH–$CHCl_3$ (1:1)]. This mixture comprised 0.3% of the 1-butanol partition.

Several low-molecular-weight phloroglucinal polymers were identified in this mixture (Figure 2) by comparing spectral data with published values (Glombitza et al., 1975; Glombitza, 1977; Ragan and Craigie, 1976; Ragan, 1978). Spectral data of the mixture included IR absorptions (THF soln) at

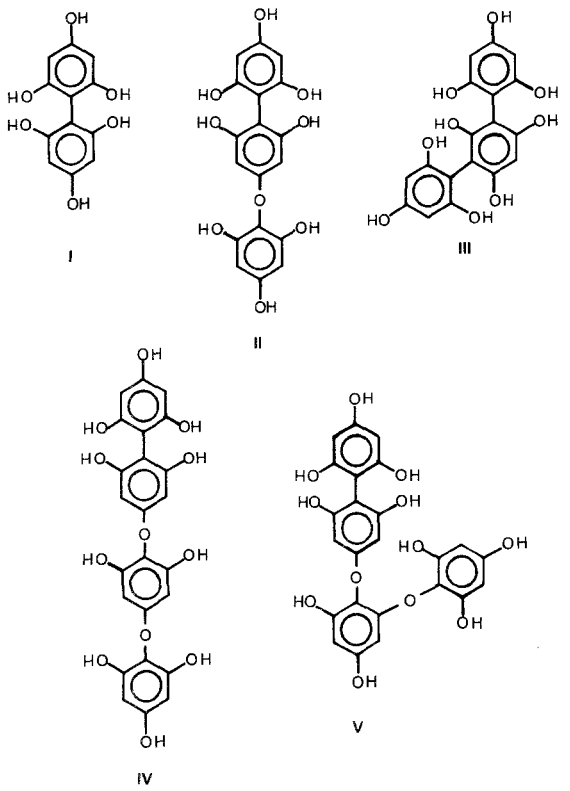


FIG. 2. Low-molecular-weight phloroglucinol polymers from *F. vesiculosus* (dry) isolated and bioassayed.

3495 and 3205 cm^{-1}, aromatic [^{1}H]NMR absorptions (60 MHz, acetone-d_6, TMS = 0) at δ 5.95–6.1 (apparent singlets) and phenolic hydroxyl [^{1}H]NMR absorptions at δ 6.88–8.20 (D_2O exchangeable). The UV absorption spectrum gave λ_{max} (MeOH) – 267 nm ($E^{1\%}_{1cm}$ 96), 375 nm (16), changing the λ_{max} (MeOH + NaOH) – 266 nm (298), 357 nm (34) on addition of 1% NaOH. From GC-MS analysis of the trimethylsilyl derivatives (Ragan, 1978), molecular ions or fragment ions of greatest mass of the major components were 682 ± 0.1, 932 ± 3, 998 ± 5, and 1110 ± 5, suggesting the presence of 2,2′,4,4′6,6′ -biphenylhexol (**I**) [MW(TMS) = 682]; 2,2′,4,6,6′ -pentahydroxy-4′-(2,4,6-trihydroxyphenoxy)biphenyl (**II**) [MW(TMS) = 950 ($-CH_3\cdot$ = 935)]; 1,5-di-(2,4,6-trihydroxyphenyl)-2,4,6-trihydroxybenzene (**III**) {MW(TMS) = 1022[$-CH_3\cdot$ = 1007, $-2(CH_3\cdot)$ = 992]}; and either 2,2′,4,6,6′ -pentahydroxy-4′ -[4-(2,4,6-trihydroxyphenoxy)-2,6-dihydroxyphenoxy]biphenyl (**IV**), or 2,2′,4,6,6′ -pentahydroxy-4′ -[2-(2,4,6-trihydroxyphenoxy)-4,6-dihydroxyphenoxy]biphenyl (**V**) [MW(TMS) = 1218, – (TMSOH + $CH_3\cdot$) = 1113] (Figure 2). The relative abundance of **I**, **II**, **III**,

and either **IV** or **V** in the mixture was 38% 7%, 45%, and 7%, respectively. Acetylation of the mixture (AC_2O-pyridine, room temperature, 1.5 hr) yielded products with [^{1}H]NMR absorptions (270 MHz, acetone-d_6, TMS = 0) at δ1.58, 1.95–2.10, 2.27, 6.7, and 7.01 which correlate well with published values of the assigned structures (Glombitza et al., 1975; Glombitza, 1977).

High molecular weight polyphenols were isolated for bioassay from the 1-butanol partition by passing the EtOAC–acetone (1:1)-insoluble, but MeOH-soluble and vanillin reactive material through BioBeads SX12 [MeOH–THF–benzene (1:10:10)]. Material that eluted with the void volume was triturated into acetone-soluble (Fraction 2) and acetone-insoluble, MeOH-soluble material (Fraction 3). Attempts to resolve the triturated fractions into components using silica gel, alumina, paper (cellulose), and Sephadex gel chromatography were unsuccessful. Poor recoveries resulted when Sephadex LH20 in MeOH was employed (see also Ragan and Craigie, 1976).

These fractions yielded spectral data characteristic of phenolic compounds.

Fraction 2 (*Acetone Soluble*). IR (Nujol): 3400 cm^{-1} (broad), IR (THF soln): 3488 and 3278 cm^{-1} (strong, broad); [^{1}H]NMR (60 MHz, acetone-d_6, TMS = 0): δ6.02, 6.12, 6.34 (relative ratio 4:2:1), and 6.83 and 7.13 (D_2O exchange); [^{13}C]NMR (50 MHz, acetone-d_6, TMS = 0): δ95.4–97.7 (relative intensity 2–10), 123.4–129.9 (1–2), 149.8–158.9 (1–4); UV-visible: λ_{max} (MeOH)-266 ($E^{1\%}_{1cm}$ 112), 372(9), 405(10), 439 sh(5), 494 sh(2), 665 nm(3); 1% NaOH, 272(201), 358(21), 395 sh(19), 495 sh(3), 665 nm(3).

Fraction 3 (*MeOH soluble*). IR (Nujol): 3350 cm^{-1} (very br.); [^{1}H]NMR (60 MHz, D_2O, DSS = 0); δ5.95, 6.08, 6.25 (4:3:2 H); [^{13}C]NMR (50 MHz, MeOH-d_4-D_2O, DSS = 0); δ98.3–105.5 (relative intensity 3–8), 126.6–130.5 (2), 152.6–159.3 (2–10); UV-visible: λ_{max} (MeOH) 281 ($E^{1\%}_{1cm}$ 64), 401(3), 448 sh(2), 664 nm (0.5); 1% NaOH – 275 (105), 404 sh(6), 448 sh(3), 510 sh(1), 664 nm (1).

From dose–response experiments (Figure 3), Fraction 3 was most active, reducing feeding by 50% (ED_{50}) at a concentraton of 0.2 mg/g media (=0.2% dry wt). Fractions 1 and 2 reduced feeding by 50% at the higher concentrations of 4.0–4.6 mg/g media (4.0–4.6% dry wt). Since much less fraction 1 was present in *F. vesiculosus* extracts than the acetone and methanol fractions (ratio of weights, fraction 1:fractions 2 + 3 = 1:13), the compounds in fractions 2 and 3 accounted for most of the chemical resistance of *F. vesiculosus* to *L. littorea*.

To determine whether higher-molecular-weight polyphenols were more active than lower-molecular-weight compounds, ultrafiltration of the triturated fractions was next employed. Triturated acetone and MeOH fractions for ultrafiltration were obtained from a new batch of dried *F. vesiculosus* (642 g) by benzene extraction followed by MeOH extraction

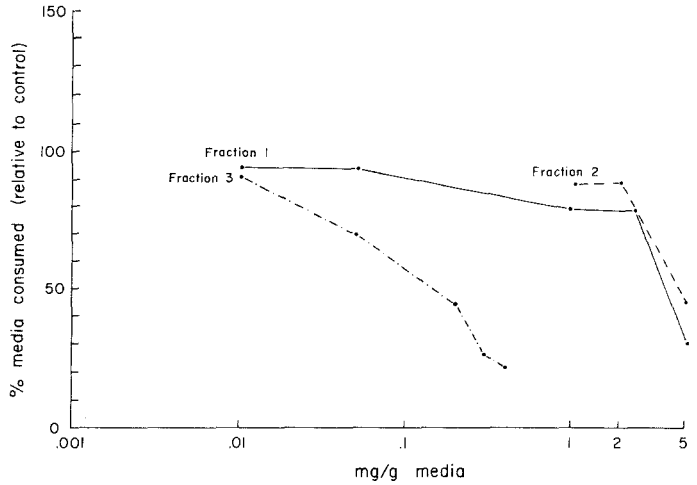


FIG. 3. Dose-response curve for the effect of fractions 1, 2, and 3 from *Fucus vesiculosus* on feeding by *L. littorea*: % media consumed = (experimental media consumed/control media consumed) × 100% ($N = 1$).

which yielded 12.4 g (1.9% dry wt) and 24.1 g (3.8% dry wt) of extract, respectively, and finally sequential trituration of the MeOH residue with EtOAc, acetone, and MeOH which yielded 12 g (1.9%), 3.6 g (0.6%), and 8.4 g (1.3%), respectively. A 400-ml Amicon stirred cell with 76-mm membranes was used for all molecular-weight separations. Before separation, the membranes were soaked in distilled water for at least 1 hr and then washed in the cell with approximately 150–250 ml water under pressure. Approximately 100 ml of deionized and twice-distilled water were used per 100 mg of sample. Each filtrate was subsequently passed through a new membrane. The high molecular weight or retained fractions were combined. Water was removed by freeze-drying. Adsorption of the polyphenols on the Amicon membranes was significant but acceptable (Table 1).

Fractions 2 (acetone) and 3 (MeOH) were separated for bioassay into those with molecular weights more than or less than 100,000 using an XM100A Amicon membrane. Recovery was 98% and 85%, respectively (Table 1). Fraction 2 had a higher percentage of molecular weight components over 100,000 (58%) than did fraction 3 (38%). All four molecular weight fractions were then bioassayed (Table 2). Each reduced feeding (compared to control) by more than 50%. The most active fraction was the acetone soluble material with mol wt $>$ 100,000.

Using similar extraction schemes and molecular-weight fractionations (Figure 4, Table 3), polyphenols were next isolated from fresh *F. vesiculosus* and *A. nodosum* and then bioassayed. Freshly collected *F. vesiculosus* (1595 g) and *A. nodosum* (1405 g) were each homogenized and extracted with 7

TABLE 1. MOLECULAR WEIGHT DATA FROM ULTRAFILTRATION OF DRIED *F. vesiculosus*

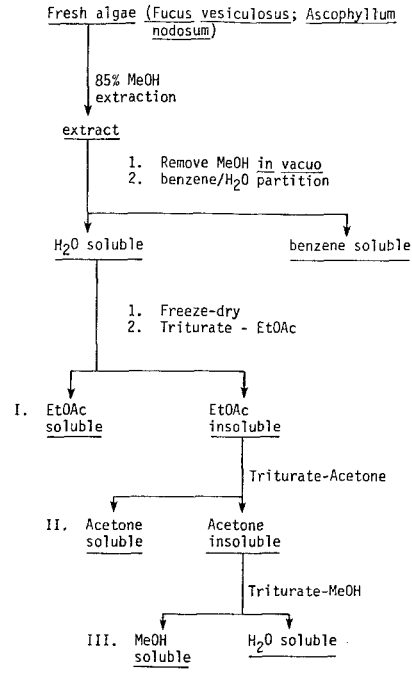
	Molecular weight range	% of fraction
Fraction 2, acetone-soluble	>300,000	27
	100,000–300,000	31
	50,000–100,000	13
	<50,000	29
Fraction 3, MeOH-soluble	>100,000	38
	50,000–100,000	7
	<50,000	55

	% Recovery-Avg. (range)	
Membrane (mol wt cut-off)	Acetone soluble	MeOH soluble
XM 300 (300,000)	75	
XM 100 (100,000)	96 (86–100)	85
XM 50 (50,000)	61	76
YM 30 (30,000		93 (85–100)

liters 85% aqueous methanol. Solvent and algal grounds were placed in flasks, flushed with nitrogen, stoppered, stored at 10°C in the dark, and swirled periodically. After two days, the solvent was filtered, and the methanol removed in vacuo and recycled. The algae were extracted two additional times under the same conditions, but for 1-day periods. The residual aqueous layer (1–1.2 liters) from the first extraction was shaken twice with 0.5 liter benzene and the benzene layers combined. Water was removed from the aqueous phase by freeze-drying. The residue was successively triturated with 1 liter EtOAc, 1 liter acetone, and 1 liter MeOH. The second and third extractions

TABLE 2. BIOASSAY OF *F. vesiculosus* (DRIED) ACETONE-SOLUBLE AND METHANOL-SOLUBLE FRACTIONS WITH MW CUT-OFFS AT 100,000

Addition to media (1.2% dry wt)	% media consumed (relative to control) (mean ± SE, $N = 2$)
Fraction 2, acetone-soluble	
<100,000	7.5 ± 0
>100,000	2 ± 0
Fraction 3, methanol-soluble	
<100,000	31 ± 7
>100,000	25 ± 7



I. Phloroglucinol + Low MW phloroglucinol polymers

II. High MW phloroglucinol polymers

III. High MW phloroglucinol polymers

FIG. 4. Extraction scheme for fresh *Fucus vesiculosus* and *Ascophyllum nodosum*.

were handled similarly. The triturated fractions were then combined and ultrafiltered. Ultrafiltration data are given in Table 3 and the bottom of Table 1. NMR data for the *F. vesiculosus* and *A. nodosum* trituration and molecular-weight fractions are given in Tables 4 and 5 and are similar to data of fractions obtained from dried algae (see above). The [^{13}C]NMR values in Tables 4 and 5 compare well to calculated values (Table 6). IR data of fractions from both algae are identical.

Aliquots of the MeOH-soluble fractions from *F. vesiculosus* and *A. nodosum* were oxidized with alkaline $KMnO_4$ (see Ragan and Craigie, 1976, for procedure). A 1% (w/w) yield of phloroglucinol was obtained from each reaction and its TMS derivative was analyzed by FID-GC (retention time = 9 min, or 6 ft 10% SE-30, 100–280°C, 1 min initial delay, 10°C/min, He flow = 45 ml/min).

Bioassay results of trituration and molecular-weight fractions from *F. vesiculosus* and *A. nodosum* were similar to those of dried algal fractions. All

TABLE 3. MOLECULAR WEIGHT DATA FROM ULTRAFILTRATION OF TRITURATED POLYPHENOL FRACTIONS FROM *F. vesiculosus* (FRESH) AND *A. nodosum* (FRESH)

	Molecular weight	Avg. %	(range, %)
Fucus vesiculosus:			
Combined EtOAc,[a] acetone,	>100,000	19	(17–21)
and MeOH fractions	30,000–100,000	41	(38–44)
	<30,000	40	(36–44)
Fraction II			
Acetone soluble	>100,000	29	(23–33)
(0.5% wet wt)	<100,000	71	(67–76)
Fraction III			
MeOH soluble	>30,000	55	(48–65)
(0.9% wet wt)	<30,000	45	(35–52)
A. nodosum			
Fraction II			
Acetone soluble	>100,000	32	(32–33)
(0.5% wet wt)	<100,000	68	(67–68)
Fraction III			
MeOH soluble	>30,000	35	—
(1.2% wet wt)	<30,000	65	—

[a]EtOAc soluble material from *F. vesiculosus* and *A. nodosum* comprised 0.2% and 0.6% wet weight of algae, respectively.

triturated fractions from both algae caused feeding reduction of more than 50% relative to control at 5.0% concentration; the acetone and MeOH fractions were active at lower concentrations of 0.1% and 1.0% (Table 7). The more active acetone and MeOH fractions from *F. vesiculosus* and *A. nodosum*, which were divided into molecular-weight ranges by ultrafiltration (Figure 4 and Table 3), were then bioassayed at concentrations of 0.1%, 1.0% and 2.0%. All molecular-weight ranges of acetone and MeOH fractions from both algal species reduced feeding by more than 50% relative to control at 1.0% and 2.0% (dry wt media) (Table 8). At the lower concentration of 0.1%, both molecular-weight ranges (less than, greater than 100,000) of the *F. vesiculosus* acetone fraction were more active than methanol-derived molecular-weight fractions. The *A. nodosum* acetone fraction with mol wt > 100,000 was more active than that with mol wt < 100,000 and both molecular-weight fractions of the methanol fraction. *F. vesiculosus* EtOAc, acetone, and MeOH partitions were also combined, divided by ultrafiltration into molecular-weight ranges, and bioassayed to determine if molecular weight alone determined the activity of compounds. All fractions inhibited feeding at 1.0% and 2.0% without relation to molecular weight; at 0.1% fractions with mol wt < 100,000 appeared more active than those with mol wt > 100,000.

TABLE 4. NMR DATA OF TRITURATION AND MOLECULAR WEIGHT FRACTIONS CONTAINING POLYPHENOLS FROM *F. vesiculosus*

Triturated fraction and molecular weight range	^1H δ (relative No. H)[a]	^{13}C δ (relative intensity)[a]
Combined solvent partitions (EtOAc, Acetone, MeOH)[b]	5.91–6.08 (2–10), 6.16–6.4 (2–3), 3.59–3.82 (3–7)	
Combined: mol wt > 100,000[b,c]	5.91–6.07 (4–10), 6.15 (4), 6.32–6.34 (4)	98.9–102.3 + 104, 106 [max at 98.6 (7), 98.7 (8), 99.2 (10)] 127.7–128.0 + 131 (1–2), 151.8–163.3 [max at 153.0 (5), 159.9 (7)]
Combined: mol wt < 100,000[b]	Same as above except decrease in relative No. H, at δ5.94 and 5.99	
I. EtOAc soluble[d]	5.83 (1), 6.00–6.03 (10), 6.13 (7), 6.33 (6)	
II. a. Acetone soluble[d,f]	5.80 (2), 5.97–6.0 (10), 6.07–6.1 (6), 6.67 (4)	
b. Acetone: mol wt > 100,000[b,c]	Same as combined solvent partitions with mol wt > 100,000	97.0–103.8 + 105 [max at 98.9 (10), 99.1 (9), 99.3 (9)], 127.3–128.1 + 131 (1–2), 151.9–163.4[max at 153.1 (7), 160.0 (7)]
III. a. MeOH soluble[d,e]	5.85 (2), 5.97–6.02 (10) 6.13–6.2 (8), 6.3 (5)	
b. MeOH: mol wt > 30,000[b,c]	5.91–6.07 (4–10) 6.15–6.2 (4), 6.33–6.37 (3–4)	96.7–102.2 [max at 98.6 (9), 99.0 (10)], 126.9–127.4 (1–2), 152.9–163.2 [max 152.9 (5) 159.9 (7)]
c. MeOH: mol wt < 30,000[b]	6.05 (10), 6.18 (4)	

[a] Scale: 0–10.
[b] 400-MHz ^1H spectrum, MeOH-d_4, TMS = 0.
[c] 100-MHz ^{13}C spectrum, D_2O, DSS = 0.
[d] 60-MHz spectrum acetone-d_6, TMS = 0.
[e] D_2O added.
[f] MeOH-d_4 added.

Because phloroglucinol is present in *F. vesiculosus* both by itself (Glombitza, 1977; Ragan and Craigie, 1976) and as a constitutent of the high-molecular-weight polyphenols in *F. vesiculosus* and *A. nodosum* (Ragan and Craigie, 1976; present study), this compound was bioassayed to see if the polymer precursor inhibits feeding by snails. Phloroglucinol is a constituent of brown algal physodes (Ragan and Craigie, 1976; Glombitza, 1977) and is also found in terrestrial plants. Gallotannin (Pfaltz and Bauer, $C_{76}H_{52}O_{46}$,

TABLE 5. NMR DATA OF TRITURATION AND MOLECULAR WEIGHT FRACTIONS CONTAINING POLYPHENOLS FROM *A. nodosum*

Triturated fraction and molecular weight range	^1H δ (relative No. H)[a]	^{13}C δ (Relative intensity)[a]
I. EtOAc soluble[b]	5.88 (3), 5.98–6.02 (10), 6.17 (7), 6.32 (5), 7.87–8.03 (D_2O exchange)	
II. a. Acetone soluble[b,c]	5.87 (4), 5.98 (10), 6.13 (7) 6.3 (4), 7.97–8.2 (D_2O exchange)	
b. Acetone: mol wt > 100,000[d,e]	Same as combined solvent partitions in *F. vesiculosus* with mol wt > 100,000	97.8–103.9 + 107 [max at 98.6 (7), 98.9 (10)], 127.3–128.0 (2–3), 153.1–161.0 [max at 153.2 (7), 157.4 (3), 159.9 (3)]
III. a. MeOH soluble[b,f]	5.93 (9), 6.10 (10), 6.23 (6), 6.33 (5)	
b. MeOH: mol wt > 30,000[d,e]	5.91–5.94 (6–10), 6.03–6.08 (5–6), 6.15–6.18 (5–6) 6.35 (3), 3.35–4.05 (1–5)	Same as IIb above except for presence of upfield absorptions at δ54–86

[a] Scale: 0–10.
[b] 60-MHz spectrum acetone-d_6, TMS = 0.
[c] MeOH-d_4 added.
[d] 400-MHz ^1H spectrum, MeOH-d_4, TMS = 0.
[e] 100-MHz ^{13}C spectrum, D_2O, DDS = 0.
[f] D_2O added.

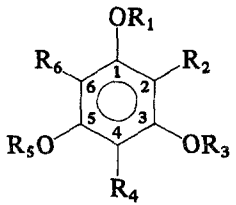
mol wt 1700), a higher-molecular-weight polyphenol common in terrestrial plants with demonstrated antiherbivore activity against insects (Todd et al., 1971), was also bioassayed. Results are summarized in the dose–response curves of Figure 5. Phloroglucinol inhibited snail feeding by at least 50% in concentrations of 0.2–0.5% fresh wt of media (2–5% dry wt). Gallotannin inhibited snail feeding by at least 50% at concentrations as low as 0.001% fresh wt (0.01% dry wt). The effective concentration (ED_{50}) of phloroglucinol is similar to that of *F. vesiculosus* polyphenolic fractions (Figure 3). Gallotannin apparently inhibits feeding more than phloroglucinol and *F. vesiculosus* polyphenolic fractions.

DISCUSSION

Stahl (1888) long ago suggested that plants produce compounds that deter animal feeding and showed that terrestrial snails ate certain leaves only after tannic substances were removed by alcoholic extraction. Hunger (1902)

TABLE 6. CALCULATED ^{13}C NMR VALUES OF PHLOROGLUCINOL AND DERIVATIVES[a]

(Range of values in interest: δ95–107, 123–131, and 149–164)



	Compound						Chemical shift[b]					
	R_1	R_2	R_3	R_4	R_5	R_6	C-1	C-2	C-3	C-4	C-5	C-6
1	H	H	H	H	H	H	158.2	95.8	158.2	95.8	158.2	95.8
2	H	Ar	H	H	H	H	157.1	108.9	157.1	96.2	157.0	96.2
3	H	Ar	H	Ar	H	H	155.9	109.3	156.0	109.2	155.9	96.6
4	H	Ar	H	Ar	H	Ar	154.8	109.7	154.7	109.2	154.8	109.7
5**	H	OR	H (R = H, φ)	H	H	H	145.5–149.2	122.7–125.1	145.5–149.2	97.2–97.8	150.9–153.2	97.2–97.8
6	H	OR	H (R = H, φ)	OR	H	H	138.2–144.2	124.1–126.8	132.8–140.2	124.1–126.8	138.2–144.2	98.6–99.8
7	H	OR	H (R = H, φ)	OR	H	OR	125.5–135.2	125.5–129.8	125.5–135.2	125.5–129.8	125.5–135.2	125.5–129.8

[a] See F.W. Wehrli and T. Wirthlin (1978) and Levy and Nelson (1972) for reference.
[b] Calculated values do not include steric effects = upfield shifts. (Compare calculated values above with observed values in Glombitza, 1977.)
[c] Changing Hs to Rs in 5–7 results in, at most, a 1% change in the range of values.

TABLE 7. CONSUMPTION BY *L. littorea* OF MEDIA TREATED WITH VARIOUS SOLVENT PARTITIONS OF *F. vesiculosus* AND *A. nodosum* (MEAN ± SE; $N = 2$)

Triturated fraction	Media consumed (% relative to control)		
	0.1[a]	1.0[a]	5.0[a,b]
F. vesiculosus			
I. EtOAc	50	55	45
II. Acetone	17 ± 6	16 ± 5	13
III. MeOH	12 ± 1	12 ± 0	0
A. nodosum			
I. EtOAc	59 ± 13	41 ± 3	40
II. Acetone	33 ± 3	38 ± 5	13
III. MeOH	20 ± 6	20 ± 8	10

[a]Concentration of partition in media (%).
[b]$N = 1$ for all bioassays of this highest concentration because of limited amounts of material obtained from extraction scheme.

TABLE 8. CONSUMPTION BY *L. littorea* OF MEDIA TREATED WITH VARIOUS MOLECULAR WEIGHT FRACTIONS OF *F. vesiculosus* AND *A. nodosum* SOLVENT PARTITIONS (MEAN ± SE, $N = 2$)

Molecular weight fractions	Media consumed (% relative to control)		
	0.1[a]	1.0[a]	2.0[a,b]
F. vesiculosus			
II. Acetone			
Mol wt < 100,000	7 ± 2	8 ± 7	
Mol wt > 100,000	7 ± 1	4 ± 1	
III. MeOH			
Mol wt < 30,000	69 ± 0	30 ± 0	0
Mol wt > 30,000	75 ± 0	14 ± 5	9
EtOAc (I), Acetone (II), and MeOH (III) partitions combined			
Mol wt < 30,000	27 ± 5	34 ± 9	3
Mol wt < 100,000	28 ± 8	32 ± 7	11
30,000 < mol wt < 100,000	73 ± 13	20 ± 8	15
Mol wt > 100,000	66 ± 13	14 ± 10	6
A. nodosum			
II. Acetone			
Mol wt < 100,000	28 ± 8	15 ± 5	
Mol wt > 100,000	8 ± 1	3 ± 1	
III. MeOH			
Mol wt < 30,000	23 ± 10	33 ± 8	17
Mol wt > 30,000	60 ± 7	38 ± 8	33

[a]Concentration of mol wt fractions in media (%).
[b]$N = 1$ for all bioassays of this highest concentration because of limited amounts of material obtained from extraction/molecular wt. separation.

suggested that some marine algae contain compounds that protect the plants against herbivores. He found that the sea hare *Aplysia* would eat the brown alga *Dictyota dichotoma* only after alcoholic extraction. Since these early studies, most investigations of plant chemical defenses against herbivores have been directed in terrestrial environments. However, evidence has accumulated that physodes and their phenols in brown algae are important antifouling, antialgal, and antibacterial agents (Conover and Sieburth, 1964; Sieburth and Conover, 1965; McLachlan and Craigie, 1966; Al-Ogily and Knight-Jones, 1977). Our investigations provide evidence that two dominant, perennial brown algae, *F. vesiculosus* and *A. nodosum*, contain compounds that inhibit feeding by the major herbivore, *Littorina littorea*, found in their community.

As shown by [^{1}H]- and [^{13}C]NMR, UV, IR, and TLC data, the compounds in *F. vesiculosus* and *A. nodosum* that inhibited feeding by snails are clearly polymeric phenols (polyphenols). The most diagnostic structural information came from [^{13}C]NMR absorption values which occur in the ranges δ95–107, 123–131, and 149–164. This information compared well to observed values of monomers, dimers, trimers, tetramers, etc., of phloroglucinol identified in brown algae by Glombitza and coworkers (1973, 1975, 1977) and to calculated values (Table 6), thus indicating the presence of phloroglucinol polymers with carbon–carbon and ether linkages. Calculated values of all carbons of phloroglucinol, and phenyl-, diphenyl-, triphenyl-, trihydroxy-, and triphenoxyphloroglucinol lie in or very close to these ranges (Wehrli and Wirthlin, 1978; Levy and Nelson, 1972). Most carbon values for hydroxy-, phenoxy-, dihydroxy-, and diphenoxyphloroglucinol lie outside these ranges, e.g., δ131–148. Even if upfield shifts observed in acetylated low-molecular-weight polyphenols (Glombitza, 1977), due to steric effects, are considered, calculated values for the latter compounds do not fall in the observed ranges. Because phloroglucinol itself is liberated from oxidation of the high-molecular-weight material, and because low-molecular-weight compounds consist of phloroglucinol units, the heterogeneous high-molecular-weight material most likely contains phloroglucinol units. Phloroglucinol polymers in the less polar acetone triturated fractions presumably contain a lower ratio of hydroxyl to ether oxygen per polymer than components in the MeOH fraction, but the [^{13}C]NMR data do not confirm this, and component overlap assuredly exists between triturated fractions.

Ragan and Craigie (1976) isolated a series of polyphenols with molecular weights as high as 650,000 from *F. vesiculosus*. Nondialyzable high-molecular-weight polyphenols, which constitute 15–25% of the total extractable polyphenols (Ragan et al., 1979), consist of approximately 45% carbon and 49% oxygen and contain only traces of amino acids and no low-molecular-weight carbohydrates (Ragan and Craigie, 1976). Because the

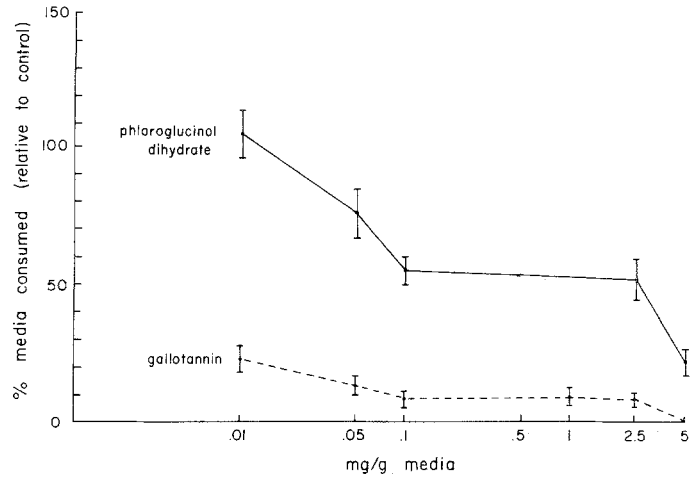


FIG. 5. Dose–response curve for the effect of phloroglucinol dihydrate and gallotannin on feeding by *L. littorea*: % media consumed = (experimental media consumed/control media consumed) × 100% (mean ± SE, $N = 4$).

linkages are chemically stable, Ragan (1976) hypothesized that in vivo polymerization could be irreversible, giving rise to physodes increasingly rich in polymeric phenols ("aging" of physodes).

Our bioassays of polyphenols from *F. vesiculosus* and *A. nodosum* showed that a series of polymers with a wide range of molecular weights and polarity inhibit feeding by snails as does phloroglucinol. The presence of these compounds in concentrations as low as 1% dry weight of media reduced feeding by more than 50% (polyphenol contents in *F. vesiculosus* and *A. nodosum* are in excess of 1% dry weight in apices, receptacles, fronds, and holdfasts throughout the year) (see Geiselman, 1980, 1981). Therefore chemical protection of these algae from herbivores is afforded by a heterogeneous mixture of polymeric phenols rather than by a single compound. The same is true for many terrestrial plants (Rhoades and Cates, 1976). Other similarities between polyphenols in brown algae and those in terrestrial plants include: (1) the general structure and activity of the compounds, including the effective concentrations against herbivores; (2) the sequestering of compounds in special vacuoles to prevent autotoxicity to the plant (Ragan, 1976); and (3) the seasonal and within-plant variations in tissue polyphenol contents (Fritsch, 1945; Levin, 1976; Feeny, 1976; Geiselman, 1980). It has been suggested for terrestrial plants that attacks by herbivores and pathogens stimulate a defense mechanism involving the release of and immediate oxidation and/or polymerization of phenols and polyphenols (Miles, 1969). These compounds then bind to amino acids, proteins, carbohydrates,

vitamins, and other plant nutrients (e.g., iron), making them unavailable to herbivore digestive processes (Van Sumere et al., 1975; De Alarcon et al., 1979). In addition, polyphenols complex with the herbivores' digestive enzymes, inactivating them, and with the herbivores' salivary proteins, causing astringent, bitter sensations which are presumably unpleasant and repellent. Previous studies have shown that polyphenols from *F. vesiculosus* are capable of denaturing proteins and inactivating enzymes (Esping, 1957a,b). We conclude that polyphenols in *F. vesiculosis* and *A. nodosum* are functionally similar to terrestrial plant polyphenols in their roles as chemical defenses against herbivores. Our findings from marine plants provide support for the "plant apparency" and antiherbivore chemistry theory (Feeny, 1976; Rhoades and Cates, 1976), derived entirely from terrestrial systems, which suggests that (1) dominant, perennial plants are defended primarily by chemicals that reduce their digestibility to herbivores, and (2) that sympatric perennial dominants can be expected to have converged on chemically similar defensive systems.

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EFFECT OF MATING ON TERMINATING AGGREGATION DURING HOST COLONIZATION IN THE BARK BEETLE, *Ips paraconfusus*

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Abstract—Attraction of male *Ips paraconfusus* to male-infested ponderosa pine logs was inhibited by volatiles from logs infested with mated males and females over an 8-day period in the field. The response of females during this time was not significantly inhibited by these volatiles. Synthesis of the male-specific pheromones, ipsenol and ipsdienol, appeared negligible after 8 days in males allowed to mate with 3 females in these logs while males alone contained levels of these pheromones at about half their maximum rate. The decline in pheromone production in mated males appears to result, at least in part, from a reduction in the activity of the biosynthetic system that converts the host monoterpene, myrcene, to ipsenol and ipsdienol. Mating and feeding have apparently no effect on the biosynthetic system that converts the host monoterpene, (−)-α-pinene, to the pheromone, *cis*-verbenol in either males or females. The reduced production and release of pheromones by males after mating appears to play a major function in the process of terminating the aggregation phase of host colonization.

Key Words—*Ips paraconfusus*, Coleoptera, Scolytidae, *Pinus ponderosa*, pheromone biosynthesis, bark beetle, myrcene, α-pinene, ipsenol, ipsdienol, *cis*-verbenol, myrtenol, attractants, pheromones.

INTRODUCTION

The male *Ips paraconfusus* initiates an entrance tunnel and excavates a "nuptial" chamber primarily in the phloem layer of ponderosa pine. After host material has passed through his digestive tract, the fecal pellets become attractive (Wood and Bushing, 1963; Vité et al., 1963; Pitman et al., 1965; Wood et al., 1966). Male frass (fragments of host tissue and fecal pellets)

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contains 3 pheromones, ipsenol, ipsdienol, and *cis*-verbenol, which together are attractive to males and females (Silverstein et al., 1966a,b; Wood et al., 1968). Ipsenol and ipsdienol are synthesized from the host plant chemical, myrcene, in males only (Hughes, 1974; Byers et al., 1979; Hendry et al., 1980). *cis*-Verbenol is synthesized from another host plant chemical (−)-α-pinene, in both sexes (Renwick et al., 1976). These pheromones accumulate in the hindgut upon exposure to vapors of the host plant chemicals. However, only ipsenol and ipsdienol can be readily detected by GLC in hindguts of males feeding in the host (Vité et al., 1972; Byers, 1981a). Logs infested with males and exposed to attacking beetles cease to be attractive after 10–18 days in the field (Wood and Vité, 1961; Vité and Gara, 1962; Vité et al., 1963) and virgin male frass produced after about 18 days is not attractive in the laboratory (Wood and Bushing, 1963; Borden, 1967).

The male *I. paraconfusus* is polygamous, permitting entry of about three females into the nuptial chamber, after which others seeking entry are not accepted (Barr, 1969). The attractiveness of logs infested with males may decrease when females join the male. For instance, Borden (1967) found in the laboratory that females were less responsive to gut extracts of fed males mated with two or three females than to gut extracts of fed unmated males. In addition, the normal decline in attraction of *I. paraconfusus* to naturally colonized logs (Wood and Vité, 1961) was found to decrease more rapidly when females were allowed to join nuptial chambers (Wood, personal communication). This is analogous to the observation of Anderson (1948) that the attraction of *I. pini* to logs with attacking males ceased "immediately" when an "excess" of females were supplied.

The previous studies did not determine whether the decline in response to male frass or infested logs was due to a decrease in production and release of attractants or to the release of inhibitors or both. The possibility that mated females may produce inhibitors has not been investigated nor has their production of *cis*-verbenol during the colonization period. Furthermore, the effect of mating and/or feeding on the biosynthetic systems that produce the pheromones in males and in females has not been established. Thus, the objectives of this study were (1) to determine if the response of *I. paraconfusus* to male-infested logs in the field could be inhibited by volatiles from logs infested with mated males and females, (2) to quantify the pheromones produced in these mated males and females and in unmated males during the field attraction period, and (3) to compare the pheromone biosynthetic systems in mated feeding beetles of both sexes to emergent beetles.

METHODS AND MATERIALS

Test for Inhibition of the Response of I. paraconfusus *to Male-Infested Logs by Mated Males and Females.* A paired-trap test was performed in the

Sierra National Forest, California, (900–1100 m elevation) (August 25–September 7, 1978). The catch of *I. paraconfusus* on a sticky trap containing a log infested with 30 males and an uninfested log was compared to the catch on a sticky trap 11–12 m away containing a log infested with 30 males and a log infested with 30 males and 90 females that were allowed to mate. The trap consisted of two 6-mm mesh screen cylinders (19 cm diam × 30.5 cm high) coated with Stickem Special® (Bedard and Browne, 1969) placed side by side 1.2 m above the ground.

I. paraconfusus adults were reared from naturally infested logging debris obtained from the Sierra National Forest at about 1000 m elevation (Browne, 1972). Logs about 14 cm diam × 28 cm long were cut from a tree and stored less than one month at 4° C before use. Males were introduced head-first into holes drilled in logs. Each log was wrapped with aluminum window screen which, in designated logs, allowed introduced females free access to all male holes but prevented their escape from the log. The screen also prevented the possibility of attack by beetles attracted to the trap. The uninfested log was drilled and screened.

Twenty-four hours after introducing the males, females were added to the appropriate logs at 8 AM on the first trapping day and allowed to join the males in the nuptial chambers. The uninfested log and the male- and female-infested log were exchanged daily between traps of each pair while the male-infested logs remained in place. Three pairs of traps were tested initially but two pairs were removed after 4, 8, and 12 days to obtain beetles for GLC analysis. *I. paraconfusus* and *Enoclerus lecontei* (Coleoptera: Cleridae), a predator, were collected from the traps each day, cleaned of Stickem with mineral spirits, and their sex was determined in the laboratory. The paired trap catches of each day (August 26–September 2) were analyzed with the Wilcoxon test for combining data from several experiments or blocks (Lehmann, 1975) because the 20 trap pairs were not independent replicates. Binomial confidence limits for sex ratios of catch were determined (Byers and Wood, 1980).

Pheromones in Unmated Males and Mated Males and Females of I. paraconfusus *Feeding in Logs*. Mated males and females (as judged by brood larvae in 12-day-old logs) and unmated males were removed from under the bark of the logs used in the test for inhibition after 4, 8, and 12 days. The mid-and hindguts of two groups of 12–15 unmated males, two groups of 12–15 mated males, and two groups of 20–30 mated females were dissected out on each date and each group was extracted with 0.2 ml diethyl ether. Another log infested with 30 unmated males at the same time as the other logs, but not field tested, was dissected after 80 hr and two groups of 12–15 hindguts were extracted as above.

The amounts of pheromones present in these gut extracts were analyzed by GLC (3.6 m × 2 mm ID glass column of Ultrabond II on 100/120 mesh at

105° C and N_2 flow of 30 ml/min; 1.8 m × 2 mm ID glass column of 3 % Apiezon L on 100/120 Gas Chrom Q at 100° C and N_2 flow of 12 ml/min). For quantification, authentic ipsenol, ipsdienol (each >97%) and *cis*-verbenol (>95%) (all Chem. Samples Co.) and myrtenol (>99%, Aldrich) were compared to gut extracts for retention time and peak area. Linear regression and analysis of covariance (Snedecor and Cochran, 1967) were performed on the amounts of ipsenol and ipsdienol found in feeding males on days 4, 8, and 12 where linear relationships were indicated (Figure 1). The presence of host material in the gut was noted, and the number of males and females per nuptial chamber was recorded except after 12 days when galleries became intermixed. Confirmation of mating was noted in the 12-day male-female logs by the presence of brood larvae (1–2 stage instar).

To determine if plant terpenes or other factors in 12 to 14-day-old logs became limiting to pheromone biosynthesis, unmated males that had fed 12 days in logs in the field were dissected out and discarded. Newly emerged males then were introduced into adjacent unattacked areas of these logs for 3 days. These males were dissected out and four groups of 13 were extracted and analyzed by GLC as described above.

Biosynthesis of Pheromones from Host Plant Terpenes in Mated and Emergent I. paraconfusus. The abilities of mated and emergent males and females to synthesize ipsdienol, ipsenol, *cis*-verbenol, and myrtenol from host plant chemicals were compared (August 11–25, 1979). Thirty males were introduced into each of two logs, the logs screened, and 48 hr later each log

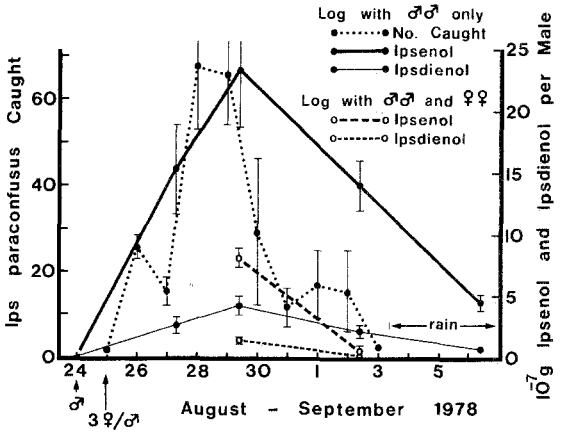

FIG. 1. Ipsenol and ipsdienol produced in mated and unmated male *I. paraconfusus* during feeding in logs and the average number of *I. paraconfusus* attracted to traps with logs infested with 30 unmated males in the Sierra National Forest. Brackets represent ± SE $\bar{X}$.

received 90 females as described above. The infested logs remained at 21 ± 1.7° C for a total of 13 days after male introduction under natural light conditions. The beetles then were removed from the logs and four groups of 9 males and four groups of 9 females that had presumably mated were exposed to vapors of myrcene and (−)-α-pinene, [$\alpha_D^{22} = -41.6°$ (both GLC purified >99.8%) in amber bottles for 18 ± 0.5 hr at 21 ± 1.7° C (Byers et al., 1979). Ten μl of each plant terpene was placed on glass filter paper inside each bottle and GLC analysis on the Apiezon L column showed that the headspace contained 1.7 ± 0.2 (±SE $\bar{X}$) × 10^{-6} g myrcene/ml and 4.2 ± 0.3 × 10^{-6} g (−)-α-pinene/ml. Mated males were not compared to unmated males that had fed for 13 days since these beetles contain even larger quantities of ipsenol and ipsdienol (Figure 1) than emergent beetles exposed to concentrations of precursor vapors similar to those tested above (Byers et al., 1979). Four groups of 9 unmated males and four groups of 9 unmated females that were refrigerated at 4° C during the 13-day period were exposed to terpene vapors as described above. Two additional control groups, 9 each of mated males and mated females from the logs, were placed in bottles for an equal time but not exposed to plant terpenes. All groups were extracted and analyzed by GLC for pheromones as described above. The *t*-tests were used to determine if differences in the quantity of ipsenol, ipsdienol, *cis*-verbenol, and myrtenol existed between mated and unmated beetles within each sex.

RESULTS

Test for Inhibition of the Response of I. paraconfusus *to Male-Infested Logs by Mated Males and Females*. The response of males, but not females, to a male-infested log was significantly lowered by the presence of a log infested with mated males and females (Table 1). However, the sex ratios of female to male catch on the paired traps were not significantly different, indicating that the inhibition of males was rather weak. The response of *E. lecontei* was not significantly affected by volatiles released from the logs infested with mated males and females (Table 1).

Pheromones in Unmated Males and Mated Males and Females of I. paraconfusus *Feeding in Logs*. Emergent males introduced into 12-day-old logs contained 19.7 ± 2.4 (±SE $\bar{X}$) × 10^{-7} g ipsenol/male and 3.7 ± 0.5 × 10^{-7} g ipsdienol/male 3 days after introduction. These quantities are similar to those produced by other unmated males in fresh logs after about 3 days (80 hr) (Figure 1). The increase in ipsenol and ipsdienol production in males feeding in logs was correlated with higher catches of *I. paraconfusus* (Figure 1). The catch may not have been proportional to the concentrations of pheromones in the guts because of variations in the daily abundance of responding beetles, differences in release of pheromones (defecation), and/or differences in wind

TABLE 1. CATCH OF *Ips paraconfusus* AND *Enoclerus lecontei* ON 20 PAIRED TRAPS BAITED WITH LOGS INFESTED WITH MALE AND WITH MALE AND FEMALE *I. paraconfusus* (AUGUST 26–SEPTEMBER 2, 1978) IN THE SIERRA NATIONAL FOREST

	Catch			
		I. paraconfusus		
Sticky traps containing	*E. lecontei*	♂	♀	Sex ratio (BCL)[a]
30 ♂ log plus blank log	231	176	492	2.79[b] (2.35–3.32)
30 ♂ log plus 30 ♂ + 90 log	291	115	405	3.52[b] (2.87–4.33)
P value of inhibition[c]	0.253	0.039	0.451	

[a]Upper and lower 95% binomial confidence limits for sex ratio (♀/♂).
[b]Sex ratios were not significantly different P = 0.092 (Chi-square).
[c]*P* values < 0.05 (two-sided) indicate a significant difference between the catch on the two treatments (Wilcoxon test).

and weather conditions (rained after September 3). *cis*-Verbenol was not detected in either males or females ($<0.5 \times 10^{-8}$ g/beetle) feeding in logs.

Regression analysis of depletion of pheromone quantities in guts of unmated males indicated that they would produce and release negligible levels of ipsenol and ipsdienol after 14 days of feeding, assuming that production continued in a linear decline (from regression, Table 2). On the other hand, regression analysis of pheromones in guts of mated males showed that ipsenol and ipsdienol production and release was negligible only 8.3 days after females joined their nuptial chambers (Table 2). The mated males did not contain detectable levels ($<0.5 \times 10^{-8}$ g/male) of any pheromone after 12 days of feeding. The quantities of ipsenol and ipsdienol produced by the mated males were significantly less than the unmated males during the period of observation as shown by the elevation difference of the respective regression lines (Table 2). All but one or two nuptial chambers were occupied by 1 male and 1–5 females with an average of 2.9 ± 0.2 and 2.8 ± 0.2 ($\pm$SE $\bar{X}$) females/male after 4 and 8 days, respectively. Mating was confirmed in the 12-day-old logs in which almost all females had produced larvae. There were no significant differences in the rate of decrease (slope) in production of ipsenol or ipsdienol between unmated males and males with females (Table 2). The ratio of ipsenol–ipsdienol production in unmated males, 6.3 ± 0.3 ($\pm$SE $\bar{X}$), was nearly identical to the ratio in mated males, 6.5 ± 0.5. These ratios are

TABLE 2. COMPARISON OF LINEAR REGRESSION LINES OF QUANTITIES OF IPSENOL AND IPSDIENOL ($Y \times 10^{-7}$ G/MALE) IN HINDGUTS OF MALE *Ips paraconfusus* FEEDING IN LOGS WITH OR WITHOUT FEMALES 4–12 DAYS (X) AFTER INTRODUCTION OF FEMALES[a]

	Equation	r^2	*P* value[b]	
			Slope difference	Elevation difference
Ipsenol— male only log	$Y = -2.35X + 32.7$	0.87	0.62	<0.001
Ipsenol— male with 3 females log	$Y = -1.92X + 15.9$	0.97		
Ipsdienol— male only log	$Y = -0.44X + 5.9$	0.86	0.48	<0.001
Ipsdienol— male with 3 females log	$Y = -0.32X + 2.6$	0.95		
Ipsenol— male only log	$Y = -2.35X + 32.7$	0.87	0.003	0.001
Ipsdienol— male only log	$Y = -0.44X + 5.9$	0.86		

[a]See Figure 1.
[b]A *P* value < 0.05 indicates a significant difference between slopes or elevations of regression lines of pheromones produced in feeding males (Analysis of Covariance).

similar to those reported in previous studies in which emergent males were exposed to myrcene vapors (5.9, Byers et al., 1979; 6.0, Byers and Wood, 1981). The significant difference in the decline in amounts of ipsenol compared to ipsdienol in unmated males (Table 2) is the result of the consistent ratio of synthesis of these pheromones at any precursor concentration, as shown by Byers et al. (1979).

Biosynthesis of Pheromones from Host Plant Terpenes in Mated and Emergent I. paraconfusus. Ipsenol and ipsdienol production was significantly less in fed, mated males than in unfed, unmated males when both were exposed to myrcene and (−)-α-pinene (Figure 2). The mated males and females that had fed for 13 and 11 days, respectively, did not contain detectable levels of any pheromones after 18 hr in a bottle without host terpenes. Unmated, emergent males that were refrigerated, instead of unmated, fed males, were compared to the mated, fed males because both groups do not contain detectable amounts of pheromone prior to terpene vapor exposure (Figure 1) (Byers et al., 1979). Mating and/or feeding appeared to have no effect on *cis*-verbenol and myrtenol production in either males or females (Figure 2).

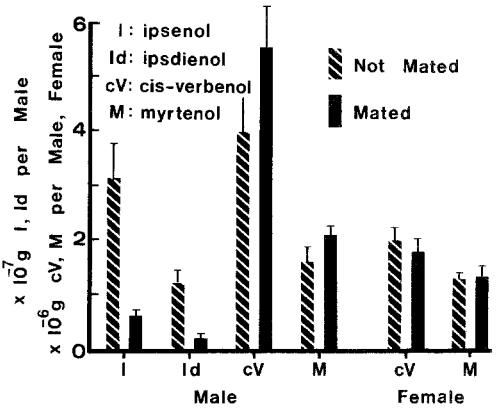

FIG. 2. Ipsenol, ipsdienol, *cis*-verbenol, and myrtenol produced in mated, fed males and in unmated, emergent males and females exposed to myrcene and (−)-α-pinene vapors (August 25, 1979). Brackets above the bars represent + SE $\bar{X}$. A t test showed that unmated males produced significantly more ipsenol ($P = 0.007$) and ipsdienol ($P = 0.005$) than mated males. There were no significant differences in the production of either *cis*-verbenol or myrtenol between mated and unmated males or between mated and unmated females ($P > 0.1$).

DISCUSSION

The decline in production of ipsenol and ipsdienol in both mated and unmated males (Figure 1) could be caused by one or more of the following mechanisms: (1) a reduction in the concentration of terpene precursors in aged logs, (2) a reduction in feeding, or (3) a change in the physiological condition of the beetle. The concentration of terpene precursors in 12- to 14-day-old logs may not be limiting pheromone production since newly emerged males feeding in these logs produced amounts of pheromone comparable to males in 3-day-old logs. However, the concentration of terpenes immediately surrounding nuptial chambers after 12–14 days might possibly have become limiting compared to adjacent areas because of volatilization and the action of microorganisms. A reduction in feeding by mated males compared to unmated males and a decline in feeding over time also might explain the decrease in production of pheromones, although all males and females, whether mated or unmated, had phloem in their hindguts. Wood et al. (1966) showed that the daily frass output of virgin males was about the same after 10–12 days as that after 2–3 days. However, the fecal pellet production may vary independently from frass output during the decline in attraction. Female defecation would make the quantification of fecal pellet production of mated males very difficult if not impossible.

The decrease in the amounts of pheromones in mated and unmated males feeding in logs could also result from a change in the physiological condition during feeding and after mating. The abilities of the biosynthetic systems in mated and virgin, emergent males to produce pheromones were compared by exposure to vapors of (−)-α-pinene and myrcene. This method circumvents possible feeding control over entry of these precursors into the intestine. Since mated males did not produce as much ipsenol and ipsdienol as unfed virgin males, a physiological change was indicated that could have been induced by effects of feeding and/or mating. However, physiological effects from feeding appear less likely since Byers and Wood (1981) found that male *I. paraconfusus* fed on a ground phloem and cellulose diet, low in plant terpenes, produced ipsenol, ipsdienol, and *cis*-verbenol in quantities not significantly different from that in unfed males when both groups were exposed to myrcene and α-pinene vapors. Hughes and Renwick (1977) reported that feeding on host tissue may even enhance the biosynthetic system, although this effect may be due, at least in part, to increased availability of precursors (Byers, 1981a). Therefore, the reduction of ipsenol and ipsdienol production observed in mated males compared to unmated males during feeding in logs might be explained, at least in part, by a reduction in the activity of the mated male's biosynthetic system since physiological effects from feeding would be expected to be similar. Possible effects of mating and feeding did not appear to significantly influence the conversion of (−)-α-pinene to *cis*-verbenol and myrtenol, so it may be that production of these compounds during host colonization does not decline after mating. However, *cis*-verbenol alone is not attractive to *I. paraconfusus* (Wood et al., 1968).

The bacterium, *Bacillus cereus*, isolated from hindguts of male and female *I. paraconfusus*, has been shown to synthesize *cis*-verbenol and myrtenol from α-pinene (Brand et al., 1975). In the present study, the quantities of *cis*-verbenol and myrtenol produced during terpene exposure were similar in beetles before and after feeding, indicating that *B. cereus* was not affected by feeding or it is not the major producer of *cis*-verbenol in the insect. Byers and Wood (1981) indicated the possibility of another symbiotic microorganism within the beetle since the antibiotic streptomycin, when fed to males, inhibited the production of ipsenol and ipsdienol during exposure to myrcene vapors, while the conversion of α-pinene to *cis*-verbenol did not appear to be inhibited. Thus, further information on the presence, location, and metabolism of the suspected microsymbionts is needed to explain the effects of mating and feeding on pheromone production.

Hughes and Renwick (1977) hypothesized that gut stretching due to feeding stimulates the release of juvenile hormone (JH) which was found to enhance ipsenol and ipsdienol synthesis during exposure of male *I. paraconfusus* to myrcene vapors. However, as noted earlier, Byers and Wood (1981) found that feeding on diets low in plant terpenes did not significantly

affect pheromone production, indicating that gut stretching/feeding may not be required for pheromone synthesis. If JH is involved in pheromone synthesis, then JH levels may decline in males after mating, since mated, fed males did not produce as much ipsenol and ipsdienol as unmated, fed males (Figure 1) and unmated, unfed males (Figure 2). The capacity of the biosynthetic systems to produce ipsenol and ipsdienol in unmated, fed and in unmated, unfed males may be difficult to determine because fed males contain appreciable amounts of pheromones while unmated, unfed males contain no pheromones prior to vapor exposure (Byers et al., 1979).

The reduction in male catch from logs infested with mated males and females suggests that inhibitors may be released that lower the attraction of males, but not females, to their pheromones. If an as yet undiscovered inhibitor is involved in terminating aggregation, then *I. paraconfusus* would need to release it for at least several days after mating to inhibit response to the still significant amounts of ipsenol and ipsdienol in mated males during this period (Figure 1). Therefore, the test for inhibition was performed during the first 8 days when inhibitors, if they exist, would be required. On the other hand, Byers (1981b) found that males, but not females, were inhibited by higher release rates of ipsenol, ipsdienol, and *cis*-verbenol in the laboratory and that male response became proportionately less than female's as the beetles approached a natural pheromone source in the field. Therefore, in the present study, it may be that the males were inhibited by a higher release rate of male pheromone from the trap with 60 males (30 mated and 30 unmated) compared to the other trap with 30 unmated males.

After the female bark beetle, *Dendroctonus pseudotsugae*, is joined by a male, she may release 3-methyl-2-cyclohexene-1-one (MCH) which inhibits response to her attractants (Rudinsky and Michael, 1972; Rudinsky, 1973). In contrast, Pitman and Vité (1974) found that males contain considerably more MCH than females, and they believe the male predominates in the release of the inhibitor upon joining the female in her gallery. The attraction elicited by female ambrosia beetles of *Trypodendron lineatum* is inhibited by volatiles released from the male (Nijholt, 1973). These "antiattractants" or inhibitors lower the attraction of beetles to their pheromones but new attacks would still continue indefinitely unless attractant production declined or other mechanisms that limit density of attack operated.

The decline in pheromone production after mating in *I. paraconfusus*, which appears to function as part of the mechanism for terminating the aggregation phase of host colonization, also occurs in the smaller European elm bark beetle, *Scolytus multistriatus* and the southern pine beetle, *D. frontalis*. Coster and Vité (1972) found that the pheromones, frontalin and *trans*-verbenol, in the hindgut of *D. frontalis* declined rapidly during the first 48 hr of feeding. Although statistical tests were not performed, mated females appeared to contain smaller amounts of these pheromones after 48 hr than

virgin females while attractive responses to these females were not significantly different. The attraction of *S. multistriatus* to female-infested elm logs declined after males were introduced (Peacock et al., 1971; Elliott et al., 1975). Elliott et al. (1975) provided evidence (but presented no statistics) that male- and female-infested elm logs did not appear to inhibit the attraction of *S. multistriatus* to female-infested logs. Gore et al. (1977) then showed that females ceased to produce (−)-4-methyl-3-heptanol, one of its pheromones, after mating. In *I. paraconfusus*, *D. frontalis*, and *S. multistriatus*, a decline in pheromone production and release after mating would not ensure the termination of mass attraction because later arriving beetles that had not mated yet would sustain the release of attractants. Therefore, additional mechanisms that regulate attack density such as territorial behavior or close-range inhibitors that would continue to be released after attractant production ceased are required to explain the entire process of termination of aggregation. These hypothetical mechanisms remain to be identified.

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ANTIFEEDANT ACTION OF *Z*-DIHYDROMATRICARIA ACID FROM SOLDIER BEETLES (*Chauliognathus* SPP.)[1]

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Abstract—The acetylenic acid, *Z*-dihydromatricaria acid (DHMA), previously isolated from the defensive secretion of *Chauliognathus lecontei*, and now shown to occur also in *C. pennsylvanicus*, is a potent feeding deterrent to jumping spiders (*Phidippus* spp.). A simple bioassay with *Phidippus* is described, which is generally applicable to studies dealing with the isolation and evaluation of feeding deterrency of natural products from insects. By use of this assay, *Phidippus* were shown to be sensitive to as little as 1 μg DHMA, an amount equivalent to less than 2% of the DHMA content of *C. pennsylvanicus*.

Key Words—Dihydromatricaria acid, antifeedant, acetylenic acid, *Chauliognathus pennsylvanicus*, Coleoptera, Cantharidae, soldier beetles, *Phidippus*, jumping spider, bioassay, defensive secretion, defensive behavior.

INTRODUCTION

An earlier paper of this series (Meinwald et al., 1968) dealt with the identification of an acetylenic acid, *Z*-dihydromatricaria acid (I; henceforth abbreviated DHMA), from the defensive secretion of a soldier beetle (Cantharidae: *Chauliognathus lecontei*). Although the beetle was shown to be

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unacceptable to a diversity of predators (ants, carabid beetles, blue jays, grasshoppers mice), it was not determined whether the unacceptability was attributable to DHMA itself, since the compound was not isolated in amounts sufficient for testing.

$$CH_3—CH=CH—C\equiv C—C\equiv C—CH_2—CH_2—COOH$$
I

Working with another species of *Chauliognathus* (*C. pennsylvanicus*), we have now reinvestigated the defensive role of DHMA and were able to show by use of a bioassay with jumping spiders (Salticidae: *Phidippus* spp.), that the acid does indeed have potent antifeedant activity. We here present the results of this assay, together with data on DHMA isolation from *C. pennsylvanicus*, and observations on the defensive behavior of the beetle.

METHODS AND MATERIALS

Chauliognathus pennsylvanicus is one of the commonest North American soldier beetles, ranging eastward of the Rocky Mountains and southward to Arizona. In the environs of Ithaca, New York, where our specimens were taken, the beetles sometimes aggregate by the hundreds in mid- to late summer on goldenrod (*Solidago* spp.).

Jumping spiders coexist ecologically with soldier beetles, and as nonspecific aggressive insectivores are a major potential threat to *Chauliognathus*. The specimens of the two species studied by us, *Phidippus audax* and *P. regius*, had been maintained in the laboratory for a period of at least several weeks prior to testing. They were kept individually in plastic Petri dishes and fed mostly houseflies. The *P. audax* were taken from the same field sites as the *Chauliognathus*.

Mass spectra were obtained using a Finnigan 3300 gas chromatograph–mass spectrometer coupled with a System Industries 150 computer. Nuclear magnetic resonance spectra were recorded at 90 MHz on a Varian EM 390 instrument. Chemical shifts (δ) are reported in ppm downfield from internal tetramethylsilane. The HPLC analyses were conducted with a Waters model 6000 solvent delivery system and a Waters model 440 ultraviolet detector. Gas chromatographic analyses were carried out using a Varian Aerograph 2100 instrument. Ultraviolet spectra were recorded in methanol solution on a Cary 14 spectrophotometer.

RESULTS

Glands and Defensive Behavior of C. pennsylvanicus. Dissection of freshly killed specimens of *C. pennsylvanicus* revealed that this beetle, like its congener *C. lecontei* (Figure 1), has nine pairs of defensive glands. Eight pairs

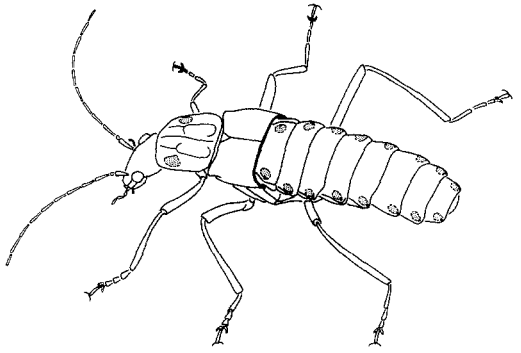

FIG. 1. *Chauliognathus lecontei*, with prothoracic and abdominal defense glands indicated by outlines. The wings are shown cut away near their bases. The drawing applies as well to *C. pennsylvanicus*.

are in the abdomen and consist of small pouches, enveloped by compressor muscles, opening near the posterolateral corners of the tergites. The ninth pair, seemingly devoid of surrounding muscles, has its openings in the pleural region beneath the pronotal shield.

The defensive behavior of the beetles was studied by subjecting individuals to various types of disturbance in the laboratory. Typically, when a beetle was picked up by hand or in forceps, it emitted droplets of secretion from the gland openings (Figure 2A). The droplets were usually milky white in appearance, but occasionally turbid brown or almost clear. Localized stimulation tended to evoke responses from only a few glands at a time. This occurred, for example, when forceps were used to pinch single legs or antennae or restricted regions of the abdomen. The glands first discharged under such circumstances were almost invariably those closest to the site stimulated. It was also noted that the beetle frequently brought its legs into play to transfer secretion from the gland openings to the offending forceps. When, for example, a beetle was held by its front end, it flexed its abdomen forward beneath the body, emitted droplets of secretion from its glands, and then, if held persistently, proceeded to brush its hindlegs alternatively against secretion on the flanks of its abdomen (Figure 2B) and against the forceps. The latter were often visibly wetted as a result. The midlegs also occasionally took part in the brushing activities.

Persistent disturbance commonly caused the beetles to regurgitate droplets of enteric fluid. As the animal struggled, the dark effluent seeped from the mouth onto legs and portions of the body, becoming mixed at times with secretion from the defensive glands. It was therefore occasionally this mixture, rather than pure glandular fluid, that was administered to the forceps by leg-dabbing.

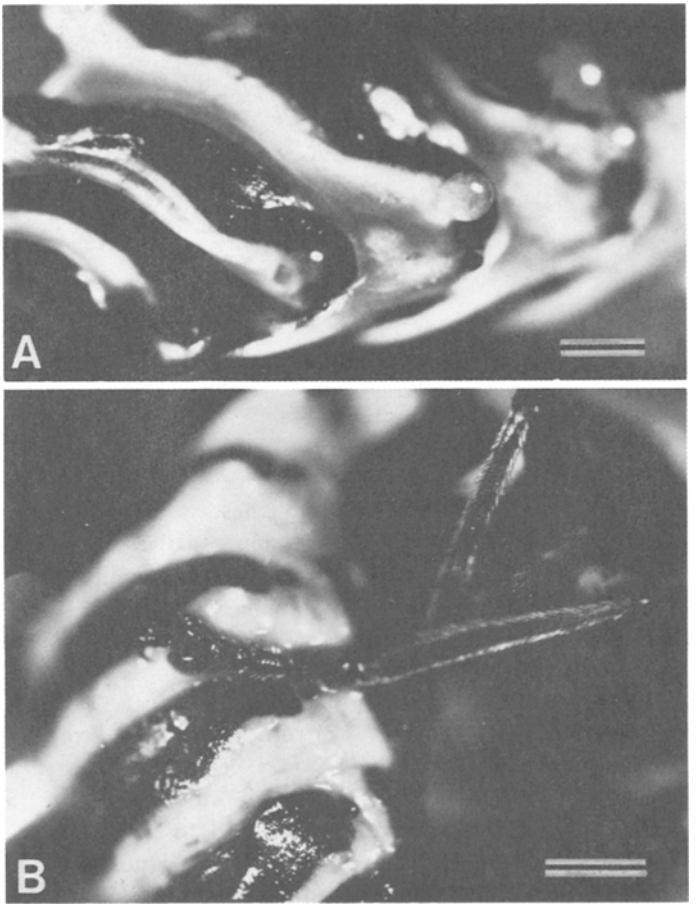

FIG. 2. (A) Close-up view of right flank of abdomen of *C. pennsylvanicus*, showing droplets of secretion emerging from defensive glands. (B) Comparable view, showing hindleg wiping against abdominal flank. Note side of abdomen wetted by smeared secretion. (Reference bars = 1 mm.)

Acceptability of C. pennsylvanicus *to Jumping Spiders.* Twenty individual *C. pennsylvanicus*, freshly collected in the field, were presented to single *Phidippus* in Petri dishes. The results are summarized in Figure 3.

Each of the eight *Chauliognathus* offered to *P. audax*, the species of jumping spider collected from the same sites as the beetles, was rejected outright, and so were 7 of 12 beetles offered to *P. regius*. The spiders pounced on the beetles, held them briefly (less than 5 sec) and then released them. Droplets of secretion were sometimes emitted by the beetles during this period. The beetles were uninjured in the encounters and were lively when examined the next day.

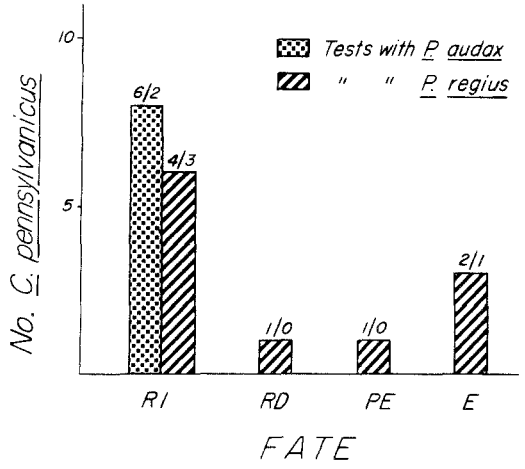

FIG. 3. Fate of individual *Chauliognathus pennsylvanicus* offered to jumping spiders (*Phidippus audax*, *P. regius*). RI = rejected immediately (<5 sec), uninjured; RD = rejected after delay (3 min), uninjured; PE = killed and partly eaten; E = killed and eaten. Male and female beetles in each column are given by the ratio (♀/♂) above the column.

One of the five additional beetles offered to *P. regius* was also released unharmed, but only after being held for 3 min. During this time the beetle emitted secretion, regurgitated, and dabbed fluid onto the spider with its hindlegs. The other four beetles were killed. One was seized by the head and, before dying minutes later, also noticeably engaged in leg-dabbing activities (Figure 4A). It was only partly eaten: the head and thorax were chewed up into small pieces and sucked dry (as spiders typically do with their prey), but the abdomen was left intact. The remaining three beetles were killed and eaten (secretory emission was noted in one; leg-dabbing in two). Their entire bodies were chewed up and only legs and wings remained intact.

Isolation of DHMA from C. pennsylvanicus. Extraction of 600 female *C. pennsylvanicus* with dichloromethane, followed by gel filtrations on Sephadex LH-20 (dichloromethane–methanol, 1:1, as eluent), gave 47.2 mg (79 μg/beetle) of a UV-active compound, proven homogeneous by silica gel TLC ($R_f = 0.3$; hexane–acetone, 2:1), by GC of its TMS derivative (3% OV-1), and by HPLC (μ-Porasil, hexane–chloroform–acetic acid, 225:75:1, detection at 280 nm). This compound was identified as *Z*-dihydromatricaria acid (DHMA) on the basis of its melting point, its ultraviolet absorption [λ_{max} (log) 233 (3.03), 240 (3.45) 254 (3.78), 267 (3.99), and 282 (3.90) nm], the chemical ionization mass spectrum of its TMS derivative [CH_4 reagent, $M+1^+$ at *m/e* 235 (17)], its infrared absorption (3500, 2904, 1745, 1710, and 1135 cm^{-1} in $CHCl_3$), and its 90 MHz [^1H] NMR spectrum in $CDCl_3$ [δ 1.85 (3, d), 2.75 (4, s), 5.50–6.00 (2, m)]. Comparable extraction of 250 male beetles gave 12.5 mg of DHMA (50 μg/beetle).

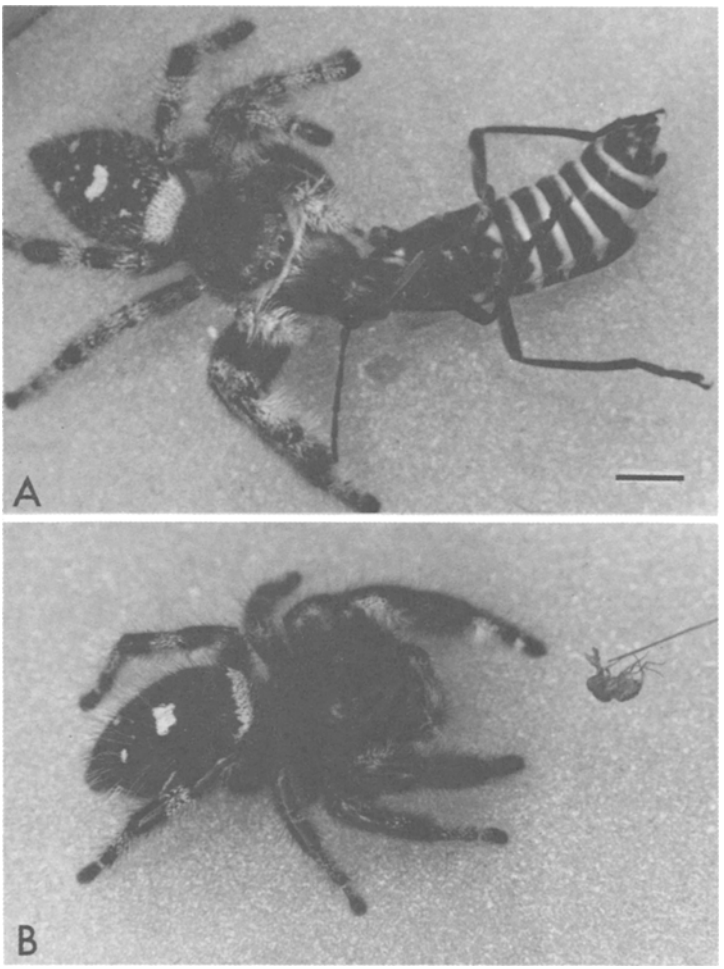

FIG. 4. (A) *Phidippus regius* holding *C. pennsylvanicus*. Note that beetle is wiping posterior margin of abdomen with left hindleg. This beetle was eventually killed but only partly eaten. (B) *P. regius* about to take bait (freshly killed *Drosophila*, treated by topical application of chemical) presented to it on tip of hair. (Reference bar in A = 2 mm.)

Separate examination of blood and secretion showed DHMA to be present in both fluids. A small sample of blood (1.54 mg) from two beetles (male and female), uncontaminated by secretion or oral effluent, was obtained by taking up in microcapillary tubes the droplets of fluid that oozed from the cut ends of antennae snipped off near the tips. The blood was extracted with dichloromethane, and the dichloromethane layer was extracted with 10% aqueous sodium bicarbonate. After washing the bicarbonate extract with

ether, it was acidified with hydrochloric acid and reextracted with ether. The ethereal extract was evaporated and taken up in methanol for ultraviolet assay. On the basis of intensity of absorption at 264 nm, the DHMA content of the blood sample was calculated (by comparison with absorption intensities of standardized DHMA solutions) to be 11 μg, or 7 μg/mg blood.

Secretion was "milked" from several hundred beetles (both sexes) by holding these individually in forceps and wiping away with small pieces of filter paper the droplets that emerged from the glands. Only papers that were clearly uncontaminated with the dark oral effluent were collected. Extraction of the papers, followed by ultraviolet assay, showed the extract to contain 14.3 μg/beetle or (since there are 18 glands) 0.8 μg/gland.

Antifeedant Action of DHMA. A special bioassay was developed to test for the feeding deterrency of chemical substances to jumping spiders. It was found that *Phidippus* can be readily enticed to take freshly killed fruit flies (*Drosophila melanogaster* killed by freezing) that are presented to them at close range, suspended from a human hair affixed to the end of an applicator stick (Figure 4B). Such flies are attacked, taken in the chelicers, chewed up, sucked dry, and reduced to a small mass of solid remains. Experimental items, consisting of fruit flies treated by topical addition of a given test substance in solution, are either rejected immediately (in less than 5 sec) after attack and physical contact by the spider, or rejected after some time (5 sec to usually no more than 3 min, during which period the item may or may not be partially eaten) or entirely eaten (reduced to a particulate mass in 6 or more minutes).

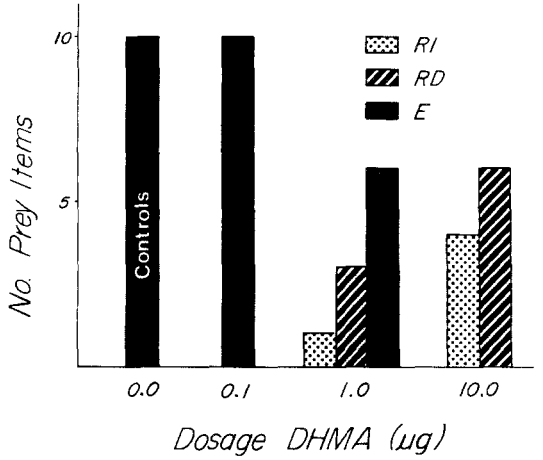

FIG. 5. Fate of individual food items (freshly killed *Drosophila*), pretreated by topical addition of DHMA at dosages indicated, and offered to jumping spiders *P. regius*). RI = rejected immediately (<5 sec); RD = rejected after delay (>5 sec); E = eaten. Results (eaten versus rejected) for either the control or the 0.1 μg group differed significanlty ($P < 0.0001$) from the effect of the 10.0-μg dosage.

Test substance is administered at a desired dosage of 1 μl of solvent directly onto the fruit fly with a microsyringe. A few minutes are allowed for evaporation of solvent before a fly is given to a spider. The fly is suspended by inserting the hair loosely between its folded legs; it thus detaches readily from the tether when seized by the spider. For control purposes flies are also offered that are treated by topical addition to 1 μl of solvent alone. We have used this bioassay for evaluation of antifeedant potency of a variety of chemical defensive agents from insects.

We tested DHMA at three dosages (0.1, 1.0 and 10 μg; dichloromethane as solvent), with 10 flies per dosage, and an additional 10 control flies treated with dichloromethane alone. A total of 29 *Phidippus regius* were used; in cases where spiders were used more than once, consecutive tests were spaced at least one day apart. It is clear from the results (Figure 5), that even at a dosage as low as 1.0 μg, DHMA proved deterrent in 4 out of 10 tests. A dosage of 10 μg proved absolutely deterrent, while 0.1 μg was inactive.

DISCUSSION

The defensive potential of DHMA appears established, certainly vis à vis jumping spiders. As little as 1 μg DHMA may be deterrent to a *Phidippus*, an amount equivalent to less than 2% of the total DHMA content of *Chauliognathus* and not much greater than the estimated average output (0.8 μg) of a single gland of the beetle. Whether DHMA is active also against other predators remains to be determined. We have preliminary evidence indicating that the compound is deterrent to some vertebrates (white-footed mice, *Peromyscus leucopus*) but not to others (Swainson's thrushes, *Hylocichla ustulata*).

In its defensive behavior, *C. pennsylvanicus* shares characteristics with other chemically protected arthropods. It is not unusual for these to have serially arranged glands and for the glands to discharge singly or in groups depending on the magnitude and degree of localization of an offense. Nor is it unusual for legs to be used in the spreading and administration of secretion or for the animal to disgorge enteric fluids when attacked. Leg-dabbing has been noted, for example, in tenebrionid beetles (Tschinkel, 1975; Eisner et al., 1974), *Hemiptera* (Remold, 1962) and opilionids (Eisner et al., 1971, 1977), while regurgitation under stress occurs in caterpillars, grasshoppers, and many other larval and adult insects (Eisner, 1970). Mixing enteric fluids with defensive secretion and administration of the mixture by leg-dabbing has been observed in certain opilionids (Eisner et al., 1971, 1977).

One wonders about the origin of DHMA in *Chauliognathus*. Is the compound made by endogenous synthesis or do the beetles obtain it from an exogenous source? The latter alternative is at least within the realm of possibility since a variety of acetylenic compounds (including the methyl ester

of DHMA) occur in fungi and higher plants (Sørensen, 1963). Among the many Compositae known to contain the compounds are species of *Solidago* (Gibbs, 1974), the genus that includes the goldenrods on which our beetles were most commonly found in large numbers. But whether portions of plants containing acetylenic compounds are actually ingested by *Chauliognathus* (and whether as a consequence their oral effluent also contains the substances) remains unknown.

Extraction of whole *Chauliognathus* yielded ca. 2–5 times more DHMA than the beetles seemed capable of ejecting from their glands. This in itself was indicative of extraglandular presence of DHMA, which was confirmed by the finding of DHMA in the blood of the beetles. One could easily envison such systemic DHMA having supplementary defensive potential. *Chauliognathus* do not bleed spontaneously (as do meloid and other beetles that "reflex bleed"), but they bleed when injured and can readily survive the loss of legs or antennae in the laboratory. Defensive use of blood could therefore be a reality for the beetles in nature. Interestingly, it is not unusual to find a *Chauliognathus* in the field, particularly later in the season, with a leg or portion of an antenna missing.

The fact that DHMA plays a protective role in soldier beetles offers at least some support to the notion that acetylenic compounds also serve for defense in the plants that contain these substances. However, experimental evidence to that effect is lacking.

The bioassay with *Phidippus* is potentially broadly applicable. The spiders are easily reared and maintained, are sensitive to a wide range of chemicals, and are reliable in performance. In the two species used by us, sensitivity varies little from individual to individual. We are currently using the assay in studies of a number of defensive chemicals from insects and have found it particularly useful in efforts to "home in" on active chemicals present in complex mixtures, as for example in whole insect extracts. For such purposes we simply fractionate the mixture by extraction with different solvents, test the individual fractions with *Phidippus*, and then proceed by subfractionation and testing of subfractions, until activity is found to be restricted to one or more pure compounds, which can then be identified. Given the sensitivity of the spiders and the fact that activity ratings of test samples can often be obtained on the basis of only 10 individual *Drosophila* offerings, very little material may be used in an assay. The testing procedure therefore lends itself to the study of samples which by virtue of rarity of source organism may be available in only small quantities.

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Journal of Chemical Ecology, Vol. 7, No. 6, 1981

Book Review

Review of Olfaction in Mammals. Edited by D.M. Stoddart, Academic Press. 363 pp. Illus. U.S. $63.50.

This volume represents the edited version of a conference held under the auspices of The Zoological Society of London in 1978. It is a valuable addition to the literature of mammalian olfaction. One major caution should be noted, however. According to the editor, the objectives of the symposium and this book were "to review the progress made in recent years in the main fields of mammalian olfaction and to produce a volume which represents a distillation of this progress" (p. ix). The title of the book "Olfaction in Mammals" mirrors this goal. However, instead of reviewing the total range of this topic, the volume itself is concerned mainly with mammalian chemical communication. Large areas of mammalian olfaction are not covered (e.g., structure, electrophysiology, and biochemistry of olfaction are discussed in 17 pages, and nothing is said on the central nervous system). Even for the more restricted area of communication, the book is not really a review and distillation of progress. For example, little is included on the chemistry of mammalian signals (except for the pig). Recent studies on the development of olfactory attachment in rodents are not considered in any detail and, with the exception of one paper, the role of the vomeronasal organ, an area of great contemporary interest, is hardly addressed. However, if this volume is approached for what it is, a set of chapters, many of which are of considerable interest, on topics in mammalian olfaction with great emphasis on communication, the reader will find rewarding, stimulating ideas.

Following an interesting, speculative introductory chapter by the editor, D.M. Stoddart, the next two chapters deal with some general topics of mammalian olfaction. A.J. MacLeod has the difficult task of discussing the chemistry of odors entirely from the human perspective; he emphasizes the relative lack of information on structure–activity relationships. G.H. Dodd and D.J. Squirrell deal with morphology, electrophysiology, and ideas about transduction mechanisms.

The remainder of the book is devoted to mammalian chemical communication. M.G. Adams provides a thorough and valuable review of odor-producing organs of mammals. Following this are two chapters dealing with carnivores. M.L. Gorman argues that territorial and nonterritorial species

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mark throughout their range while the former, in addition, mark intensively at borders. He emphasizes the importance of information on individual and group identity. In an unusually thorough and thoughtful chapter, D.W. Macdonald discusses the interaction between carnivorous species' ecology and their patterns of scent marking. D.J. Bell reviews and describes new studies on lagomorphs, mainly the European wild rabbit. Emphasized is the variety of sources and messages available to rabbits. In a chapter on territoriality in prosimians, A. Schilling presents some fascinating data on discrimination of individual identity as well as an attempt to integrate field observations with experimental results. One is frustrated here and in some subsequent chapters, however, by the lack of details of experimental methods. This problem is likely inherent in an edited volume which presents unpublished data. The chapter by L.M. Gosling on savannah ungulates continues an emphasis of the preceding four chapters in attempting to relate field observations to the functional analysis of chemosensory signals. Furthermore, this chapter makes a useful theoretical approach to the neglected area of interspecific communication.

Following this are three chapters which deal with external chemical messengers (presumably acting via chemosensory channels) and reproduction in small rodents, mainly mice. J.J. Cowley discusses the interaction of physical growth and reproductive maturation. S.R. Milligan then considers the endocrine basis for reproductive effects of chemical signals. The multifaceted, interactive nature of stimuli influencing maturation are ably described, and a central role for prolaction is proposed. The next chapter, by A. Marchlewska-Koj, while interesting, is somewhat out of place in this volume since it is mainly a very specific report of several experiments.

The last two chapters deal with important issues in mammalian chemical communication. W.D. Booth reviews studies on pigs with particular emphasis on the 16-androstene steroids. For a long time the specificity apparently evident in the chemistry and behaviors of this system provided the least ambiguous evidence among mammals for a direct chemical-behavioral pathway. Booth's review, however, emphasizes the complexity evident even here. Finally, E.B. Keverne's essay deals with the role of olfaction in the behavior of nonhuman primates. This chapter is not in any sense a review of progress in this area, and it will probably be difficult to follow and evaluate for those not familiar with the controversy in this area. Keverne argues that the role of olfaction may be subtle and difficult to uncover, and he presents portions of a study with talpoin monkeys which suggest such subtlety.

The steep price (U.S. $63.50) is regrettable.

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Erratum

FEEDING PREFERENCES OF SPRUCE BUDWORM (CHORISTONEURA FUMIFERANA CLEM.) LARVAE TO SOME HOST-PLANT CHEMICALS

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On page 392, Methods and Materials section, "penultimate (fifth) instar" should read "ultimate (sixth) instar" on line 4, and "fifth-instar" should read "sixth-instar" on line 5.

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