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PORAPAK-Q COLLECTION OF AIRBORNE ORGANIC COMPOUNDS SERVING AS MODELS FOR INSECT PHEROMONES

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Abstract—Organic compounds with properties resembling those of insect pheromones can be removed from an airstream by absorption on Porapak Q and recovered from the absorbent by extraction. When this procedure is applied to aeration of live insects, the Porapak extract represents a concentration of the chemical message.

Key Words—Porapak Q, pheromones, volatile organic compounds, chemical message.

INTRODUCTION

Insect pheromones have most frequently been isolated from body and by-product extracts that contain many nonvolatile compounds and require considerable purification prior to gas chromatographic (GLC) fractionation. Such extracts provide information about the amounts of components present in the insect or by-products at the moment of extraction, but they do not provide a reliable estimate of the amounts and proportions of the components that comprise the chemical message. Aeration of living insects over their span of pheromone production and trapping of the volatile components from the airstream is an alternative procedure; the trapped material represents a concentration of the chemical message. A recent summary, *Insect Sex Pheromones* (Jacobson, 1972), lists more than a dozen examples of pheromone collection with cold traps, which require frequent attention and produce a dilute aqueous condensate that must be extracted. A novel, very effective "total condensation cold trap," which draws by condensing

air with liquid N_2 and requires no pump, has been used to collect insect pheromones (Browne et al., 1974). Charcoal (Keller et al., 1964), powdered cellulose (Tunstall, 1965), lanolin (Brady and Smithwick, 1968), and triglycerides (Röller et al., 1968) have been used to trap pheromones. The recoveries from all of these trappings were reported to be biologically active, and use of the last absorbent resulted in identification of one component of a pheromone.

A previous paper (Silverstein and Rodin, 1966) reported the use of gas chromatographic liquid phases for collecting organic compounds serving as models for insect pheromones. Gas chromatographic solid phases have since been used for analytical determinations in which organic compounds were trapped from the headspace of orange juice (Schultz et al., 1971) and urine (Zlatkis et al., 1973); however, use of solid absorbents for the continuous removal of volatiles from large volumes of air over extended time periods has not been reported to our knowledge. This paper reports the collection and recovery of model compounds with Porapak Q as a solid phase absorbent, and provides the basis for a practical, efficient method for pheromone collection from live insects or insect-host combinations. The methodology has been used extensively over the past two years in pheromone studies in this laboratory and in those of our collaborators (J. H. Borden, unpublished[1]; J. W. Peacock, unpublished[2]; and R. G. Yarger et al., unpublished[3]). Recently Porapak Q has been used to collect pheromones on a small scale (Rudinsky et al., 1973).

METHODS AND MATERIALS

General

The sorbent used to collect the aerated model compounds was Porapak Q (Ethylvinylbenzene-divinylbenzene copolymer, 50/80 mesh; Waters Associate, Inc., Framingham, Massachusetts). Porapak was conditioned by heating it in a N_2 stream (3 liters/min) at 180°C for 24 hours and 110°C for 24 hours (Krumperman, 1972). The tube holding the Porapak (25 g) was 20 cm × 2.2 cm (OD). Both ends were constricted to 6 mm, and the Porapak was held in place by a glass wool plug at each end. A 24/40 $\bar{S}$-joint near one end permitted access. House vacuum provided an air flow, which was regulated by a screw clamp on rubber tubing on the vacuum side to a flow

[1] Sixth and Seventh Annual Research Report, Simon Fraser University, 1972 and 1973.
[2] Presented at the Annual Meeting of the Entomological Society of America, Dallas, November, 1973.
[3] Presented at the 166 Annual Meeting of the American Chemical Society, Chicago, August, 1973.

TABLE 1. RECOVERIES FROM PORAPAK OF 40-MG SAMPLES OF MODEL COMPOUNDS AERATED FOR ONE WEEK

Compound	% Recovered from Porapak Q	% Recovered from evaporation chamber
Butanol	0	0
Nonanol	89	8.5
Dodecanol	69	25
Octadecanol	1.5	100
Octane	9.5	0
Tetradecane	68	8.7
Tetracosane	0	101
Pentyl acetate	65	0
Methyl caproate	66	0
Methyl oleate	3	84
Frontalin[a]	85	0
Myrcene[b]	75	0

[a] 1,5-Dimethyl-6,8-dioxabicyclo[3.2.1]octane, an aggregation pheromone of *Dendroctonus frontalis*.
[b] 2-Methyl-6-methylen-2,7-octadiene, a tree terpene.

TABLE 2. RECOVERIES FROM PORAPAK OF 0.5-MG SAMPLES OF MODEL COMPOUNDS AERATED FOR ONE WEEK

Compound	% Recovered from Porapak Q	% Recovered from evaporation chamber
Pentyl acetate	58	0
Dodecyl acetate	81	0
Hexalure[a]	84	0
Sulcatol[b]	83	0
Ipsenol[c,e]	89	0
Ipsdienol[d,e]	77	0

[a] *cis*-7-Hexadecenyl acetate, synthetic attractant for pink bollworm males.
[b] 6-Methyl-5-hepten-2-ol, aggregation pheromone of *Gnathotrichus sulcatus*.
[c] 2-Methyl-6-methylen-7-octen-4-ol.
[d] 2-Methyl-6-methylen-2,7-octadien-4-ol.
[e] Member of three-component aggregation pheromone of *Ips paraconfusus*.

of 2 liters/min for the break-through studies and for the recoveries in Table 1, and 1 liter/min for the recoveries in Table 2; flow rates were measured at the inlet of the Porapak tube. All tubing connecting glassware in contact with the model compounds was Teflon to obviate sorption or contamination. All experiments were done at room temperature.

Breakthrough of Model Compounds

A 50-μl sample of a solution, 3.80 mg/ml each, of propanol, butanol, hexanol, octanol, and decanol in ethanol was injected into the inlet end of the Porapak tube. The flow rate was 2 liters/min. To trap compounds breaking through the Porapak, a 3-mm U-shaped glass tube in a liquid air bath was placed between the Porapak tube and the vacuum source for half-hour periods at the 4th and 8th hours, and then every 8 hours to the 168th hour. A drierite tube was attached to the inlet of the Porapak tube a half hour before the trapping period in order to avoid plugging of the U-tube with ice. The cold traps were rinsed with 25–50 μl ethanol, and the solution was examined by GLC.

Recoveries of 40-mg Samples of Model Compounds

The aeration apparatus had an evaporation chamber attached to the inlet end of the Porapak tube. The evaporation chamber was a glass tube (16 cm × 6 mm) lined with filter paper. Solutions of model compounds were prepared by adding 120 μl of each compound in a group to 1 ml of hexane, and were stored at −20° in vials with Teflon-lined caps. A 0.50-ml aliquot was applied to the filter paper in the evaporation chamber with a syringe. After one week, the aerated Porapak was transfered to a Soxhlet thimble and extracted with redistilled pentane for 24 hours. This extract was concentrated to about 5 ml by distilling the pentane slowly through a 25-cm × 1-cm column packed with glass beads. The evaporation chamber was rinsed with 10 ml of pentane.

Recoveries of 0.5-mg Samples of Model Compounds

The Porapak used for the recoveries of small quantities was further conditioned by Soxhlet extraction with pentane for 24 hours and dried in a N_2 stream for 4 hours at 100°C. An atmosphere scrubber consisting of a length of 6-mm glass tubing packed with 5 cm of Porapak was placed at the inlet end of the evaporation chamber. Solutions were prepared by adding 6 μl of each compound in a group to 5 ml of hexane, and 0.5 ml-samples were applied to the filter paper. Because of scheduling problems, the collection

tubes containing the sorbed compounds were capped and refrigerated for two weeks.

Analyses of Model Compounds

All analyses were done by temperature-programmed GLC. The cold trappings from the breakthrough study were examined for the presence of eluting model compounds on a Carbowax column (4% on Chromosorb G AW DMCS 60/80, 6.1 m × 3.17 mm [OD] ss, 25 ml He/min). For the recoveries from Porapak, a SE-30 column (4% on Chromosorb G AW DMCS 60/80, 1.83 m × 4 mm [ID] glass, 50 ml He/min) was used for all analyses except for the group of pheromonal alcohols for which a Carbowax 20 M column (4% on Chromosorb G AW DMCS 60/80, 6.1 m × 4 mm [ID] glass, 50 ml He/min) was used. Measured amounts of the stock solution, the Porapak extract, and the evaporation chamber rinse were injected into the gas chromatograph, and recoveries of the model compounds were calculated.

RESULTS AND DISCUSSION

Model compounds were selected to cover a wide range of volatility and stability in several chemical classes. Several components of insect pheromones were included. In general, results were similar to those reported in an earlier study (Silverstein and Rodin, 1966), but Porapak is a much more convenient substrate than those previously used. Aerations were carried out continuously for one week at a flowrate of 2 liters/min for the compounds in the breakthrough study and for the compounds in Table 1, and at 1 liter/min for those in Table 2. Recoveries of 40-mg samples are given in Table 1, and of 0.5-mg samples in Table 2.

The breakthrough studies showed that propanol had broken through the Porapak by the 4th hour and was gone by the 8th hour. Butanol had also broken through by the 4th hour, and it was gone by the 16th hour. Hexanol, octanol, and decanol did not appear in the cold U-tube during the monitoring period of 168 hours.

Two of the model compounds are quite sensitive to air oxidation: myrcene, a triunsaturated hydrocarbon, and ipsdienol, a triunsaturated allylic alcohol. Both compounds were recovered from the Porapak in reasonably good yield.

The data in Table 2 show that trapping efficiencies remained high for the submilligram samples. Two precautions were necessary to measure submilligram recoveries by gas chromatography. The thermally conditioned

Porapak was extracted with pentane to remove thermolysis products, and an air scrubber was used to remove atmospheric contaminants. Use of extracted Porapak and an air filter, such as activated charcoal or Porapak, is recommended in practical applications of this technique.

The practical lower and upper limits of volatility of candidate compounds are suggested by the data of Tables 1 and 2. A check for complete extraction of ipsdienol from Porapak by pentane was made by a subsequent extraction with benzene; no additional ipsdienol was found in the benzene extract.

Insect pheromones in general appear to fall within the range of volatilities and stabilities in which aeration through a Porapak column can be applied. The authors recognize several advantages and disadvantages. The advantages are:

1. Aeration can be carried out over long periods while the insects are continuously producing pheromone, and large volumes of air can be utilized without involving operator time.

2. Pentane extraction of the Porapak in an unattended Soxhlet apparatus removes the sorbed compounds. After the Porapak is dried, it is ready for reuse. Extraction of large volumes of water is avoided, and the extract requires no purification prior to gas chromatographic fractionation.

3. Used Porapak tubes can be capped and shipped or stored for future use. Some of the tubes in this study were capped and refrigerated for two weeks.

4. The actual composition of the chemical message can be determined from the trapped volatiles.

5. Extraction of insect bodies or glands frequently involves maceration of tissue with possible enzymatic action on the gland contents. Aeration avoids this possible complication.

The principal disadvantages of the aeration-Porapak trapping procedure are the possibility of loss by breakthrough of very volatile compounds and of air oxidation of very sensitive compounds. Further, pheromone components of low volatility, which may operate over a short range, will not be effectively collected by any aeration procedure. We have not encountered either problem to our knowledge in our work to date on insect pheromones; Porapak collections show high levels of biological activity. In comparison studies, several species of bark beetles boring in logs were aerated, and the volatile compounds were trapped on Porapak and in the total condensation cold trap (Browne et al., 1974). The amounts of recovered material, appearance of chromatograms, and levels of biological activity were about the same for both methods.

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SPECIFICITY OF RESPONSE TO PHEROMONES IN THE GENUS *Ips* (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—Seventeen species of *Ips* were laboratory or field tested for the specificity of their response to male-produced aggregating pheromones. In the laboratory, some species appeared not to differentiate among their own pheromones and those of closely related species, whether the pheromones were bioassayed individually or in direct competition. Other species showed strong preference for their own pheromones in competition with those of closely related species, even though they had demonstrated strong attraction to pheromones of the related species. Cross-responsiveness among *I. confusus*, *I. montanus*, and *I. paraconfusus*, and between *I. mexicanus* and *I. concinnus*, was confirmed in field tests. Moreover, wild *I. paraconfusus* females entered the nuptial chambers of males of *I. montanus* and *I. confusus* but not those of the more distantly related *I. mexicanus*. It is hypothesized that specificity of response to aggregating pheromone is important in the maintenance of reproductive isolation among sympatric *Ips* and that the lack of specificity among closely related species enforces the parapatric distributions characteristic of these species.

Key Words—*Ips*, Scolytidae, aggregating pheromone, reproductive isolation, specificity, cross-attraction.

INTRODUCTION

In the pioneering study by Silverstein et al. (1966), the aggregating phero-

[1] Study supported in part by the National Science Foundation (GB 2972), U.S. Forest Service (No. 5), California Division of Forestry, T. B. Walker and Surdna Foundations, and various forest industries.

mones produced by male *Ips paraconfusus* Lanier [formerly *I. confusus* (Leconte)] were identified as three synergistic terpene alcohols. Characterization of the aggregating pheromones of *I. grandicollis* (Eichhoff) (Vité and Renwick, 1971), *I. calligraphus* (Germar) (Renwick and Vité, 1972), and *confusus* (Young et al., 1973) resulted in the discovery of no additional biologically active compounds; each species utilized one or more of the compounds described from *paraconfusus*. Furthermore Vité et al. (1972) reported various combinations and quantities of the *paraconfusus* pheromones in gas chromatographs from the hindguts of 9 additional *Ips* species. In general, the distribution of the three pheromones was consistent within Hopping's (1963) groups of closely related species although several species in different groups also possessed the same combinations of the three components.

Despite the parsimony in attractants, rigid response specificity has been demonstrated for several species of *Ips* which are sympatric in California (Vité and Gara, 1962; Wood, 1970; Lanier et al., 1972), the southeastern states (Wilkinson, 1964; Vité et al., 1964), and the Rocky Mountains (Lanier, 1970*a*). On the other hand, strong response of *grandicollis* to *paraconfusus* (reported as *confusus*) has been demonstrated by Vité et al. (1964). Vité and Renwick (1971) reported "interspecific attraction" among *paraconfusus*, *confusus*, *I. montanus* (Eichhoff), *grandicollis*, and *I. cribricollis* (Eichhoff), but they did not state which combinations were tested and what levels of response were observed. All of these species are in Hopping's (1963, 1965) group IX, are allopatric or parapatric with respect to each other, and have been subjected to exhaustive biosystematic study (Lanier, 1970*b*). Lastly, reciprocal field tests in California, Idaho, and New York demonstrated geographic variation in the pheromone system of *I. pini* (Say) (Lanier et al., 1972).

This paper presents the results of extensive tests of response specificity among 17 of the 27 species of North American *Ips* which were conducted at Berkeley from 1964 to 1967 and in Calgary, Alberta from 1968 to 1970. Part of this work was presented orally at the 1966 meeting of the Entomological Society of Canada in Banff, Alberta, and some specific results have been cited by Lindquist (1969), Wood (1970), Lanier (1970*b*, 1972), and Lanier et al. (1972) as contributions to the biosystematics of the *Ips* and the mites that are parasitic on the eggs of this genus.

METHODS AND MATERIALS

Females used in the laboratory olfactometer were collected as they emerged from naturally infested logs or from ponderosa pine bolts in which

aboratory colonies had been reared (Footnote, Table 1). Sexes were separated by characters summarized by Lanier and Cameron (1969). No attempt was made to separate emerging parents from brood adults. Beetles were tested immediately after emergence or following storage at about 5°C for 10 or fewer days. Storage is known to have little or no effect on the response behavior of *paraconfusus* (Borden, 1967), and it did not appear to influence other species used in these tests.

Pheromone-laden frass for bioassay was collected in gelatin capsules affixed over the entrance tunnels of individual males. Host material for most tests was ponderosa pine (*Pinus ponderosa*). Jeffrey pine (*Pinus jeffreyi*) was used in tests of cross-attraction between eastern and western *pini*. Because *I. tridens* (Mannerheim) did little boring in ponderosa pine, frass for the *tridens* vs. *paraconfusus* test was produced from Sitka spruce (*Picea sitchensis*). A test sample of about 0.1 g was withdrawn from an aggregate produced by 10 or more males that had been excavating for 3–5 days. Samples of frass were usually produced from the same host log for each series of tests. Frass was presented either in its natural state or as an ethanol extract. The 5-port multiple-choice olfactometer described by Wood and Bushing (1963) was used for all tests. Attraction was assessed in two ways: (1) a single sample of frass was placed under one of the inner three airstreams; (2) samples of frass from two species were assayed in airstreams number 2 and 4 simultaneously, and the positions of the samples were reversed after half of the females were exposed. In all tests, groups of 10 or fewer females were released at a point where the 5 airstreams converged. Individuals walking upwind to a port containing a frass sample were scored as positive responders. All beetles that did not exhibit a positive response in the first trial were collected and released once more. Therefore, the response figures reported here include the total number of females walking to a frass sample on the first and second trials (Wood et al., 1966). Generally, insects were used only once. Occasionally, beetles were held at room temperature in petri dishes for 15 or more minutes and tested again. Indices of attraction were computed as follows: interspecific attraction (%)/conspecific attraction (%) × 100 (Lanier, 1970*a*).

Interspecific attraction within groups I and IX was assessed in the field by trapping beetles responding to male-infested bolts. All bolts were wrapped with metal screen to prevent volunteer attacks and placed in sticky traps consisting of ¼-inch-mesh screen cylinders (20 cm diameter × 30 cm height) on a platform supported by a 2½-m pipe standard driven into the ground (Bedard and Browne, 1969). These traps were placed at intervals of 75–100 m in a line at various California localities. Bark beetles were picked from the traps, washed in solvent, identified, and classified by sex. For tests of group I species, traps containing 20 males of *I. mexicanus* (Hopkins) or *I. concinnus*

(Mannerheim) boring in Monterey pine (*Pinus radiata*) were set out in Berkeley, Alameda County, where *mexicanus* naturally occurs. For tests of group IX species, 40 males each of *paraconfusus*, *montanus*, and *confusus* boring in the principal host of the local species were tested in the following localities: *paraconfusus* area, Blodgett Experimental Forest, El Dorado County (host, ponderosa pine); *montanus* area, Donner Summit, Nevada County (host, western white pine, *Pinus monticola*); *confusus* area, Frazier Park, Kern County (host, single-leaf pinyon pine, *Pinus monophylla*). An uninfested bolt of the local host species served as a control in each test.

In addition to the assay of attraction, mate preference was assessed in the laboratory and field. In the laboratory, two species of males (12 each) were introduced into preformed entrance tunnels on a ponderosa pine log. The two species of males and the unoccupied holes were alternated so that each occurred twice per row and column in a 6×6 Latin-square design. After allowing 2 days for the males to excavate nuptial chambers, 36 females were released on the log. Four days later, bark was stripped from the bolts and the location of the females recorded. For field tests, males of each species were introduced into separate logs and, together with uninfested logs, were placed in a transect or Latin square in an area where only *paraconfusus* occurred. The presence of wild females in the nuptial chambers of marked males was a measure of mate preference.

RESULTS

Olfactometer Tests

Between 42% and 76% of the females responded to frass produced by males of their own species. This conspecific attraction is, by definition (Lanier, 1970*a*), index 100 (Table 1). Indices of attraction between species of different groups ranged from 0 to 38. The response of *paraconfusus* to frass produced by species of groups 0, I, III, VI, and VIII was not different from its response to air alone. However, a low-level cross-attraction between species of different groups appeared to exist in some of the tests involving groups III, IV, IX, and X. This is best illustrated by reciprocal tests between *pini* (group IV) and *paraconfusus* (group IX) and by tests of *paraconfusus* (group IX) females exposed to *calligraphus* (group X) frass.

Indices of attraction between species of the same group ranged from 25 to 125 when frass samples were presented separately (Tables 2, 3). Except for those tests with females of *I. lecontei* Swaine (group IX) and some of the tests with *lecontei* frass, interspecific response was not significantly different from conspecific response. For example, indices for four tests of the response of *paraconfusus* females to *confusus* frass were 129, 90, 80, and 82. These data are combined to show an index of 97 in Table 2.

TABLE 1. SPECIFICITY OF RESPONSE TO ATTRACTANT PHEROMONES PRODUCED BY *Ips* OF DIFFERENT SPECIES GROUPS,[a] AS MEASURED IN THE LABORATORY OLFACTOMETER

Group	Species exposed (♀♀)	Species producing frass (♂♂)[b]	Group	Number tested	Percent positive	Index of response[c]
III	*plastographus maritimus*[d]	*p. matitimus*	III	131	76.3	100
		pini (W)	IV	97	18.6	24[c]**
		paraconfusus	IX	25	12.0	16
		calligraphus	X	47	17.0	22
		air only	—	61	6.6	9
IV	*pini* (W)	*p. maritimus*	III	168	8.3	14
		pini (W)	IV	396	60.9	100
		paraconfusus	IX	87	23.0	38[c]**
		calligraphus	X	10	10.0	16
		air only	—	50	8.0	13
VIII	*tridens*	*tridens*	VIII	295	45.4	100
		paraconfusus	IX	65	6.2	14
		air only	—	50	8.0	18
IX	*paraconfusus*	*latidens*	0	30	6.7	11
		sabinianae	0	40	7.5	13
		concinnus	I	19	5.3	9
		mexicanus	I	19	5.3	9
		p. maritimus	III	143	9.8	16
		pini (W)	IV	159	18.2	30[c]***
		utahensis	VI	37	0	0
		woodi	VI	19	0	0
		tridens	VIII	57	1.7	3
		paraconfusus	IX	776	59.7	100
		calligraphus	X	56	16.0	27[c]**
		air only	—	313	5.4	9
X	*calligraphus calligraphus*	*p. maritimus*	III	42	11.9	18
		pini (W)	IV	50	12.0	19
		paraconfusus	IX	55	9.1	14
		c. calligraphus	X	81	64.2	100

[a] Groups of closely related species are arranged as designated by Hopping (1963) and Lanier (1967).

[b] Data from male frass and ETOH extracts of frass are combined.

[c] Indices of response compare percent positive responses based upon conspecific attraction as base 100. Indices for most interspecific comparisons are based on single tests. Data for conspecific attraction is usually a summation of results from several tests run as controls to the tests of interspecific attraction. Interspecific responses are significantly less ($P < 0.01$) than conspecific responses in all cases. Differences between interspecific response and response to air only are significant at 0.05,* 0.01,** and 0.001*** confidence levels, based upon chi-square test of raw data.

[d] Collection data for the insects used in these tests and those in Tables 2, 3, and 4 (from

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TABLE 2. INDICES OF RESPONSE TO ATTRACTANT PHEROMONES AMONG *Ips* SPECIES OF GROUP IX, AS MEASURED IN THE LABORATORY OLFACTOMETER

Species exposed (♀♀)	Species producing frass (♂♂)					
	para.[a]	*conf.*	*hopp.*	*mont.*	*lec.*	*crib.*
Presented separately						
paraconfusus	100	97	—	86	76[b]**	—
	(776)[c]	(494)		(487)	(171)	
confusus	99	100	—	111	64	—
	(80)	(166)		(78)	(79)	
montanus	122	125	—	100	25[b]***	—
	(186)	(180)		(275)	(65)	
lecontei	63[b]**	65[b]**	—	65[b]**	100	—
	(154)	(194)		(164)	(186)	
Presented simultaneously						
paraconfusus	100	55[b]***	63[b]**	35[b]***	59[b]***	114
		(1010)	(183)	(689)	(480)	(556)
confusus	111	100	—	—	78	—
	(81)				(77)	
hoppingi	144	—	100	—	—	—
	(99)					
montanus	83	119	—	100	—	—
	(234)	(173)				
lecontei	51[b]***	47[b]***	39[b]***	45[b]***	100	10[b]***
	(88)	(100)	(87)	(123)		(117)
cribricollis	—	—	—	—	113	100
					(578)	

[a] Abbreviations: *para.*, *paraconfusus*; *conf.*, *confusus*; *hopp.*, *hoppingi*; *mont.*, *montanus*; *lec.*, *lecontei*; *crib.*, *cribricollis*.

[b] Differences with conspecific response are significant at 0.05,* 0.01,** and 0.001*** confidence levels, based upon chi-square test of raw data.

[c] Aggregate number of insects used in the various comparisons is in parentheses.

Table 1. (continued)

California unless otherwise specified): Group 0: *latidens* (LeConte)—Calaveras County, *Pinus jeffreyi*; *sabinianae* (G. Hopping)—Calaveras County, *P. sabiniana*. Group I: *mexicanus* (Hopkins)—Alameda County, *P. radiata*; *concinnus* (Mannerheim)—Del Norte County, *Picea sitchensis*. Group III: *plastographus maritimus* Lanier—Marin County, *Pinus muricata*. Group IV: *pini* (Say) (W-western)—Calaveras County, *P. jeffreyi*; *pini* (Eastern) Sault Ste. Marie, Ontario, *P. resinosa*; *bonanseai* (Hopkins)—Pima County, Arizona, *P. ponderosa*. Group VI: *utahensis* Wood—Salt Lake County, Utah, *Picea engelmannii*; *woodi* Thatcher—Glacier County, Montana, *Pinus flexilis*. Group VIII: *Ips tridens*, Klamath County, Oregon, *Picea engelmannii*. Group IX: *paraconfusus* Lanier—Calaveras, Nevada, and Placer Counties, *P. ponderosa*; *confusus* (LeConte)—Los Angeles and Ventura Counties, *P. monophylla*; *hoppingi* Lanier—Chochise County, Arizona, *P. cembroides*; *montanus* (Eichhoff)—Placer County, *P. monticola*; *lecontei* Swaine—Pima County, Arizona, *P. ponderosa*; *cribricollis* (Eichhoff)—Lincoln County, New Mexico, *P. ponderosa*. Group X: *calligraphus calligraphus* Germar)—Calaveras County, *P. ponderosa*; *c. ponderosae* Swaine—Lincoln County, New Mexico, *P. ponderosa*.

TABLE 3. SPECIFICITY OF RESPONSE TO ATTRACTANT PHEROMONES WITHIN *Ips* GROUPS I, IV, AND X,[a] AS MEASURED IN THE LABORATORY OLFACTOMETER

Group	Species exposed (♀♀)	Species producing frass (♂♂)	Number tested	Percent positive	Index[b]
Presented separately					
I	*concinnus*	*concinnus*	51	49.0	100
		mexicanus	54	40.7	83
I	*mexicanus*	*mexicanus*	32	75.0	100
		concinnus	28	78.6	105
IV	*pini* (W)	*pini* (W)	396	60.9	100
		pini (E)	42	64.3	105
		bonanseai	58	43.1	71
IV	*pini* (E)	*pini* (E)	41	65.9	100
		pini (W)	75	42.7	65
Presented simultaneously					
I	*mexicanus*	*mexicanus*	59	64.4	100
		concinnus		18.6	29[b]***
IV	*pini* (W)	*pini* (W)	125	68.0	100
		pini (E)		32.0	47[b]**
IV	*pini* (E)	*pini* (E)	82	47.6	100
		pini (W)		45.1	95
X	*c. calligraphus*	*c. calligraphus*	112	59.8	100
		c. ponderosae		32.2	48[b]***
X	*c. ponderosae*	*c. ponderosae*	140	50.7	100
		c. calligraphus		42.2	82

[a] Data for groups IV and X were presented previously (Lanier, 1972).
[b] Differences within tests are significant at 0.01** and 0.001*** confidence levels, based upon chi-square test of raw data.

Despite their high cross-attractiveness, differences between pheromone systems of these species were frequently revealed in choice tests which simultaneously presented the frass from two species. For example, *paraconfusus* females were significantly ($P<0.05$) more responsive to conspecific frass than to *confusus* frass in 6 of 7 choice tests, even though *paraconfusus* and *confusus* frass were nearly equally attractive to *paraconfusus* females when presented separately (Table 2). Female *mexicanus* exhibited the same pattern in their response to *mexicanus* and *concinnus* frass (Table 3).

The above-mentioned preference patterns exhibited in the simultaneous comparisons did not occur in all reciprocal tests. Within group IX, *paraconfusus* and *lecontei* usually exhibited a strong preference for conspecific frass, while *confusus*, *I. hoppingi* Lanier, *montanus*, and *cribricollis* did not (Table 2).

Discrimination was not apparent in several tests in which frass from

TABLE 4. RESPONSE OF *Ips* SPECIES OF GROUP IX TO ATTRACTANT PHEROMONES OF TWO DIFFERENT SPECIES PRESENTED SIMULTANEOUSLY IN THE LABORATORY OLFACTOMETER

Species exposed (♀♀)	Species producing frass (♂♂)	Number tested	Percent positive[a]
paraconfusus	*montanus*	286	30.8
	lecontei		26.6
paraconfusus	*confusus*	67	28.3
	lecontei		26.9
paraconfusus	*montanus*	212	25.5
	confusus		23.1
paraconfusus	*cribricollis*	160	26.9
	lecontei		24.4
lecontei	*paraconfusus*	98	17.3
	confusus		13.3
confusus	*lecontei*	160	24.4
	cribricollis		26.9
cribricollis	*lecontei*	103	21.4
	hoppingi		27.2

[a] Differences within all tests are not significant ($P > 0.05$), based on chi-square test of raw data.

two species of group IX was presented simultaneously to females of a third species (Table 4). Especially noteworthy is the test in which *paraconfusus* responded equally to frass of *cribricollis* and *lecontei*; in tests of individual frass samples *paraconfusus* was strongly attracted to *cribricollis* but only moderately attracted to *lecontei* (Table 2).

Infraspecific differences were also noted in the simultaneous tests (Table 3). Western *pini* showed a preference for frass from males of the

TABLE 5. RESPONSE OF *I. mexicanus* TO ATTRACTANT PHEROMONES PRODUCED BY MALE *I. mexicanus* AND *I. concinnus* BORING IN *Pinus radiatae*, BERKELEY, CALIFORNIA, SEPTEMBER 28–OCTOBER 10, 1967

Species of males producing pheromone[a] (♂♂)	Number trapped			Sex ratio (♀♀/♂♂)
	9/28–10/4	10/4–10/10	Total	
mexicanus	25	136	161	2.3
concinnus	0	13	13[a]	3.3

[a] Attractiveness of *concinnus* pheromone is probably underestimated because teneral adults of this species bored less than did the more mature *mexicanus*.

TABLE 6. INTERSPECIFIC RESPONSE TO BORING MALES BY *I. confusus*, *I. montanus*, AND *I. paraconfusus* IN THE FIELD

		Species producing pheromone (♂♂)					
		paraconfusus[b]		montanus		confusus	
Test[a]	Species responding	Number trapped	Sex ratio (♀♀/♂♂)	Number trapped	Sex ratio (♀♀/♂♂)	Number trapped	Sex ratio (♀♀/♂♂)
1	*paraconfusus*	152	5.6	72	3.5	144	3.8
2	*montanus*	122	2.6	2	1.0[c]	14	2.5
3	*confusus*	210	6.0	109	2.1	416	4.4

[a] Data for tests are as follows: Test 1, September 13–15, 1967, Blodgett Forest, El Dorado County, California, males boring in *Pinus ponderosa*; Test 2, September 18–27, 1967, Donner Summit, Nevada County, California, males boring in *P. monticola*; Test 3, October 11–November 7, 1967, Frazier Park, Kern County, California, males boring in *P. monophylla*.

[b] Collection data for the males tested are the same as that for the respective tests.

[c] Attractiveness of *montanus* pheromone is probably underestimated because the teneral adults of this species bored less than more mature *confusus* and *paraconfusus*.

TABLE 7. FEMALES OF GROUP IX SPECIES ENTERING NUPTIAL CHAMBERS OF CONSPECIFIC AND INTERSPECIFIC MALES IN THE LABORATORY

Species released (♀♀)	Females per nuptial chamber excavated by ♂♂[a]			
	lecontei	*cribricollis*	*paraconfusus*	*montanus*
cribricollis	0	1.71	—	—
cribricollis	—	1.08	0	—
paraconfusus	—	—	0.60	0.53
montanus	—	—	1.50[b]	0.67[b]

[a] The number of nuptial chambers per species of male ranged from 10 to 16. Some of the 12 males introduced failed to bore while others abandoned their first nuptial chamber and reattacked the log.
[b] Differences are significant ($P<0.05$, chi-square test).

western population while eastern *pini* did not exhibit preference. A similar pattern occurred between the subspecies of *calligraphus* (Table 3).

Field Trapping Tests

In the field, both sexes of *mexicanus* responded in considerably higher numbers to logs infested with *mexicanus* males than to logs containing *concinnus* males (Table 5). In reciprocal field tests, both sexes of *paraconfusus*, *confusus*, and *montanus* were attracted to boring males of each of these three species (Table 6). *I. paraconfusus* responded in lowest numbers to *montanus* males and equally to *paraconfusus* and *confusus* males. *I. confusus* showed a definite preference for conspecific males and, surprisingly, *montanus* was least attracted to conspecific males and most attracted to *paraconfusus*. Both sexes were attracted to all treatments, but the sex ratios within tests were significantly different ($P<0.05$) in some cases. Uninfested (control) logs attracted no *Ips* in any of the tests.

Entrance of Females in Nuptial Chambers

I. paraconfusus released on a caged log containing boring *montanus* and *paraconfusus* males showed no preference for the nuptial chambers of either species of males (Table 7). In all cases the females constructed normal-appearing egg galleries and, based upon previous experience (Lanier, 1967, 1970*b*), it can be assumed that insemination occurred. *I. montanus* females showed a significant preference for the nuptial chambers of *paraconfusus* males over those of *montanus* males. However, *cribricollis* females did not enter the nuptial chambers of *paraconfusus* or *lecontei* males.

TABLE 8. *Ips paraconfusus* FEMALES IN NUPTIAL CHAMBERS OF INTRODUCED MALES, BLODGETT FOREST, EL DORADO COUNTY, CALIFORNIA

Test[a]	Introduced ♂♂	Number of logs	Nuptial chambers	♀♀ per nuptial chamber	Volunteer attacks[b]
I	*paraconfusus*	5	48	2.17	107
	confusus	4	33	0.30	3
	mexicanus	2	20	0.00	0
	uninfested	4	0	0	0
IIA	*paraconfusus*	3	15	0.40	0
	confusus	3	15	0.07	0
	montanus	3	15	0.33	0
IIB	*paraconfusus*	3	15	0.13	0
	confusus	3	15	0	0
	montanus	3	15	0	0

[a] Specific conditions for each test were as follows: I. Logs containing 10 males each were placed in an east-west transect 25 M apart at the University of California's Blodgett Forest (El Dorado County) on July 7 and picked up on July 9, 1966. II. Logs containing 5 males each were placed in 3 × 3 Latin squares designated A and B. These were placed near the site of test I on August 9 and picked up on August 16, 1966.

[b] All volunteer attacks were by *paraconfusus* males.

In the field, *paraconfusus* showed a strong preference for the nuptial chambers of *paraconfusus* males (Table 8). This result was consistent with olfactometer choice tests (Table 2) but in conflict with the apparent lack of discrimination by *paraconfusus* in field trapping experiments (Table 6).

DISCUSSION

These data show that attractant pheromones produced by *Ips* of the same species group are generally cross-attractive, while pheromones of *Ips* of different species groups are minimally or not at all cross-attractive. Interspecific attraction was confirmed in the field for *paraconfusus*, *confusus*, and *montanus* of group IX, as was attraction of *mexicanus* to *concinnus* of group I.

Infraspecific variation in pheromone systems was indicated by differential attraction of *c. calligraphus* to the frass produced by the two subspecies and by the discrimination of western *pini* in favor of its own pheromone over that of eastern *pini*. In the field, both eastern and western populations of *pini* showed strong preference for pheromones of males of the same population (Lanier et al., 1972; Piston and Lanier, 1974).

Differences in attraction indices for assays in which pheromones were presented individually and in competition with conspecific pheromones expose the fallacy of equating attractants on the basis of data from individual tests. The choice test is obviously more powerful than individual tests for determining preferences for two attractant pheromones in the laboratory, and it is probably more indicative of the relative attractiveness of pheromones in the field.

Results of our mate selection test (Table 8) were consistent with laboratory assay results showing preference by *paraconfusus* for conspecific pheromone over that of *confusus* and *montanus* (Table 2). However, in field trapping tests it was *confusus* rather than *paraconfusus* which showed preference for the pheromone of conspecific males (Table 6). These field results were confounded by position effects and insufficient replication. Furthermore, the inferior attractiveness of boring *montanus* males, even to the same species, was possibly an effect of removal of *montanus* males prior to emergence. Similarly, the preference by *mexicanus* for conspecific over *concinnus* pheromones in the field (Table 5) may have been exaggerated by the immaturity of the *concinnus* males and/or reluctance of these males to bore in *Pinus radiata*. Borden (1967) found that callow *paraconfusus* were less attractive than older males boring in the same host.

Specificity of pheromone systems among sympatric species is undoubtedly an important mechanism for minimizing competition for host material and preventing interspecific mating, especially for species such as *paraconfusus* and *pini* or *grandicollis*, *calligraphus*, and *I. avulsus* (Eichhoff), which breed in the same host species and may coinhabit the same tree. Conversely, lack of pheromone specificity among closely related parapatric species such as *montanus*, *paraconfusus*, and *confusus* may prohibit introgression of their ranges. Our field tests, together with a previous observation by Lanier (1970*b*), show that cross-attractiveness can promote interspecific matings among these species. Specificity of pheromones and/or alternate premating isolating mechanisms must evolve before these species can coexist without mutually disadvantageous wastage of gametes through interspecific inseminations that are inviable (Lanier, 1970*b*). Thus *lecontei*, which overlaps the ranges of *cribricollis*, *hoppingi*, and *confusus*, appeared to have the most unique pheromone system among the species of group IX (Table 2). Pheromones, however, cannot be the only means by which *lecontei* maintains premating isolation with sympatric species of group IX. That *cribricollis* females did not enter the nuptial chambers of *lecontei* males to which they were strongly attracted in olfactometer tests, indicates that mechanisms other than specificity of response to pheromones are involved. The role of attractant pheromones in the maintenance of reproductive isolation is discussed in detail by Lanier and Burkholder (1974).

Lack of attraction of *paraconfusus* to *calligraphus* may be explained by the gas chromatographs of Renwick and Vité (1972) and Vité et al. (1972), which show that only two of the *paraconfusus* pheromones are produced by the other species; all three must be present for strong response by *paraconfusus* in the field (Wood et al., 1967). On the other hand, lack of response to *paraconfusus* pheromone by *calligraphus* might result from inhibition such as that known to occur in *I. latidens* (Leconte), which was attracted to the combination of two of the *paraconfusus* pheromones but not to the tripartite mixture (Wood et al., 1967; Wood 1970). Birch and Wood (1975) reported that one of the components of the *paraconfusus* pheromone bouquet, ipsenol, inhibits the response of *pini* to its own pheromone and that linalool, a compound produced by *pini*, inhibits aggregation by *paraconfusus*. A similar mechanism could account for the response specificity observed between *calligraphus* with *pini* in the laboratory (Table 1) and with *avulsus* in the field (Vité et al., 1964), despite the occurrence of the same combinations of known pheromones in gas chromatographs of these three species (Vité et al., 1972).

Host species per se did not appear to affect response specificity or the lack of it. For example, *paraconfusus* produced frass in sitka spruce (for comparison with the *tridens* pheromone) that was attractive to *paraconfusus*. Moreover, *paraconfusus* produced attractant when boring in Douglas fir (Wood and Bushing, 1963) and white fir (Wood et al., 1966), neither of which is a natural host of a North American *Ips* species. *I. montanus* and *confusus* were attracted to frass produced in ponderosa pine, even though this tree is very rarely selected as host material by these bark beetles. Similarly, Lanier (1970*c*) found that specificity between *I. plastographus* (Leconte) and *I. integer* (Eichhoff) was maintained in the field when the two species were introduced into each other's host. In nature, host specificity is probably maintained by a complex of stimuli and responses (Wood, 1972). However, if an error is made and boring is successful, pheromone will be produced and the unusual host may be colonized.

Although host species does not appear to be critical for pheromone production, differences in the attractiveness of *pini* boring in two host species have been observed (Piston and Lanier, 1974). Whether these results reflect qualitative or quantitative differences in the attractant bouquet can only be determined by systematic comparison of the volatiles emanating from beetles of the same population boring in different hosts.

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RESPONSES OF ECOLOGICALLY DISSIMILAR POPULATIONS OF THE WATER SNAKE *Natrix s. sipedon* TO CHEMICAL CUES FROM PREY

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Abstract—Three populations of the water snake, *Natrix s. sipedon*, were tested for their responses to surface water extracts prepared from various prey species. It was clearly shown that these snakes can distinguish between different prey genera on the basis of chemical cues alone. Adult snakes caught at a fish hatchery where goldfish (*Carassius auratus*) were abundant responded most to goldfish extract, as did laboratory-born snakes reared on goldfish for one year. However, snakes caught in a relatively undisturbed mountain stream environment and naive young born to a mountain female responded more to the extracts of various sympatric mountain fish and amphibians. The evidence indicates that newborn snakes have genetic predispositions for sympatric species of the classes of prey normally eaten. These preferences can be enhanced or inhibited during ontogeny.

Key Words—snakes, prey preference, chemical senses, *Natrix*, chemical cues, predator-prey, ecology, adaptation.

INTRODUCTION

It is a truism in ecology and evolution that species are adapted to their environment. Further, populations of the same species living in diverse habitats should adapt to the particular contingencies found therein, be they predators, disease, nest sites, or food. Clearly behavior is often involved in such adaptation but as Tinbergen (1963) has pointed out, the adaptiveness of behavior has rarely been assessed directly. Complicating the situation is the fact that specific adaptations may not be directly traceable to natural

selection, but involve conditioning, habituation, and other psychological processes. While biologists generally interpret all adaptations as genetic, psychologists often approach adaptation as exclusively an individual phenomenon divorced from evolution, as in the influential "adaptation-level" theory of Helson (1964). Ethologists, however, have stressed that experiential and innate factors can interact in various ways, animals can be programmed to learn specific types of skills (e.g., development of species-specific bird songs), and different genotypes can process identical experiences differently, with even opposite phenotypic results. This study was an attempt to begin such an assessment of adaptation on the chemical control of prey preferences in an obligate carnivore.

The sensory control of feeding by snakes is complex and often diverse across groups. The chemical senses, especially the Jacobson's organ-tongue system, are of considerable importance in feeding (Wilde, 1938; Burghardt, 1970*a*). A method of investigating chemical cue response has been developed by Burghardt (1970*a*), who made surface extracts of prey items so as to separate visual from chemical cues. When the extracts are presented to the snake on cotton swabs, the tongue picks up chemicals and transfers them to the Jacobson's organ, which contains sensory receptors (Wilde, 1938). The response can be measured by the number of tongue flicks per unit time in the presence of the extract or by the latency and frequency of prey attacks. Newborn snakes tested with this technique have included the natricine genera *Thamnophis*, *Storeria*, *Natrix*, and *Regina* (Burghardt, 1970*a*). All species tested from this group are born with preferences for one or more categories of prey as inferred from differential responses to various prey extracts. Such preferences may help to reduce competition in sympatric species. However, the stereotypy involved is relative and the responses to particular extracts by newborn snakes of the same species from various geographical areas may differ and probably correspond to the types of prey that are available (Burghardt, 1970*b*). Dix (1968) tested the relative prey (not prey extract) preference of naive and experienced garter snakes (*Thamnophis sirtalis*) and *Natrix sipedon* collected in Massachusetts, Maryland, and Florida and found, in some cases, significant differences. However, he did not establish that the snakes could discriminate between different species of fish or amphibians and, in fact, used a frog species available to all of his populations and a fish found only in one area.

It has also been shown that early feeding experience can alter the response of previously unfed neonate garter snakes (*T. sirtalis*) to water extracts of prey (Fuchs and Burghardt, 1971). Responses to extracts of the prey that the snakes were eating increased, while the extracts of the prey not offered declined in effectiveness. This indicates that conspecifics may develop marked differences in food preferences because of differences in prey avail-

ability. Similarly, aversions to prey and prey extracts can be induced by aversive experiences with prey (Burghardt, Wilcoxon, and Czaplicki, 1973).

We thus have at least two factors operating in chemical prey preferences of adult snakes—initial preferences and those resulting from prior feeding experience. In the present experiments, adult *N. sipedon* from two localities in East Tennessee with different prey compositions were tested on their responses to extracts prepared from prey species occurring in their home areas. Further, the role of experience in this species was studied by testing newborn and laboratory-reared snakes on similar series of prey extracts. These experiments thus represent a beginning in relating laboratory sign stimulus research in snakes to actual ecological conditions.

METHODS AND MATERIALS

Subjects

Four different groups of *N. sipedon* were used:

1. 10 laboratory-reared one-year-old snakes, born to a female obtained prior to testing from Midwest Reptile Company, Fort Wayne, Indiana. They had been fed only on feral goldfish (*Carassius auratus*).

2. 6 wild-caught snakes from Sterchi's Bait Hatchery, Knoxville, Tennessee. These were caught over a period of time and fed goldfish every 4 days.

3. 6 wild-caught snakes from Tremont, Blount County, Tennessee, in the Great Smoky Mountain National Park. These snakes were caught at different times from June until the beginning of testing in August, 1971, and were also fed goldfish.

4. A litter of 8 ingestively naive snakes born to a female from Tremont on September 15, 1971, and initially tested at 10 days of age.

Housing

The laboratory-reared and newborn snakes (Groups 1 and 4) were housed and tested in separate 23 × 14 × 17-cm glass tanks. White cards were placed between the tanks and white paper was taped to the front to insure visual isolation. Lighting was both natural and fluorescent. As the wild-caught snakes were less accustomed to the presence of humans, they were tested in larger cages (30 × 30 × 30 cm) and tested in a darkened room (see Figure 1). The cages had screen tops and one side was clear plastic. The experimenter was in shadow, and therefore less likely to be seen by the snakes. For all testing, the temperature was 23–25°C and relative humidity was 50–65%. Further details can be found in Gove (1971) and Burghardt (1968).

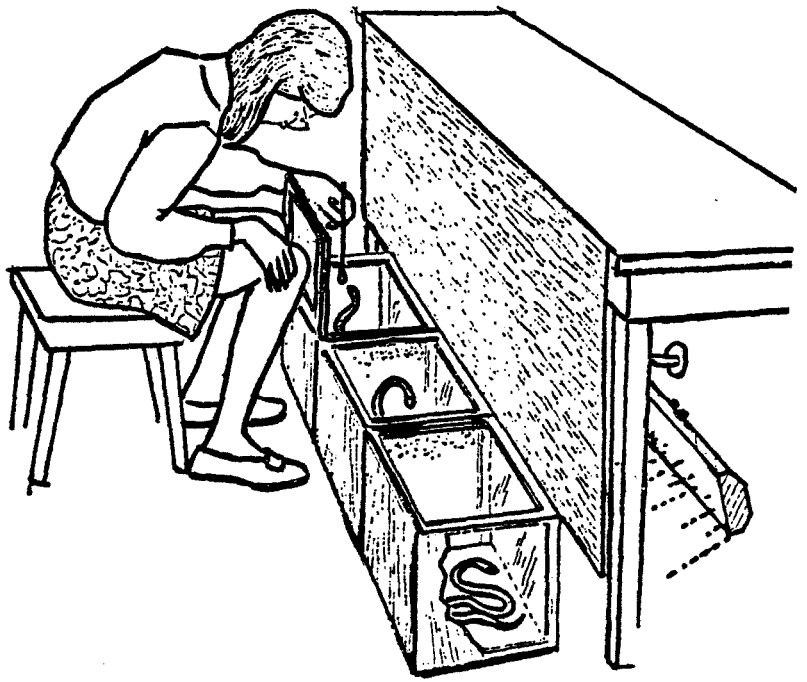

FIG. 1. Laboratory setup for testing wild-caught snakes. The fluorescent light under the table is the only light in the room, so the experimenter is in shadow. In actual testing, 4 boxes were used.

Prey Material

Prey species chosen for this experiment are listed in Table 1 and are based on reported stomach contents. Most of them were captured in areas where the snakes were found.

Stimulus Preparation

Surface extracts were prepared from live prey which were washed, dried, weighed, and then stirred in distilled water for one minute at 60°C. 20 ml of water were used for each 6 g of prey. The extract was centrifuged at 3,000 rpm, the supernatant fluid poured into tightly capped vials and kept frozen until needed. They were prepared during a 6-week period prior to first testing.

Testing Procedure

The extracts were taken from the freezer and kept at room temperature

TABLE 1. PREY SPECIES USED FOR PREPARATION OF EXTRACTS FOR TESTING FOOD PREFERENCES IN *N. sipedon*

Prey species	Symbol	Genus reported in stomach contents
Fish		
Campostoma anomalum (T)[a]	Can	Raney and Roecker, 1947
Carassius auratus (S)[b]	Cau	Brown, 1940
Cottus carolinae (T)	Cc	King, 1939
Etheostoma ruflineatum (T)	Er	Brown, 1940
Notropis coccogenis (T)	Nc	Conant, 1938
Amphibians		
Bufo fowleri (T,S)	Bf	Brown, 1940
Desmognathus fuscus (S)	Df	King, 1939
D. monticola (T)	Dm	King, 1939
Gastrophryne carolinensis (S)	Gc	
Plethodon glutinosis (T)	Pg	
Rana catesbiana (tadpole) (T,S)	Rc	King, 1939
Rana pipiens (T,S)	Rp	King, 1939
Other		
Mus musculus (baby)	Mm	
Orconectes sp. (T)	Or	Fraker, 1970

[a] Present at Tremont.
[b] Present at Sterchi's Bait Hatchery.

TABLE 2. FOOD-PREFERENCE TESTS CARRIED OUT ON WATER SNAKES *N. sipedon* FROM DIFFERENT HABITATS

Series	*N*	Locale	Extracts[a,b]	Number of sessions
1	10	Laboratory	Df, Mm, Dm, Cau, Pg, Rp.	7
2	10	Laboratory	Nc, Cc, Or, Er, Cau, Can.	7
3	2	Tremont	Dm, Cc, Bf, Cau, Rc,	8
	2	Sterchi	Nc, Rp, Can, Er, Gc.	
4	2	Tremont	Dm, Cc, Bf, Cau, Rc,	8
	2	Sterchi	Nc, Rp, Can, Er, Gc.	
5	2	Tremont	Dm, Cc, Bf, Cau, Rc,	8
	2	Sterchi	Nc, Rp, Can, Er, Gc.	
6	8	Tremont (newborn)	Dm, Cc, Bf, Cau, Rc, Nc, Rp, Can, Er, Gc.	6

[a] A distilled water control was used in all sessions of all series.
[b] Symbols for extracts as in Table 1.

for 40 minutes. Every snake was tested once with each extract in continuous trials over a period of 2–4 hours. Such sessions occurred once or twice a day. For the first snake in each series of sessions, the extracts were presented randomly with the constraint that if 2 fish stimuli were adjacent another type of stimulus, such as anuran, was inserted between them. The order for the second snake was the same, except the first extract was dropped into the last position, and so on. At each successive session, the first stimulus for each snake was dropped to last position. In a session there was at least a 20-minute interval between successive tests. The laboratory snakes were given seven testing sessions on each of 2 sets of 6 extracts plus distilled H_2O, while the newborns were exposed to 6 sessions and the wild caught snakes 8 sessions on identical sets of 10 extracts plus distilled H_2O. Table 2 outlines the tests given.

A trial lasted 2 minutes. It began when a cotton swab was dipped into the extract vial, shaken once, and positioned about 2 cm from the snake's snout, slightly to one side. The tongue flicks and latency of attack in seconds were recorded. The trial ended if the snake attacked (defined by opening its mouth and lunging toward the swab) or when 2 minutes elapsed. Opening the mouth to any angle without lunging was recorded as a gape, and testing continued. No attacks were seen with a gape angle of less than 30°. If an aggressive or defensive attack occurred, which is easily distinguishable by the flattening of the head and body, the trial was postponed for 20 minutes and then repeated. This only occurred twice, and so did not disrupt the testing schedule.

Total tongue flicks were counted with a mechanical hand counter. Tongue flicks that were not directed toward the swab were counted mentally and subtracted from the total to get the recorded figure of tongue flicks to swab. Since the swab was not directly in front of the snake, it was not difficult to determine if the tongue flick was directed toward the swab. Tongue flicks toward the swab were recorded if (1) the tongue turned toward the swab, (2) the head turned toward the swab and the tongue flicks became straight, or (3) the snake approached the swab, overshot it, and tongue flicked beyond the swab before returning to it. All others, including those in the third category if the snake did not return to the swab, were recorded as general tongue flicks. If the snake attacked the swab, the latency of attack was recorded to the nearest second.

Statistical Treatment

A snake which did not attack was given a trial score of the tongue flicks to the swab. If an attack occurred in a trial, the snake was given a score which consisted of its tongue flicks to the swab plus 120 minus the latency of

attack in seconds. The mean score and standard deviation were computed for the combined scores of all snakes in each of the four groups for each extract. A critical distance was computed using the following formula:

$$SD\sqrt{2/N} = \text{Critical distance}$$

The graphs of the mean $\pm$ critical distance indicate significant differences where the critical areas of the two means do not overlap ($P<0.05$). This method was used by Burghardt (1968) for developing extract response profiles for newborn snakes. Admittedly conservative, this allows ready visual evaluation of the results for comparing any grouping of extracts.

The data for the wild-caught snakes were given a square-root transformation. The variances for the wild-caught snakes were much greater than for the laboratory-reared snakes, probably because they were less accustomed to captivity, and the transformation reduced this variance.

RESULTS

Tests with Laboratory-Reared Snakes

Figure 2 shows the response profile for the laboratory-reared snakes to the extracts prepared from different prey species. The mean scores for goldfish, *C. auratus*, are highest and the critical distances do not overlap with the scores for other extracts, indicating that the response to goldfish was significantly greater than to all other extracts.

C. auratus received a much higher percentage of attacks than any other extract (30% of the trials), while the next was the slimy salamander, *P. glutinosus* (6%). The unexpectedly high response to baby mouse, *Mus musculus* was due to one snake which consistently responded to mouse second to goldfish. All other snakes' responses to mouse extract ranked sixth or seventh.

Tests with Snakes Caught in Mountain Habitat

The data on the snakes from Tremont (Figure 3), show a significantly higher response to the darter *E. ruflineatum*, the sculpin *C. carolinae*, the minnow *N. coccogenis*, and the stone roller *C. anomalum* than to goldfish. The water control elicited significantly less response than any other stimulus. The data show a higher percentage of attacks for the extracts which received a high score, with no attacks to goldfish. One snake from Tremont had a 100% attack score for minnow, darter, and sculpin. Another gave a 100% attack score for darter.

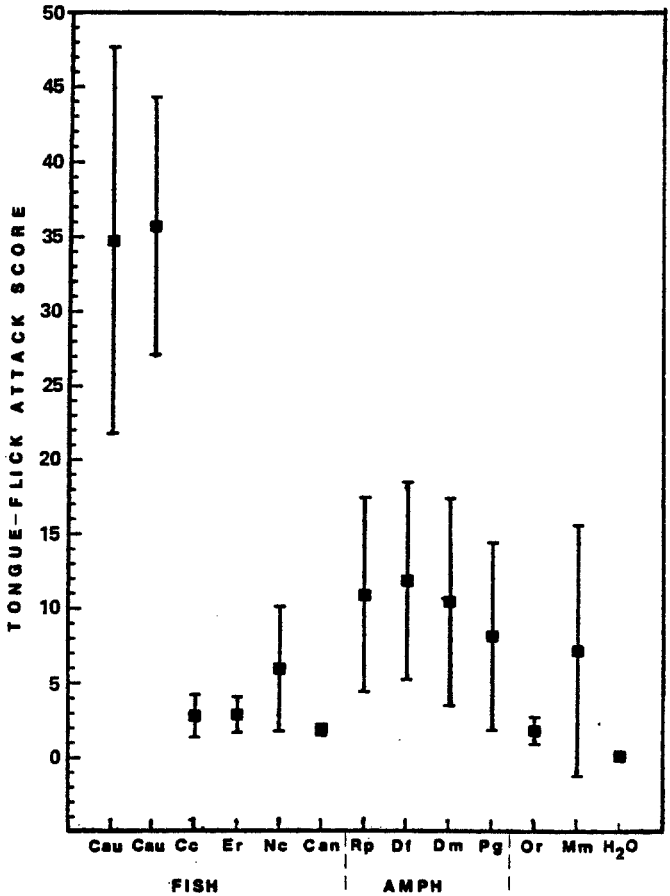

FIG. 2. The tongue-flick attack scores of laboratory-reared water snakes, *N. sipedon*, in relation to the extracts of prey species.

Tests with Snakes from the Bait Hatchery

Figure 4 shows that the bait-hatchery snakes gave a significantly higher response to goldfish than to any other fish species. Only the sympatric narrow-mouthed toad, *G. carolinensis*, found only at Sterchi's, did not elicit a significantly lower response. The bait-hatchery snakes had much lower general attack scores than the mountain snakes, and goldfish received 75% of the attacks. The general tongue flicks, which are not shown in these data, were also higher for the mountain snakes than for the bait-hatchery snakes.

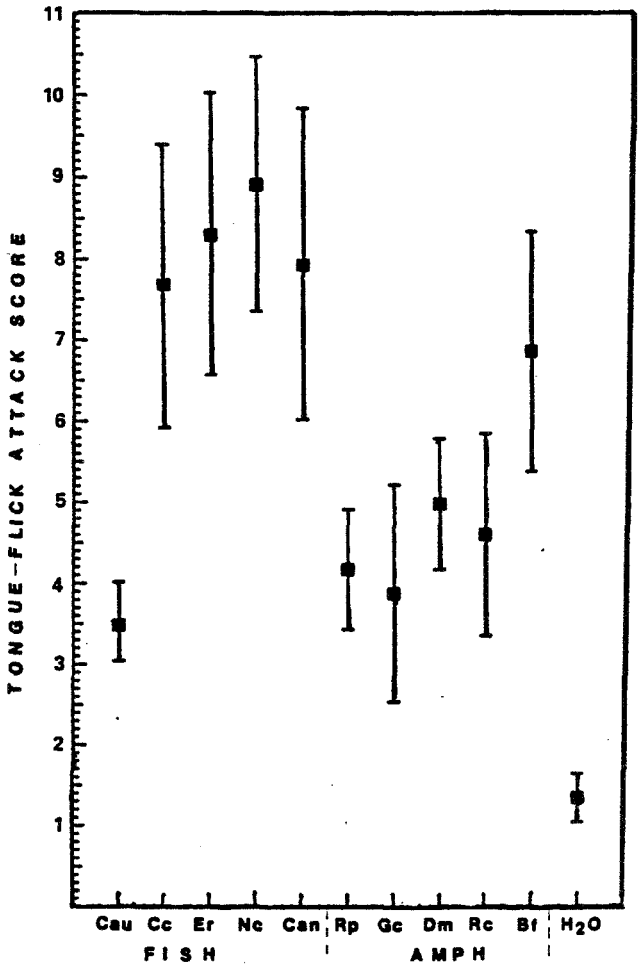

FIG. 3. The tongue-flick attack scores of the water snake, *N. sipedon*, from a mountain habitat in relation to extracts of prey species.

Tests with Newborn Snakes Born in the Laboratory to Female from Mountain Habitat

8 newborn snakes, born in the laboratory of Tremont parents, were tested at the age of 10 days with the same extract sequences as the adult Tremont and Sterchi snakes. The combined response profile is shown in Figure 5. The highest response was to extract of the salamander *D. monticola*,

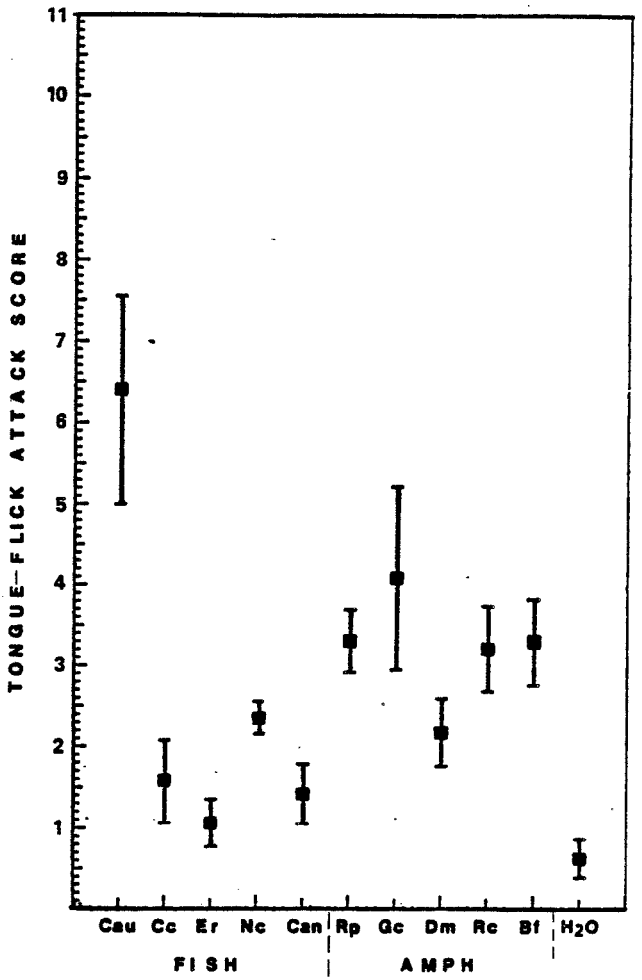

FIG. 4. The tongue-flick attack scores of the water snake, *N. sipedon*, from a bait farm in relation to extracts of prey species.

but this was not significantly different from the responses to young toads, *B. fowleri*, bullfrog tadpoles (*R. catesbiana*), sculpin, minnow, or stone roller. Darter, goldfish, and frog (*R. pipiens*) all received relatively low responses. No attacks occurred in this series.

Considerable individual variation was seen in the 8 newborn snakes, and this is shown in Figure 6. Goldfish and H_2O always elicited relatively low rates of response. This variation is greater than that of the adult snakes.

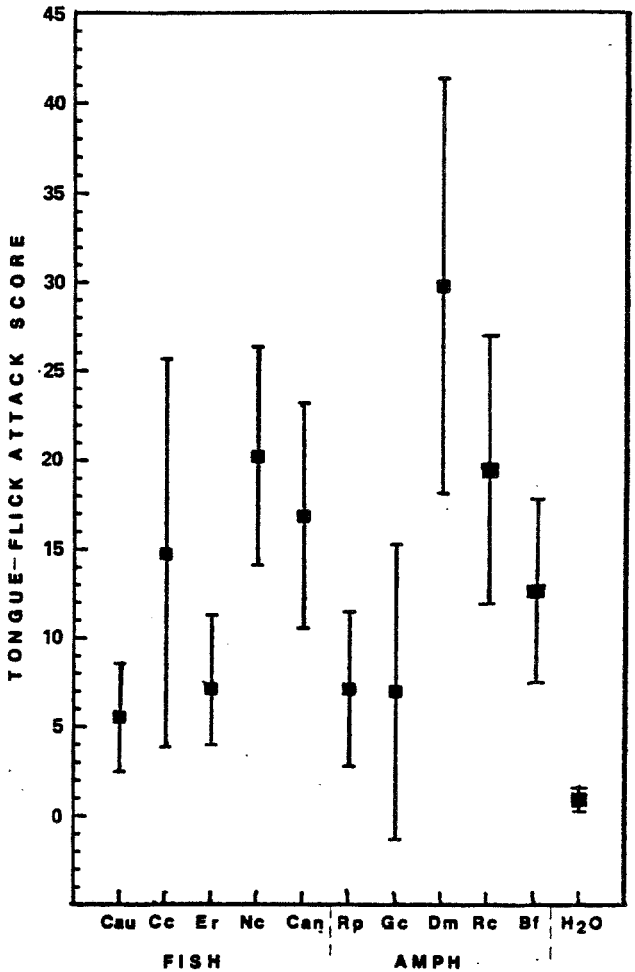

FIG. 5. The tongue-flick attack scores of 10-day-old water snakes (*N. sipedon*) born in the laboratory to parents from a mountain habitat in relation to extracts of prey species.

Comparisons Between Groups

Table 3 shows the Spearman rank order correlations for all groups of snakes (Siegel, 1956). The stimuli common to each paired group were used in computation, 8 in comparisons involving the laboratory-reared snakes, 11 in all other cases.

The results show that the only significant correlation involving either the adult or newborn mountain snakes is the one where they are correlated

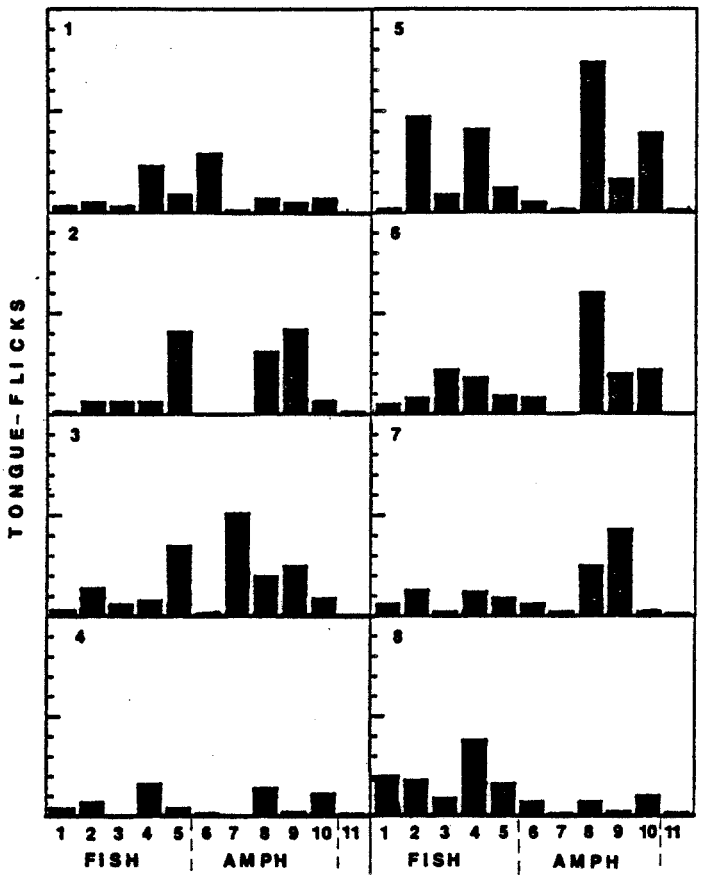

FIG. 6. The tongue-flick attack scores of individual newborn water snakes, *N. sipedon*, born to mountain parents in relation to extracts of prey species. The scale for each box is 0–50. The numbered extracts are: 1, *C. auratus*; 2, *C. carolinae*; 3, *E. ruflineatum*; 4, *N. coccogenis*; 5, *C. anomalum*; 6, *R. pipiens*; 7, *G. carolinensis*; 8, *D. monticola*; 9, *R. catesbiana*; 10, *B. fowleri*; 11, distilled H_2O.

TABLE 3. SPEARMAN RANK CORRELATIONS BETWEEN THE COMMON STIMULI PRESENTED TO DIFFERENT PAIRS OF WATER SNAKE (*N. sipedon*) GROUPS

	Newborn mountain	Bait hatchery	Laboratory-reared
Mountain	0.68[a]	−0.35	−0.11
Newborn mountain	—	−0.15	0.05
Bait-hatchery	—	—	0.90[b]

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

with each other. Similarly, the laboratory-reared and bait-hatchery snakes are significantly correlated with each other. The most negative correlation is that between the adult mountain and bait-hatchery populations.

DISCUSSION

Laboratory-reared *N. sipedon* responded more to the extract of prey that they had eaten for one year than to extracts of foods they had never eaten. The snakes were not tested before their first feeding, so it is not known conclusively that their response had changed because of their eating experience. However, Burghardt's (1968) response profile of newborn *N. sipedon* showed that a frog extract elicited a greater response than minnow or goldfish. Fuchs and Burghardt (1971) also found a change in response pattern to extracts because of feeding experience.

An intermediate response was given by the laboratory snakes to extracts of species that they were reported to eat in natural situations, such as minnow, frog, etc. Lower responses were elicited by distilled water and to extracts of prey species that the snakes do not normally eat, such as mouse and crayfish. This may indicate that the intermediate level of response to new prey extracts is due to innate preferences of the snakes. It would be advantageous to the snakes to retain the ability to respond to species-characteristic prey even while increasing the response to particular foods, in case prey availability in their activity range changes.

The wild-caught snakes with unknown experience were tested with similar extracts. Some of the extracted species are found in the mountains, some at the bait hatchery, and at least two, *R. pipiens* and *B. fowleri*, are found in both areas. A *Notropis* species, but not *N. coccogenis*, occurs in a creek at the

bait hatchery. Its population there is probably much lower than that of *C. auratus*. The highest responses of the mountain snakes were to extracts from prey found in the Tremont area; the highest responses of the bait-hatchery snakes were to extracts from prey found at Sterchi's. The attack scores were consistent with the combined scores (Gove, 1971). The mountain snakes demonstrated a relatively low response to the goldfish extract, even though they had eaten this species in the laboratory immediately prior to the testing sessions. This suggests that early experience is more important in determining the response than is recent experience. All of the adult mountain snakes had at least 1 meal of goldfish, and none had more than 4 (the meals were spaced 4 days apart, and consisted of 1 or 2 fish). These feedings were necessary to avoid possible large differences in motivation, since they were not all caught at the same time. In spite of this, responses to goldfish were low.

It would have been useful to include more fish species from the bait hatchery, but it is safe to assume that goldfish are important food items for snakes at the bait hatchery since they are abundant, even dying in overcrowded ponds. The creek minnows were observed to be less common and faster swimmers. Fraker (1970), who also worked with snakes from a fish hatchery, observed that *N. sipedon* ate equal amounts of frogs and goldfish.

The mountain snakes were much more responsive to extracts and water. This difference may be correlated with our difficulties in observing snakes at the bait hatchery compared with snakes at Tremont. The snakes which were not wary of humans may have been selected out by Mr. Sterchi's shotgun or otherwise eliminated. The snakes may consequently be more sensitive to the presence of humans and, in addition, may be more nocturnal. Both of these characteristics may have affected the degree of responding in the test setting.

Newborn snakes from the bait hatchery could not be obtained, so data from newborns of the two populations cannot be compared. The highest response of Tremont newborns is to the salamander *D. monticola*, which is common in small creeks and wet areas in the National Park. It is possible that the diets of the snakes vary with age and size, and that young salamanders may be easy to catch for young snakes. Those with an initially high response for the salamander may have an advantage to help them survive the period after birth, and thus may not be competing with older snakes. There is a greater response to the mountain fish extracts, especially significant with minnows, than to the goldfish extract; this may indicate that the later wide difference between Tremont and Sterchi adult snakes has some genetic component. Minnow species are extremely common throughout the range of *N. sipedon*, so this preference may be a selective advantage. The data from the mountain newborn snakes shows a great variability among littermates. Since the snakes were tested repeatedly with each extract, the variability

reflects real differences and is added evidence for chemical perception polymorphism found to exist in garter snakes (Burghardt, 1975).

The present experiments demonstrate that experienced snakes from separate areas have different prey-extract responses that are associated with local prey availability. They also show that the snakes can discriminate between different genera of fish on the basis of chemical cues alone. Further, the snake's feeding experience affects the responses to food extracts in different populations.

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MICROBIAL DECOMPOSITION OF FERULIC ACID IN SOIL

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Abstract—The suppression of plant growth by different phenolic acids is well known. This work was designed to determine if ferulic acid, a known phenolic inhibitor of plant growth, accumulates in the soil and if soil microorganisms could be isolated that metabolize it. Over 99% of the extractable ferulic acid was lost from decaying hackberry leaves in 300 days. During this time the amount in the top 15 cm of soil remained fairly constant at about 30 ppm, except for the March sample which was significantly higher than the rest. Addition of ferulic acid to soil caused an increase in CO_2 evolution and in numbers of a select group of microorganisms. *Rhodotorula rubra* and *Cephalosporium curtipes*, which actively metabolize ferulic acid, were isolated, but the metabolic pathways employed appear to be different from the reported one. The reported pathway for ferulic acid breakdown is ferulic acid to vanillic acid to protocatechuic acid to β-keto-adipic acid. *Rhodotorula rubra* was found to convert ferulic acid to vanillic acid, but no evidence was found for utilization of the rest of the pathway. *Cephalosporium curtipes* appears to use a different pathway or to metabolize intermediate compounds rapidly without accumulating them, because no phenolic compounds were found during the breakdown of ferulic acid. The presence in the soil of microorganisms that metabolize ferulic acid and other phenolic acids is ecologically significant because such organisms prevent long-term accumulations of these substances, which are toxic to many other microorganisms and higher plants.

Key Words—ferulic acid metabolism, vanillic acid, soil, microorganisms, *Rhodotorula rubra*, *Cephalosporium curtipes*.

INTRODUCTION

Ferulic, synapic, and *p*-coumaric acids are important in the synthesis of

lignin (Freudenberg and Neish, 1968). These same acids have been shown to be products of lignin degradation. The resistance of lignin to decomposition has been reviewed by Norman (1936), Alexander (1961), and Hurst and Burges (1967). These authors attribute this resistance to the chemicals of which it is composed and to the complex bonding of the lignin molecule. Many different organisms have been found to be involved in the decomposition of lignin (Gottlieb and Pelczar, 1951).

The products of lignin decomposition have been shown to suppress plant growth (Hennequin and Juste, 1967; Wang, Yang, and Chuang, 1967). Ferulic acid and *p*-coumaric acid have been shown to retard ion uptake and protein synthesis in plants (Croak, 1972), inhibit seed germination (Rasmussen and Rice, 1971; Wilson and Rice, 1968), and suppress plant growth (Guenzi and McCalla, 1966*a*). Degradation products have also been shown to retard growth of plant pathogens (Davey and Papavizas, 1959). Rice (1965*a*, 1968) demonstrated that phenolic acids retard the growth of several organisms involved in the nitrogen cycle.

Organic material on the soil surface is usually high in phenolic acids and especially high in acids involved in lignin synthesis (Rice, 1965*a*; Guenzi and McCalla, 1966*a*; Lodhi and Rice, 1971; Rasmussen and Rice, 1971). Therefore, it might be expected that these compounds would accumulate to high concentrations in the soil. This does not occur, however, because the amount in the soil is generally very low (Guenzi and McCalla, 1966*b*; Wang, Yang, and Chuang, 1967).

We hypothesized that lack of accumulation of high concentrations of phenolic acids in soil is due to the rapid decomposition of these acids upon entry into the soil. We also hypothesized, however, that if leaves are allowed to decompose on the surface, any loss in phenolic acids from them will cause at least a temporary increase in the amounts in the soil. Experiments were designed, (1) to test these hypotheses, (2) to determine if addition of ferulic acid to soil reduces the number and activities of microorganisms, (3) to attempt to isolate microorganisms from soil capable of utilizing ferulic acid as their sole carbon source, and (4) to determine metabolic pathways employed in the metabolism of ferulic acid.

METHODS AND MATERIALS

Disappearance of Ferulic Acid from Decomposing Leaves

In order to follow the loss of ferulic acid from a natural source, leaves of *Celtis laevigata* (hackberry) were collected immediately after leaf drop in the fall of 1971. This species was chosen because of the high amounts of ferulic acid found in its leaves (Lodhi and Rice, 1971). The leaves were oven

dried at 60°C for 24 hours and then sewn into nylon net bags which were weighed and numbered. The bags were returned to the collection area and placed on the soil surface in five rings spaced 0.75 M apart around a hackberry tree. Each ring consisted of 12 bags to permit removal of one bag from each ring every month for a year. When the bags were collected, 300 g of soil were removed from the 0–15-cm layer beneath each one and the ferulic acid concentration of this soil was determined.

The soil and leaf samples were oven dried at 60°C for 24 hours. All visible roots and debris were removed from the soil, and soil particles were removed from the leaf surfaces before soil and leaves were analyzed for ferulic acid.

Ferulic acid extractions were done by the procedure of Guenzi and McCalla (1966*b*). The samples were autoclaved in 2 N NaOH for 45 minutes at 15 psi, the debris was removed by filtration, and the pH of the filtrate was adjusted to 2 with concentrated HCl, which precipitated the humic acid fraction. This fraction was then removed by centrifugation at 12,000 *g* for 15 minutes. The resulting supernatant was extracted three times with one-third volume of anhydrous ethyl ether. The ether portions were evaporated to dryness, and the residue taken up in a known volume of 95% ethanol. A portion of the extract was then spotted on Whatman 3 MM chromatographic paper and developed by the descending technique in *n*-butanol–acetic acid–water (63 : 10 : 27) (BAW) in the first dimension, and 6% aqueous acetic acid (6% AA) in the second dimension. The developed papers were viewed under short (2537*A*) and long (3360*A*) UV light to reveal the bright blue ferulic acid spot. Other phenolics were identified, but no attempt was made to quantify them. The ferulic acid spots were eluted from the paper with 40% ethanol, and absorbance of the eluate was determined at 285 nm with a Beckman DBG Spectrophotometer. The concentration of ferulic acid was determined from a standard curve made by adding known amounts of the acid to Whatman 3 MM paper and following the same procedure as with the unknowns. The ferulic acid in the soil and in root samples was also extracted and its concentration determined by the above procedure.

Influence of Ferulic Acid on Microbial Activity

Prairie soil was air dried, passed through a 2-mm sieve, and amended with 500 μg/g ferulic acid. One hundred grams of this soil were added to a 500-ml Erlenmeyer flask with a side tube containing KOH (Bartha and Pramer, 1965). The moisture content was brought to 30% with distilled water. Carbon dioxide was trapped in 0.08 N KOH and titrated with 0.08 N HCl. The control soil was treated the same as the test soil, except it did not contain ferulic acid.

In connection with the above study, the number of microorganisms capable of utilizing ferulic acid was determined. A serial dilution was performed on a sample of this soil and 0.5 ml of each dilution was spread on agar plates containing M-9FA, a chemically defined medium. M-9FA consists of 1 g NH_4Cl, 0.13 g $MgSO_4$, 3.0 g KH_2PO_4, and 6.0 g Na_2HPO_4 per liter of water, with ferulic acid (500 ppm) as the carbon source. These plates were incubated at 30°C for 48 hours before counting.

In addition to ferulic acid (3-methoxy-4-hydroxycinnamic acid), vanillic (3-methoxy-4-hydroxybenzoic acid) and cinnamic acid (*trans*-benzenepropenoic acid) were tested to determine their influence on the soil. The evolution of CO_2 was used as an indicator of microbial activity.

Isolation and Identification of Organisms

Two methods were used to isolate organisms that decompose ferulic acid. The first was to take soil from areas with the potential for large amounts of ferulic acid, do a serial dilution on the soil samples, and plate each dilution out on the previously described medium (M-9FA). Any organism that appeared in the highest dilution was streaked on slants of M-9FA. If the organism continued to grow, it was saved; if not, it was discarded. The second method was a soil-perfusion procedure (Goswami and Green, 1971) in which ferulic acid at 1000 ppm in water was allowed to circulate through a column of soil for two weeks; a portion of the soil was then removed, diluted, and plated out on M-9FA. These two methods plus the organisms isolated from the respiration study yielded eighteen different isolates. Each isolate was inoculated into M-9FA liquid medium and placed on a reciprocating shaker for 24 hours to determine which organisms utilize ferulic acid as the sole source of carbon. The amount of growth was determined by the density of the medium. Two cultures grew significantly better in a 24-hour period; these were identified as *Cephalosporium curtipes* and *Rhodotorula rubra*.

Metabolic Studies

The optimal pH for growth in ferulic acid of these two organisms was determined by culturing them at different pHs and then determining the utilization of ferulic acid during a 24-hour period.

The growth rate of *Rhodotorula rubra* on ferulic acid was measured by following increases in absorbance and decreases in ferulic acid content of the medium. The decrease in ferulic acid was also followed with *Cephalosporium curtipes*, and biomass measurements were taken. In these studies, the inoculum was prepared by growing cells for 24 hours on a reciprocating shaker in M-9FA medium, then transferring 1 ml of inoculum to a 250-ml

Erlenmeyer flask containing 50 ml of the same medium. The absorbance of *R. rubra* cultures was determined by growing the organism in 250-ml Erlenmeyer flasks equipped with side-arms which fit into a Spectronic 20. Measurements were taken at 600 nm. Two flasks were harvested at each time interval to determine the concentration of the remaining ferulic acid. *Rhodotorula* cells were removed from the medium by centrifugation. Because *Cephalosporium curtipes* did not pellet well upon centrifugation, it was removed from the medium by filtration through a 0.45-μm Millipore filter and then dried for 24 hours at 60°C, after which the hyphal weight was determined.

The loss of ferulic acid during the growth cycle was followed by extracting ferulic acid from the cell-free spent medium. The first step of the extraction procedure was the adjustment of the medium to pH 2.0 with concentrated HCl. The ferulic acid was extracted with anhydrous ethyl ether, the ether was evaporated, and the residue taken up in a known volume of 95% ethanol. Absorbance was determined at 309 nm and amounts of ferulic acid were determined from a standard curve. This wavelength is slightly different from that cited in the decomposition section because the maximum absorption of ferulic acid varies, depending upon the concentration of water present.

It was of interest at this point to determine if other species of *Cephalosporium* and *Rhodotorula* could utilize ferulic acid as a sole source of carbon. Four species of *Rhodotorula* and three species of *Cephalosporium* were selected from the American Type Culture Collection on the basis of the original source of isolation. We specifically wanted soil isolates. All seven selected species were grown for 72 hours in shaker flasks using the M-9FA medium. Any increase in absorbance was taken as a sign of growth. As a control, the seven organisms were also grown in the same chemically defined medium with glucose as the carbon source instead of ferulic acid.

Identification of Intermediates

The growth rates of *Cephalosporium* and *Rhodotorula* were also determined on vanillic, *p*-hydroxybenzoic, cinnamic, and synapic acids. In order to determine whether intermediates were produced, a portion of the ether fraction was chromatographed on Whatman 3 MM paper. The chromatograms were developed in BAW followed by 6% AA as previously described. *Cephalosporium* and *Rhodotorula* were also grown on M-9 (same as M-9FA without ferulic acid) with glucose as the carbon source and the spent medium was extracted as previously described. Paper chromatograms were prepared of this spent medium and of that obtained when the organisms were grown on specific phenolic acids. Resulting phenolic compounds were identified only if they were produced in the phenolic acid medium

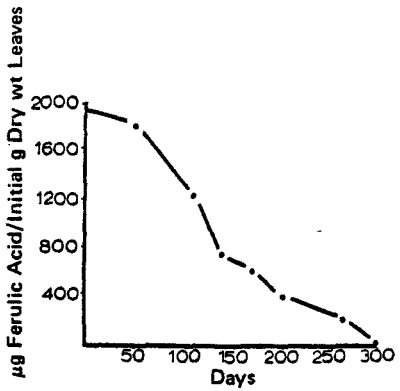


FIG. 1. Change in concentration of ferulic acid during decomposition of leaves of *Celtis laevigata*. Concentration is plotted against time in days starting from day 0, which was December 10, 1971.

and not in the glucose medium. The identification procedures used closely followed those described by Rice (1965*b*).

RESULTS

Disappearance of Ferulic Acid from Decomposing Leaves

Analysis of the decaying leaves placed around the hackberry tree showed that over 65% of the extractable ferulic acid was lost in the first 100 days, and by day 300 less than 0.1% remained in the leaves (Figure 1). The rate was much faster during the early part of the experiment (January–April) than during the last part (April–October).

A statistical comparison between the results from individual months was made with Student's *t* test, and there was a significant difference at the 0.05 level between samples for adjacent months from day 1 to day 150.

TABLE 1. CONCENTRATION OF FERULIC ACID IN SOIL UNDER BAGS OF DECOMPOSING HACKBERRY LEAVES

Days[a]	0	30	100	130	161	191	222	253	300	365
Ppm[b]	25	26	47[c]	22	22	30	34	27	32	23

[a] Number of days from start of decomposition.
[b] Each figure is average of five analyses.
[c] Difference from concentrations at day 30 and day 130 significant at 0.05 level.

TABLE 2. CHROMATOGRAPHY OF PHENOLIC ACIDS EXTRACTED FROM LEAVES AND SOIL

Compound	*Rf*s on Whatman #1[a]		Fluorescence		Reagent color[b,c]		
	BAW	6% AA	Long UV	Short UV	*p*-Nit[b]	Sulfanilic[b]	$FeCl_3$–$K_3Fe(CN)_6$[b]
Ferulic acid	0.83	0.46	bl	bl	br	org	bl
Suspected ferulic	0.80	0.44	bl	bl	br	org	bl
Vanillic acid	0.88	0.56	none	viol	viol	org	bl
Suspected vanillic	0.84	0.55	none	viol	viol	org	bl
p-Coumaric acid	0.85	0.52	none	abs	viol	org	bl
Suspected *p*-coumaric	0.85	0.48	none	abs	viol	org	bl
p-Hydroxybenzoic acid	0.88	0.66	none	abs	red	yel	dk bl
Suspected *p*-hydroxy-benzoic	0.89	0.67	none	abs	red	yel	dk bl

[a] See text for solvent systems.
[b] Diazotized *p*-nitroaniline (Bray et al., 1950), diazotized sulfanilic acid (Bray et al., 1950), ferric chloride–potassium ferricyanide (Smith, 1960, p. 329).
[c] bl, blue; viol, violet; br, brown; dk, dark; abs, absorption; org, orange.

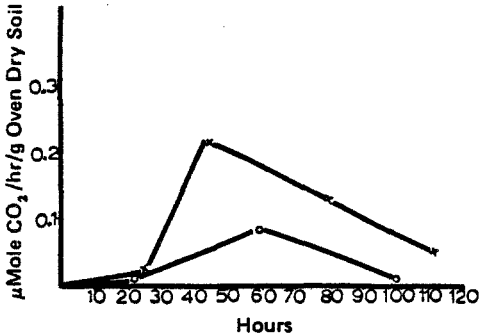


FIG. 2. Effect of ferulic and vanillic acids on CO_2 evolution from the soil: ×, effect of ferulic acid; ○, effect of vanillic acid. Amounts shown are those resulting after subtracting control values.

Furthermore, there was a significant difference at the 0.05 level between day 150 and day 300, but differences in amounts for adjacent months during this time were not significant. The experiment was terminated on day 300 because of the low amount of ferulic acid remaining in the leaves.

Concentrations of ferulic acid in the soil under the bags of decaying leaves usually remained relatively constant from month to month except for minor fluctuations which were not statistically significant (Table 1). The only exception to this was the concentration at day 100, which was significantly higher than the concentration at the previous sampling period and than that at the following sampling period.

Other phenolic acids identified from the soil were *p*-hydroxybenzoic, *p*-coumaric, and vanillic acids (Table 2). These were not found in the leaves with the Guenzi–McCalla technique.

Young roots of hackberry trees contained 2000 ppm ferulic acid.

Effect of Ferulic Acid on Microbial Activity

Similar curves of CO_2 evolution indicated that addition of ferulic and vanillic acid to soil elicited similar responses from the soil population (Figure 2). Addition of cinnamic acid resulted in a similar curve except that the lag phase was 14 days long.

The concentration of microorganisms in the soil increased greatly on addition of ferulic acid (Figure 3), and the increase was about twice as great as in unamended soil. A pseudomonad and an actinomycete-like organism (Skerman, 1967) accounted for virtually the total number of microorganisms that responded to the amended soil. These two organisms were discarded from later metabolic studies because of their slow growth on ferulic acid.

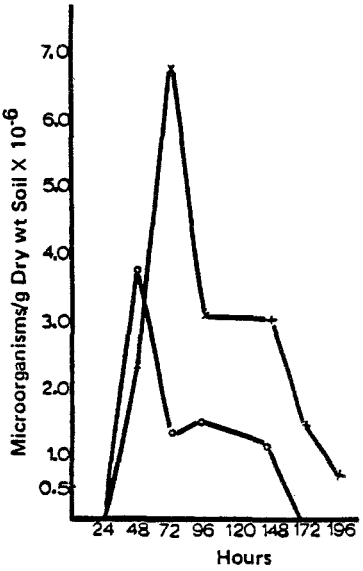


FIG. 3. Change in microbial numbers in soil amended with 500 ppm ferulic acid, ×; unamended soil, ○.

Identification of Organisms

The preliminary isolation was done with unamended soil from the climax tall-grass prairie located near the hackberry tree which was used in the decomposition experiment. It yielded eight different colony types. Three of the colonies were hard and crusty and gave a gram-negative reaction. One species of *Penicillium* (Barnett and Hunter, 1972) and *Cephalosporium curtipes* (Gilman, 1957) were also isolated. Three of the original isolates

TABLE 3. EFFECT OF pH ON UTILIZATION OF FERULIC ACID DURING A 24-HOUR PERIOD BY *Rhodotorula* AND *Cephalosporium* ON M-9FA WITH AN ORIGINAL CONCENTRATION OF 500 PPM OF FERULIC ACID

Rhodotorula rubra		*Cephalosporium*	
pH	Ppm of ferulic acid utilized	pH	Ppm ferrulic acid utilized
7.3	436	6.2	226
6.0	300	5.8	441
5.3	300	5.2	413
3.0	24	3.5	100

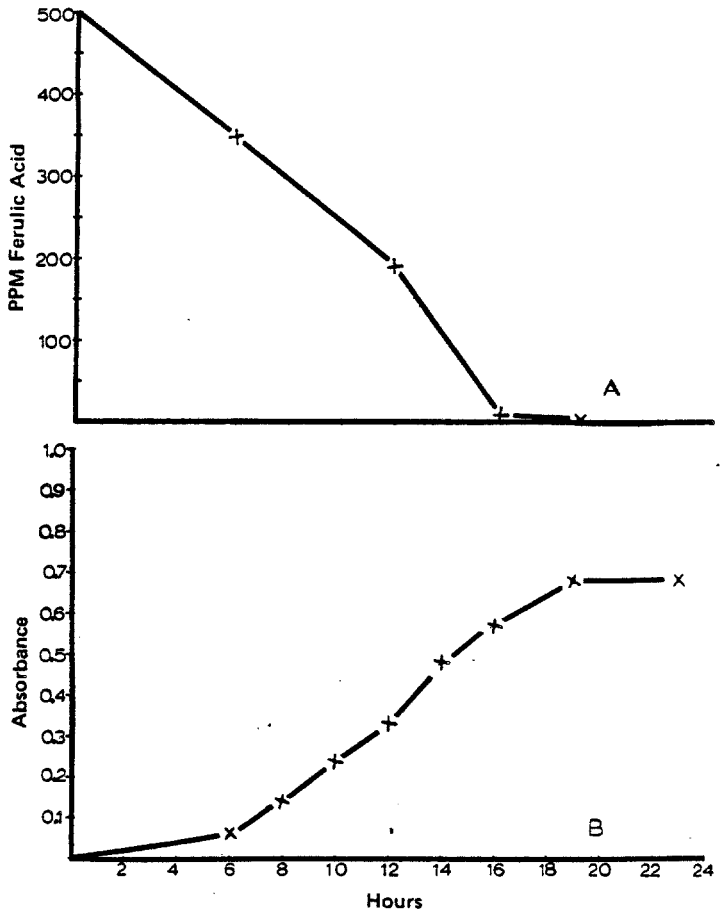


FIG. 4. Growth of *Rhodotorula rubra* in M-9 with ferulic acid as sole source of carbon: A, change in concentration of ferulic acid during growth cycle; B, change in absorbance during growth cycle.

did not grow after the initial isolation. The *Cephalosporium* was used in later studies because of its rapid growth on ferulic acid. The second isolation technique employed soil enriched with 1000 ppm ferulic acid. This procedure yielded nine morphologically different organisms, of which eight were gram-negative and one gram-positive. With physiological tests, several organisms were keyed to the genus *Pseudomonas* (Skerman, 1967). The last isolations were obtained by percolating ferulic acid through a column of soil for 14 days. This procedure yielded only one major organism, which was identified as *Rhodotorula rubra* (Lodder and Kreger-van Rij, 1952).

Further growth tests in a liquid medium with ferulic acid as the sole carbon source resulted in the elimination of all of the original isolates except *Cephalosporium curtipes* and *Rhodotorula rubra*, which were used in subsequent studies.

Metabolic Studies

Of the pHs tested, the optimum for the utilization of ferulic acid by *Rhodotorula rubra* was 7.3, whereas the optimum for *Cephalosporium curtipes* was 5.8 (Table 3). In subsequent studies, both organisms were grown at pH 5.0 because both organisms grew well at this pH with ferulic acid as the sole carbon source.

The efficiency of *Rhodotorula* in utilization of ferulic acid is indicated by almost total removal of the acid from the medium (Figure 4). *Rhodotorula* also appears to grow almost as well on vanillic, cinnamic, and

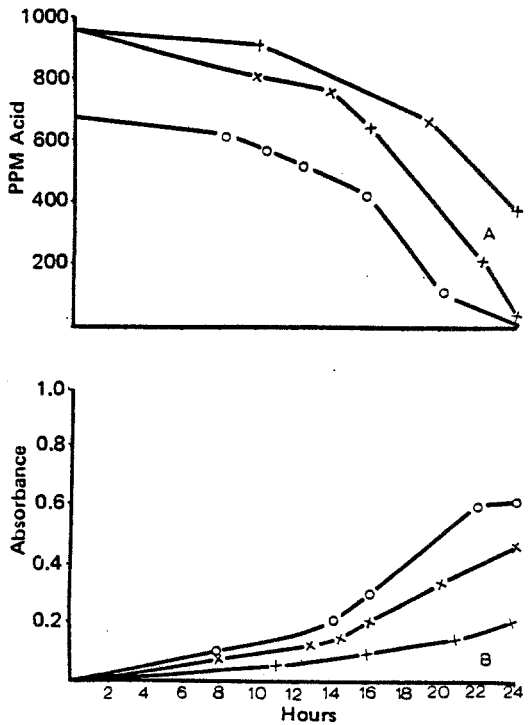


FIG. 5. Growth of *Rhodotorula rubra* in M-9 with sole source of carbon being: vanillic acid, ×; cinnamic acid, ○; or *p*-hydroxybenzoic acid, +. A, ppm of the carbon source plotted against time; B, change in absorbance during growth on different carbon sources.

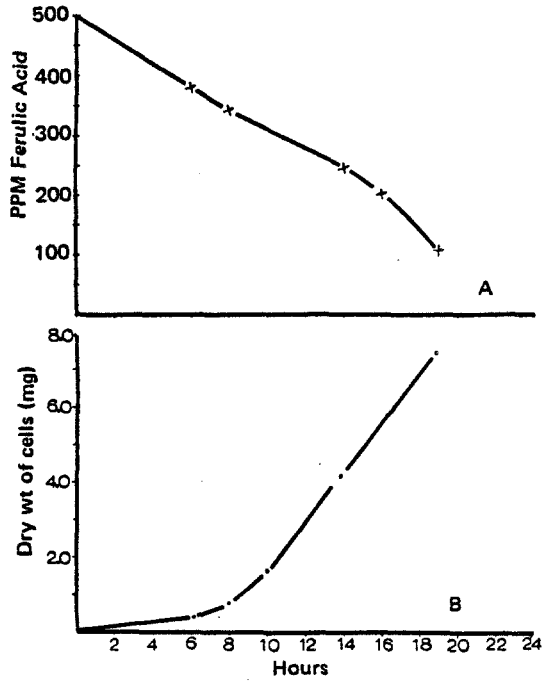


FIG. 6. Growth of *Cephalosporium curtipes* on ferulic acid as sole source of carbon; A, change in concentration of ferulic acid; B, change in biomass of culture.

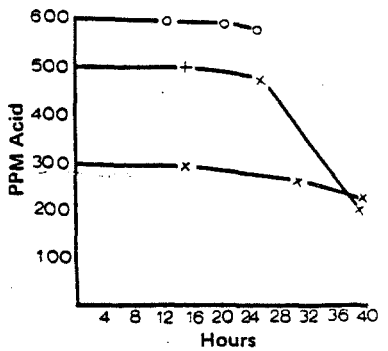


FIG. 7. Change in concentration with time of vanillic acid, ×; *p*-hydroxybenzoic acid, +; and cinnamic acid, ○; when used by *Cephalosporium curtipes* as sole source of carbon.

TABLE 4. ABILITY OF SEVERAL SPECIES OF *Cephalosporium* AND *Rhodotorula* TO UTILIZE FERULIC ACID OR GLUCOSE AS THE SOLE CARBON SOURCE

Organism	ATCC No.	Glucose	Ferulic
Cephalosporium furcatum	16218	+	+
C. khandalense	16091	+	−
C. nordinii	16236	+	−
C. roseum	16227	+	−
Rhodotorula glutinis	10788	+	+
R. rubra (mucilaginosa)	16639	+	+
R. lactosa	18177	−	−

p-hydroxybenzoic acids (Figure 5). *Cephalosporium* was not as efficient as *Rhodotorula* in removal of ferulic acid from the medium during an 18-hour period of growth (Figure 6A). When both organisms were grown for 18 hours on ferulic acid, *Rhodotorula* removed over 95% of the carbon source, whereas *Cephalosporium* removed only about 80%. *Cephalosporium* utilized ferulic acid more rapidly than it did vanillic, cinnamic, or *p*-hydroxybenzoic acids (Figures 6, 7).

One of the four species of *Cephalosporium* obtained from the American Type Culture Collection was able to utilize ferulic acid as the sole carbon source, but all could utilize glucose (Table 4). Two of the three species of *Rhodoturula* from the same source were able to utilize ferulic acid, and two were able to use glucose (Table 4). No additional experiments were performed using these organisms.

Identification of Intermediates

When *Rhodotorula* grew on ferulic acid, vanillic acid appeared in the

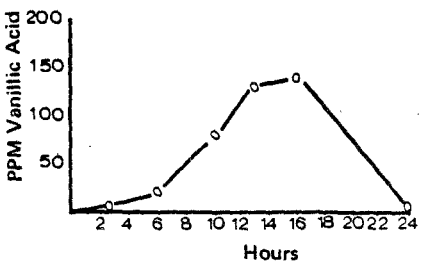


FIG. 8. Appearance of vanillic acid in medium during growth of *Rhodotorula rubra* on ferulic acid.

medium immediately, with a peak concentration appearing after 18 hours (Figure 8). No other phenolic acids were detected during this time period. When *Rhodotorula* was grown on vanillic, cinnamic, and *p*-hydroxybenzoic acid, no phenolic intermediates were detected.

There were no phenolic intermediates detected when *Cephalosporium* was grown on ferulic, vanillic, or *p*-hydroxybenzoic acid.

DISCUSSION

At the outset of this research, we suspected that ferulic acid in leaves would be lost at a rate correlated with that of the breakdown of lignin. The rate of lignin breakdown has been reported by several different authors (Phillips, Weihe, and Smith, 1930; Martin and Wang, 1944) to vary according to the type of plant material being decomposed, but in all cases, it has been shown to be very slow. On the other hand, the rate of disappearance of ferulic acid from the leaves as indicated by the Guenzi–McCalla technique seems to be much faster than any reported rates of lignin breakdown. There is some evidence for the presence of other ferulic acid containing compounds in plants, and these have been shown to be rapidly decomposed by microorganisms (Sundman, 1964*a*, 1964*b*; Tom and Wood, 1970). Among these are the lignans which have been reported to be widespread in the plant world (Robinson, 1968), but we could find no data on the amounts present in plants. Thus their possible role in explaining the rapid loss of ferulic acid from hackberry leaves is only speculative. Ferulic acid occurs as the free acid in some plants, and also in numerous glycosides which are relatively easily decomposed (Harborne, 1964).

It was assumed that ferulic acid would leach into the soil after being released from the bound form in the leaf and would thus increase the concentration of this acid in the soil. We were unable to test this assumption adequately, however, due to limitations imposed by low concentrations of ferulic acid in the soil and by available techniques. It was desirable to keep the quantity of leaves per unit area in the nylon bags similar to that on the soil elsewhere under the hackberry tree. This resulted in a very small amount of ferulic acid being released per bag compared with the amount of soil analyzed under the bag (ca. 300 g). Possibly the minor fluctuations in ferulic acid concentration in the soil from month to month could have resulted in part from the leaching of the released material from the leaves. Moreover, it is likely that the concentration of ferulic acid increased considerably in a thin layer of surface soil next to the leaves. On the other hand, the very large increase in concentration of ferulic acid in the soil down to a depth of 15 cm which was measured at day 100 (March) could not have resulted from

material leaching from the leaves. This additional ferulic acid must have been exuded from living roots of the hackberry or must have been leached from dead or decomposing roots, because the roots had an initial concentration of ferulic acid as high as the leaves.

Wang et al. (1971) found that they could not recover several phenolic compounds with cold alcoholic NaOH immediately after adding them to soil. They suggested that the added phenolic compounds were being chemically bound by humic acid. Others have reported a similar binding of phenolic compounds with organic material in the soil (Blum and Rice, 1969; Rice and Pancholy, 1973). Blum and Rice (1969) found that they had to add 400 ppm tannic acid to a prairie soil under investigation before they could recover any by immediate extraction with acetone. Nevertheless, the addition of only 30 ppm tannic acid to the same soil severely inhibited the nodulation of heavily inoculated bean plants. Thus, the binding of at least some phenolic compounds by organic matter in soil does not keep the compounds from being biologically active.

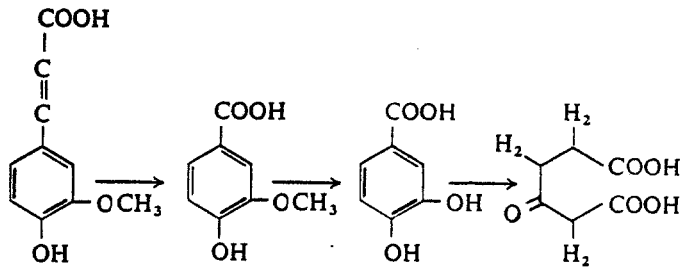
It seems doubtful that the ferulic acid level was being maintained at a relatively constant level through most of the year due to binding by soil organic matter because of the large increase in extractable ferulic acid which occurred in March. Any binding which occurs usually seems to take place rapidly so that the material cannot be recovered immediately after its addition (Wang et al., 1971). Another point of significance in the present investigation is that extraction was achieved by autoclaving the soil with 2 N NaOH for 45 minutes at 15 psi. This releases at least some phenolic compounds from the bound form (Rice and Pancholy, 1973). It seems likely, therefore, that the reduction in ferulic acid from the peak in March to the average level was due to microbial decomposition.

When ferulic acid was added to the soil, there was an increase in the evolution of CO_2 accompanied by an increase in total numbers of microorganisms in the soil. This increase in numbers was due almost entirely to two different organisms. One organism was identified as a pseudomonad, and the other appeared to be an actinomycete, but no positive identifications were made. The failure of the usual soil microorganisms to appear on sample plates indicated that they were inhibited by the ferulic acid.

Eighteen microorganisms were isolated which were able to grow in soil amended with ferulic acid. Many of these isolates were identified as belonging to the genus *Pseudomonas*; three were fungi, *Cephalosporium curtipes*, *Penicillium* sp., and *Rhodotorula rubra*; and the other organisms appeared to be actinomycetes. The isolation of bacteria, actinomycetes, and fungi which were apparently able to utilize ferulic acid as a carbon source agrees with reports of other workers (Henderson and Farmer, 1955; Henderson, 1956; diMenna, 1959). Among the isolated organisms, *Cephalosporium curtipes*

and *Rhodotorula rubra* grew best with ferulic acid as the sole carbon source. Several species of *Cephalosporium* and *Rhodotorula* were subsequently selected from the American Type Culture Collection and tested for their ability to use ferulic acid as the sole carbon source. Only one species of *Cephalosporium* and two of *Rhodotorula* were found to be able to do this, and one of the species of *Rhodotorula* was a strain of *R. rubra*.

A proposed scheme for the metabolism of ferulic acid by certain microorganisms was developed by Henderson (1963).



Ferulic Acid Vanillic Acid Protocatechuic Acid β-Keto-adipic Acid

The strain of *R. rubra* used in this study appeared to employ at least part of this scheme, because a definite conversion of ferulic acid to vanillic acid was observed. When *Rhodotorula* was grown on vanillic acid, however, no detectable amount of protocatechuic acid or any other phenolic compound was found. No attempt was made to look for nonphenolic compounds. *C. curtipes* appeared either to utilize ferulic acid directly, to use another pathway that does not involve phenolic compounds, or to break down intermediate compounds rapidly without accumulating them. Henderson and Farmer (1955) isolated many organisms that metabolized ferulic acid and found many that did not utilize the scheme as outlined above. This indicates involvement of other pathways in ferulic acid metabolism.

In conclusion, it was observed that ferulic acid was rapidly lost from decomposing hackberry leaves. On the other hand, the amount of extractable ferulic acid in the top 15 cm of soil under a hackberry tree remained relatively constant throughout the year, except for a large increase in the month of March. The isolation of two soil microorganisms that actively metabolize ferulic and other phenolic acids provides a possible explanation for the lack of a long-term accumulation of these compounds in the soil. This decomposition of ferulic and other phenolic acids in the soil is of ecological importance, because accumulation of such compounds to high concentrations could significantly reduce most plant growth.

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NORSESQUITERPENES AS DEFENSIVE TOXINS OF WHIRLIGIG BEETLES (COLEOPTERA: GYRINIDAE)

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Abstract—By use of a minnow bioassay, toxins were detected in the pygidial secretions of the gyrinid beetles *Dineutus assimilis* (Kirby) and *Dineutus nigrior* Roberts. The active agents, which may be largely responsible for the relative immunity of the Gyrinidae from predation, were isolated and identified as the norsesquiterpenes *gyrinidione* [(E)-1-methyl-2-formyl-3-(1′-methylhex-3′-ene-2′,5′-dione)-cyclopentane], *gyrinidone* [(E,Z)-2-hydroxy-5,9-dimethyl-4-(but-1′-ene-3′-one)-3-oxo-bicyclo[4.3.0]-non-4-ene], *gyrinidal* [(E,E,E)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal], and *isogyrinidal* [(E,E,Z)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal]. Since gyrinidione and isogyrinidal are being reported for the first time, their physical and chemical properties are presented and biosynthetic relationships of the four norsesquiterpene structures are discussed. About 50% of the beetle defensive material was norsesquiterpenes, 25% polar lipids, and 20% could not be extracted from water into chloroform. As quantified by gas-liquid chromatography, *D. assimilis* contained 245 ± 73 μg and *D. nigrior* 144 ± 64 μg norsesquiterpenes per individual. The average relative composition of norsesquiterpenes in the pygidial secretions of both beetle species was constant: isogyrinidal, 6%; gyrinidone, 7%; gyrinidione, 36%; and gyrinidal, 48%. When administered externally in solution to fish, isolated norsesquiterpenes possessed narcotic and toxic activity similar to that of the anesthetic steroids deoxycorticosterone (DOC) and testosterone. Minnow dose-response curves demonstrated that gyrinidione and gyrinidal (LC_{100} = *ca.* 2 μg/ml) were as toxic to fish as was DOC (LC_{100} = *ca.* 3 μg/ml). Gyrinidone was less toxic (LC_{100} = *ca.* 15 μg/ml) while isogyrinidal was relatively inactive (LC_{100} = *ca.* 90 μg/ml).

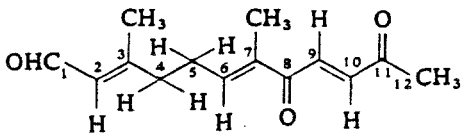
Key Words—biocommunication, defensive secretions, Gyrinidae, norsesquiterpenes, gyrinidal, isogyrinidal, gyrinidone, gyrinidione, steroid anesthesia.

INTRODUCTION

Adult whirligig beetles, family Gyrinidae, commonly inhabit the surface of ponds, small lakes, and some slow-flowing streams. Being gregarious, these beetles frequently assemble into stable clusters and at times congregate by the thousands into compact flotillas. Although they seemingly represent a food source conspicuously available to predators both from above and below the water surface, the relative immunity of gyrinids from predation by birds, amphibians, and fish has been amply documented (Wilson, 1923; Benfield, 1970 and references therein).

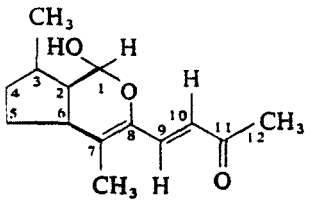
Previously, gyrinid immunity from predation has been attributed largely to morphological adaptations for escaping predators such as visually acute compound eyes adapted for vision both in air and in water (Leech and Chandler, 1956), a well developed Johnston's organ sensitive to minute disturbances in water (Rudolph, 1967), and a powerful swimming apparatus (Natchtigall, 1965). Although it had long been a matter of speculation (Wilson, 1923), the odorous secretions from the paired pygidial glands of the Gyrinidae have only recently been shown to have defensive value. In a series of feeding experiments, Benfield (1972) clearly demonstrated that gyrinid pygidial secretions served as feeding deterrents to several species of fish and to the common newt. Thus, in addition to adaptations for escape, chemical agents have been implicated as principal regulators of gyrinid predator-prey interactions.

The pygidial secretions of the Gyrinidae have attracted the attention of several groups of chemists who have identified some of the components from various species. Schildknecht and Neumaier (1970) partially characterized a major component of the pygidial secretions of *Gyrinus natator* as a C_{14} tricarbonyl compound with the molecular formula $C_{14}H_{18}O_3$ and Schildknecht et al. (1972*a*) established the complete structure of this component as the norsesquiterpene (E,E,E)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal, *gyrinal* (Structure I),



(I)

and reported its presence in *Gyrinus substriatus* and *Gyrinus minutus* as well. Meinwald et al. (1972) independently identified (I), which they named *gyrinidal*, from *Gyrinus ventralis*, *Dineutus hornii*, and *Dineutus serrulatus* and mentioned that at submicrogram levels this material was a feeding deterrent to a species of bass. The cyclopentanoid norsesquiterpene *gyrinidone* (II),



(II)

was identified by Wheeler et al. (1972) as the major component of the pygidial secretions of *Dineutus discolor* and the presence of a mixture of unidentified isomeric aldehydes was noted. The volatile odorous components of *G. natator* pygidial secretions have been identified as 3-methylbutyraldehyde and 3-methylbutan-1-ol (Schildknecht et al., 1972*b*).

Although a number of compounds have been identified from the pygidial secretions of the Gyrinidae, their biological activities have received little or no study. Some of these compounds may be primarily responsible for the repellent nature of unfractionated pygidial secretions (Meinwald *et al.*, 1972). Others may function as the water-borne gyrinid alarm substances also present in these secretions (Benfield; 1970). The identified components, as well as other unidentified pygidial agents, may possess still further biological activity.

In this study, the pygidial secretions of *Dineutus assimilis* (Kirby) and *Dineutus nigrior* Roberts were examined for agents possessing narcotic or toxic activity similar to that of the dytiscid defensive steroids (Schildknecht, 1970; Miller and Mumma, 1973). Such toxins were detected by a minnow bioassay and were isolated and identified as gyrinidal, gyrinidone, and two new norsesquiterpenes, *gyrinidione* and *isogyrinidal*. Quantitative data on the composition of pygidial secretions are presented and a comparison is drawn between the biological activity of the norsesquiterpenes and that of the anesthetic steroids.

METHODS AND MATERIALS

Collection of Beetles and Pygidial Secretions

D. assimilis and *D. nigrior* were netted by the thousands from a large irrigation reservoir at the Rock Springs experimental farms 10 miles southwest of State College, Pennsylvania. Beetle collection was greatly facilitated by use of a flotation procedure, employing a chain of logs fastened end to end, that forced the gyrinids into large assemblages near the shoreline.

The electrical shocking procedure for mass collection of water beetle defensive secretions (Miller and Mumma, 1973) was used to efficiently collect the white, paste-like pygidial secretions in aqueous solution. By shocking 500 beetles (50–100 at a time) in the same 500-ml sample of tap water, opaque milky-white suspensions of defensive materials were obtained. Aqueous

defensive suspensions that were not immediately fractionated were stored at 8°C in sealed flasks.

Bioassay for Toxicity

The bioassay animals, fathead minnows (*Pimephales promelas* Raf.) weighing between 1 and 2 grams, were purchased from a live-bait dealer and were available year-round. The physiological state of all batches of minnows was standardized by storing them in aquaria at 5°C without feed for at least 48 hours before they were used for bioassays. As needed, minnows were allowed to equilibrate from 5°C to 25°C over a period of 2–3 hr and were subsequently used for bioassays within the next few hours. All bioassays were carried out in a temperature bath at 25 ± 0.5°C. Maximal sensitivity and excellent reproducibility of the bioassay were realized when these procedures were followed.

In initial tests for toxicity of gyrinid pygidial agents, the secretions of at least 10 beetles were added to 50 ml of well aerated water containing 3 *P. promelas*. Immediately upon addition of these materials the minnows characteristically reacted violently, darting about the 250-ml beakers in which they were contained, occasionally leaping into the air. After several minutes in the defensive solutions, the swimming ability of minnows was impaired and they suffered loss of equilibrium, turning on their sides when not actively swimming. Muscular activity progressively diminished, culminating in death. Minnow survival time in solutions of defensive secretions was dependent upon the concentration of pygidial agents administered. This simple bioassay procedure was used to trace the toxic factors as the pygidial materials were fractionated by standard chemical procedures.

Dose-responses curves for the isolated toxins and several steroid standards were obtained by recording the average elapsed time from the addition of varying amounts of the pure compounds to 3 *P. promelas*, contained in 50 ml of aerated tap water in a 250-ml beaker, until the gill-pumping motions of the fish ceased. Minnows used in these experiments averaged 1.2 g (range 1.0–1.5 g). Stock solutions of the compounds to be tested were prepared in ethanol (66.7 μg/μl) and the appropriate amount of stock solution was injected into the bioassay containers. Appropriate control experiments demonstrated no additive toxic effects from ethanol. All compounds tested had solubilities greater than 120 μg/ml water.

Isolation of Toxic Agents

The defensive suspensions of *D. assimilis* and *D. nigrior* were thrice extracted with equal volumes of chloroform and the resultant fractions bio-

assayed. Aliquots of the lipid portion of the defensive solutions, dissolved in 20 μl ethanol, produced toxic effects resembling those caused by the original suspensions while the aqueous fraction was completely inactive.

When chloroform extracts of the defensive secretions were further fractionated by silicic acid column chromatography (CC) into nonpolar (eluted in $CHCl_3$) and polar (eluted in CH_3OH) lipids (Rouser et al., 1965), only the nonpolar lipids were toxic. Nonpolar lipids were fractionated by thin-layer chromatography (TLC) using the adsorbents Supelcosil 12A and 12B, containing a zinc silicate phosphor (Supelco, Inc., Bellefonte, Pennsylvania) and the solvent systems diethyl ether–petroleum ether (60–70°C) (50:50, v/v) and cyclohexane–ethyl acetate (50:50, v/v). Chromatograms were examined for bands under long- and short-wavelength ultraviolet (UV) light and by charring with H_2SO_4. All resultant bands were scraped, eluted from the adsorbent with chloroform–methanol (2:1), and bioassayed. Active materials were isolated in quantities sufficient for chemical identifications and further bioassay studies by preparative TLC, employing multiple development in the above-mentioned solvent systems, and by extensive column chromatography (CC) on Supelcosil-ATF 120 (Supelco, Inc., Bellefonte, Pennsylvania), eluting with increasing percentages of ethyl acetate in cyclohexane or increasing percentages of chloroform in benzene.

Instrumentation for Identification of Isolated Toxins

UV spectra were recorded on a Bausch and Lomb Spectronic 600 recording spectrophotometer. Infrared (IR) spectra were recorded on a Perkin-Elmer 621 IR spectrophotometer using a focusing-beam condenser and a 0.4-μl capacity microcell containing the compounds in chloroform solution. Low-resolution mass spectra were obtained on a LKB-9000 gas-liquid chromatograph (GLC) interfaced mass spectrometer using a 6-ft × $\frac{3}{16}$-in O.D. glass column packed with 2.0% OV-1 on Supelcoport 100/120 (Supelco, Inc., Bellefonte, Pennsylvania), a He flow of 30 ml/min, and a column oven temperature of 180°C. High-resolution mass spectra were taken on an AEI Model MS-902 mass spectrometer using a direct sample inlet system. All mass spectra presented in the text were recorded at an ionization potential of 70 eV. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A-60A NMR spectrometer.

GLC Analysis

The nonpolar lipid components of the gyrinid defensive suspensions were chromatographed on a MicroTek 220 gas-liquid chromatograph using a 6-ft × 4-mm I.D. glass column packed with 2.0% OV-1 on Supelcoport 100/120, a

N_2 flow of 40 ml/min and a column oven temperature of 170°C. Metal columns were avoided since they catalyzed isomerizations of the defensive compounds. The dual-flame ionization detector signal was coupled to an Aerograph 471 digital integrator for recording peak areas.

Since absolute identification of *D. assimilis* and *D. nigrior* requires microscopic examination of their genitalia, separation of the two species could not be accomplished without loss of some defensive materials due to manipulation. Therefore, in quantifying the defensive titer of these beetles it was necessary to first separately collect the pygidial secretions of each individual in the sample and then make identifications. Pygidial secretions of individual beetles were collected in 2 ml tap water by electrical shocking, then immediately extracted into 2 ml chloroform and quantified by GLC.

RESULTS

Yields from Fractionation of Defensive Suspensions

Table 1 presents the dry-weight composition of *D. assimilis* pygidial secretions upon fractionation. The major portion of the secretions (81%) including all of the toxic agents, was extractable from water into chloroform. A significant portion of the secretions (23%) was polar lipids which had no adverse effects on *P. promelas* when bioassayed. Toxic compounds constituted nearly the entire nonpolar lipid fraction and were isolated in amounts averaging nearly 50% of the starting dry weight of 0.6 mg per beetle. Relative

TABLE 1. FRACTIONATION OF THE PYGIDIAL SECRETIONS OF *Dineutus assimilis*[a]

Fraction	Toxic to fish	Dry weight (μg/beetle)	Percent of total
Aqueous defensive suspension[b]	+	602	100
Water layer after chloroform[b] extraction	−	98	16
Chloroform extract	+	490	81
Polar lipids	−	140	23
Neutral lipids	+	320	53
Bands from TLC	+	300	50

[a] Typical yields from fractionation of the excretions of 1000 beetles.
[b] Weight of residue from tap water subtracted.

yields for *D. nigrior* were similar although actual quantities of each fraction were consistently less.

TLC of Nonpolar Lipids

TLC of the nonpolar lipid portions of fresh pygidial secretions from either beetle species resolved four distinct bands, all toxic in some degree to *P. promelas*. In the solvent system diethyl ether–petroleum ether (60–70°C) (50:50, v/v), the toxic bands possessed R_f values of 0.10, 0.20, 0.44, and 0.58 and are designated Bands A–D, respectively. Bands B and D were the predominant components while Bands A and C were minor components. The eluate of pooled portions of chromatograms exclusive of Bands A–D was

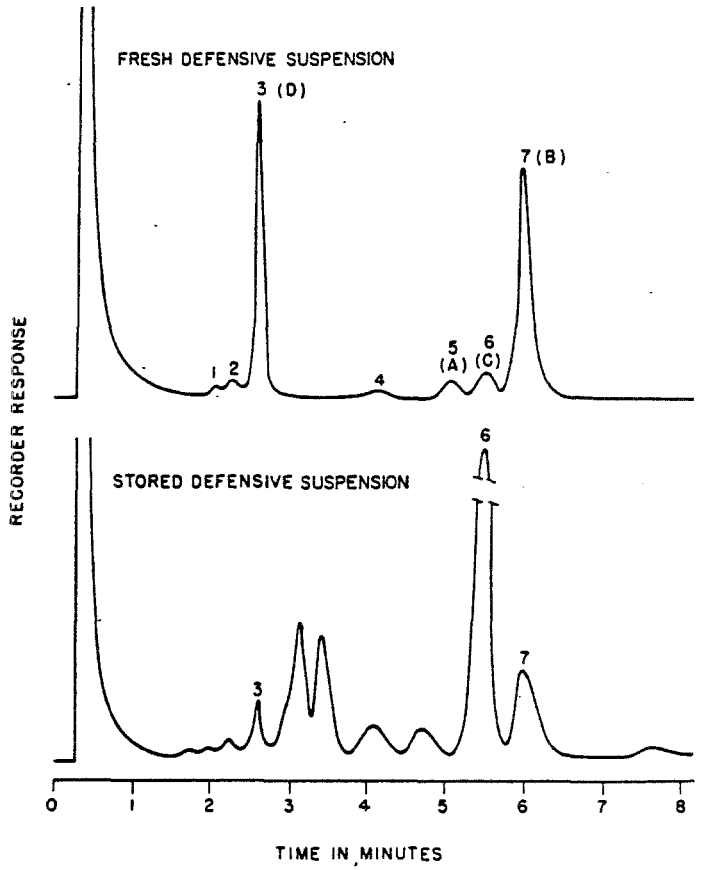


FIG. 1. Representative gas-liquid chromatograms of the chloroform extracts of fresh and stored pygidial secretions of *Dineutus assimilis* and *Dineutus nigrior*. Letters represent corresponding TLC Bands.

not toxic to *P. promelas*, although traces of further components were detected.

Numerous bands resulted from TLC of the nonpolar lipid portion of stored defensive suspensions, the mixture being so complex that compounds could not be completely resolved. The material in greatest quantity was Band $\ddot{\ddot{C}}$, which in fresh secretions was a minor component.

The chromatographic mobility of the toxic agents in various solvent systems was similar to that of testosterone and deoxycorticosterone (DOC), steroids which are representatives of the narcotizing agents from the prothoracic defensive glands of the Dytiscidae (Schildknecht, 1970; Miller and Mumma, 1973). When chromatograms were stained with I_2 or sprayed with 2,4-dinitrophenylhydrazine reagent (Stahl, 1969), Bands A–D developed a more intense color than did equivalent amounts of monounsaturated monocarbonyl standards. A positive response to potassium ferricyanide–ferric chloride spray reagent by all four of these compounds indicated that they all possessed some enol character.

GLC of Fresh and Stored Defensive Materials

Gas-liquid chromatograms of the nonpolar lipid portions of fresh and stored defensive materials (Figure 1) clearly demonstrated compositional changes upon storage. Freshly collected defensive secretions yielded seven distinct peaks with Peaks 3 and 7 making up over 80% of the total. Temperature programming starting at ambient temperature and progressing to 300°C resolved only traces of additional compounds. Numerous unresolved peaks appearing in gas chromatograms of the nonpolar lipid portions of stored defensive suspensions, demonstrated degradation of the original compounds with major changes in relative composition. Upon storage, the relative percentages and absolute quantities of Peak 3 and 7 greatly diminished while the relative percentage of Peak 6 greatly increased. The actual quantities of Peak 6 increased from less than 20 μg per beetle in fresh secretions to more than 100 μg per beetle in stored secretions, indicating that some of the original compounds were apparently being converted into Peak 6.

Characterization and Identification of Toxic Compounds

TLC Band B (GLC Peak 7), isolated by CC from fresh defensive secretions cochromatographed with an authentic sample of gyrinidal in various TLC solvent systems and on several GLC columns. Band B demonstrated UV λ_{max}^{EtOH} 238 nm (ε = 25,000); a mass spectrum identical to authentic gyrinidal, both giving prominent mass spectral peaks (relative abundance calculated from *m/e* 43 as base peak) at *m/e* 234 (3, M), 216 (6), 206 (3), 205 (4), 191 (7), 173 (17), 149 (23), 145 (14), 135 (17), 125 (21), 109 (62), 98 (24), 95 (25), 93 (18), 91 (31), 79 (30), 67 (24), 55 (42), and 43 (100); NMR ($CDCl_3$, δ in ppm

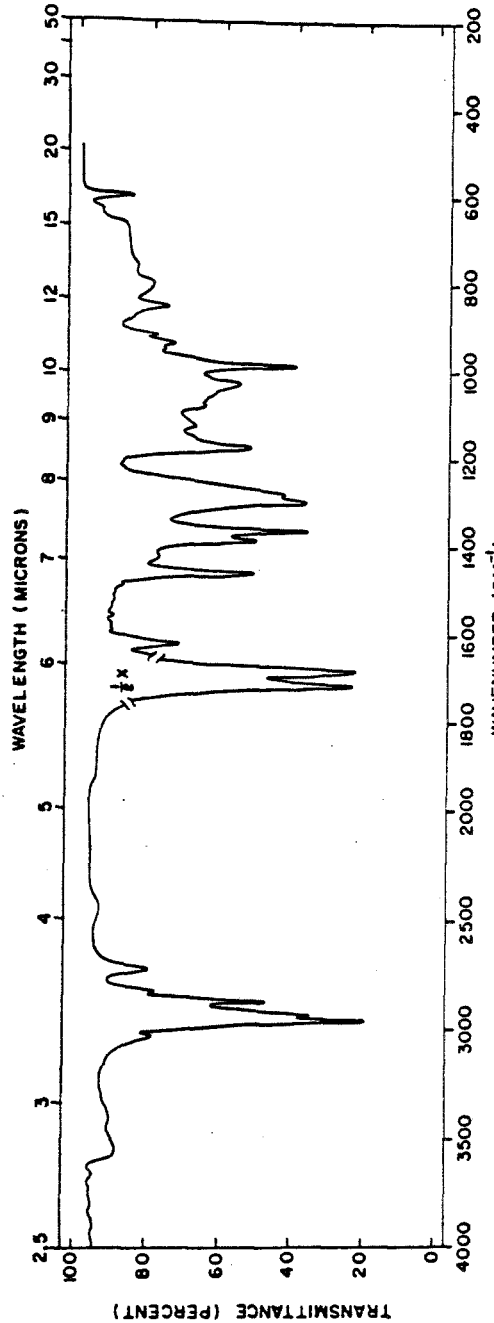


FIG. 2. IR spectrum ($CHCl_3$ solution) of TLC Band D (gyrinidione) from *Dineutus assimilis*.

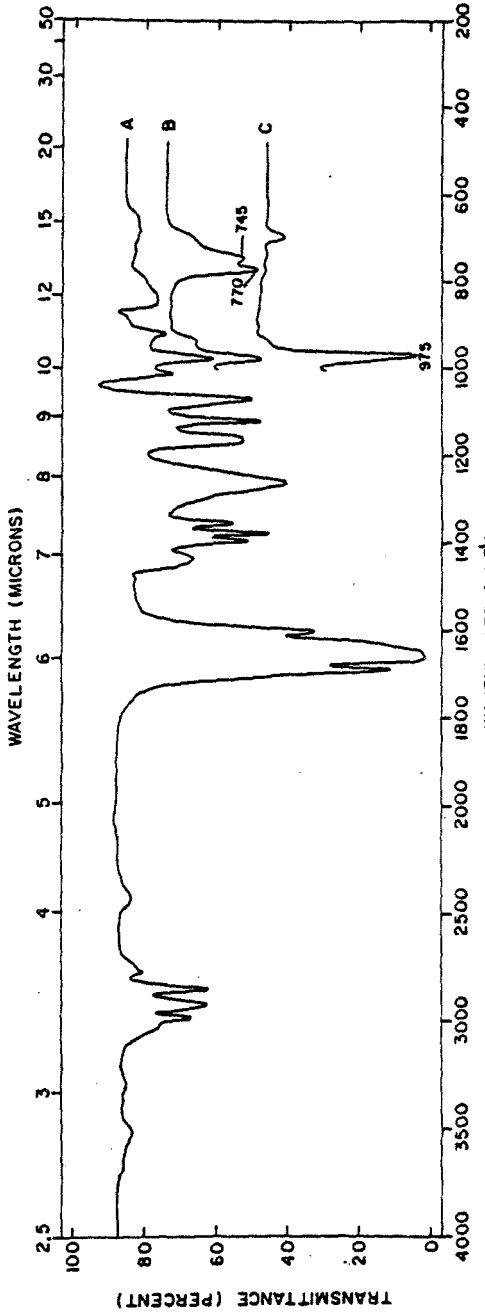


FIG. 3. IR spectrum of TLC Band A (isogyrinidal) from *Dineutus assimilis*. A = Band A in $CHCl_3$ solution; B = Band A in CS_2 solution; C = gyrinidal in CS_2 solution.

relative to internal tetramethylsilane) 1.89 (d, J = 1 Hz, 3 H), 2.24 (d, J = 1.5 Hz, 3 H), 2.38 (s, 3 H), 2.4–2.7 (m, 4 H), 5.90 (d, J = 8 Hz, 1 H), 6.5–6.8 (m, 1 H), 6.85 (d, J = 16 Hz, 1 H), 7.42 (d, J = 16 Hz, 1 H), 9.98 (d, J = 8 Hz, 1 H). Since these data are consistent with those collectively reported for gyrinidal (Schildknecht et al., 1972*a*; Meinwald et al., 1972; Miller et al., 1973), the compound represented by TLC Band B was assigned structure I, gyrinidal.

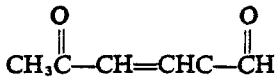
TLC Band C (GLC Peak 6), isolated in high yield from stored defensive suspensions by CC, was crystallized out of aqueous methanol and had a mp of 81–83°C. It demonstrated UV λ_{max}^{EtOH} 318 nm (ε = 13,300) and 234 (ε = 8,700); prominent mass spectral peaks (relative abundance calculated from *m/e* 43 as base peak) at *m/e* 236 (17, M), 218 (6), 207 (10), 175 (19), 151 (35), 125 (32), 111 (26), 109 (43), 98 (50), 81 (37), 55 (36), 43 (100); NMR ($CDCl_3$) δ 1.10 (d, J = 5 Hz, 3 H), 1.86 (d, J = 1 Hz, 3 H), 2.28 (s, 3 H), 4.50 (broad s, 1 H), 5.02 (d, J = 5 Hz, 1 H), 6.48 (d, J = 16 Hz, 1 H), 7.33 (d, J = 16 Hz, 1 H), and seven additional protons between 1.1 and 2.9. Since these data are in close agreement with those reported by Wheeler et al. (1972) for gyrinidone, the compound represented by TLC Band C was ascertained to be gyrinidone (II). A higher UV absorption coefficient was determined for gyrinidone than was previously reported; however, this is probably explained by the fact that our product was crystalline.

TLC Band D (GLC Peak 3), isolated by CC from fresh defensive suspensions possessed UV λ_{max}^{EtOH} 230 nm (ε = 11,200). Its IR spectrum (Figure 2) contained prominent absorptions bands (listed in cm^{-1}) indicating, among others, these structural features; 3040 (—C=C—H stretch), 2730 (aldehydic C—H stretch), 1720 (aldehydic C=O stretch), 1680 (—C=C—C(=O)—R carbonyl stretch), 1360 (—C(=O)—CH_3 methyl bending), 981 (*trans* —CH=CH— bending). High-resolution mass spectrometry demonstrated a molecular formula of $C_{14}H_{20}O_3$ (mass per unit charge [*m/e*] 236.1437, calculated 236.1412). Band D gave the following low-resolution fragmentation pattern (relative abundance calculated from *m/e* 151 as base peak and *m/e* assignments from high resolution); *m/e* 236 (3, $C_{14}H_{20}O_3$, M), 221 (2, $C_{13}H_{17}O_3$, M—CH_3), 218 (7, $C_{14}H_{18}O_2$, M—H_2O), 208 (7, $C_{13}H_{20}O_2$, M—CO), 207 (8, $C_{12}H_{15}O_3$, M—C_2H_5), 193 (4, $C_{12}H_{17}O_2$, M—$COCH_3$), 190 (5, $C_{13}H_{18}O$), 189 (7, $C_{13}H_{17}O$), 175 (10, $C_{12}H_{15}O$), 168 (14, $C_9H_{12}O_3$), 165 (20, $C_{11}H_{17}O$), 151 (100, $C_{10}H_{15}O$), 135 (18, $C_9H_{11}O$), 125 (25, $C_7H_9O_2$), 109 (34, C_7H_9O), 93 (23, C_7H_9), 83 (30, C_5H_7O), 69 (15, C_4H_5O), 55 (52, C_3H_3O, C_4H_7), 43 (71, C_2H_3O). The NMR absorptions of Band D are shown in Table 2.

TABLE 2. NMR SPECTRUM OF TLC BAND D (GYRINIDIONE) FROM *Dineutus assimilis*

δ (in $CDCl_3$)	Number of protons	Splitting pattern
1.10	3	d, J = 6 Hz
1.17	3	d, J = 7 Hz
1.2–2.7	7	complex
2.37	3	s
2.7–3.3	1	m
6.89	2	s
9.79	1	broad filled in doublet, J = 3 Hz
	—	
	20	

The spectral data of I and II as well as that of 3-hexene-2,5-dione (III)

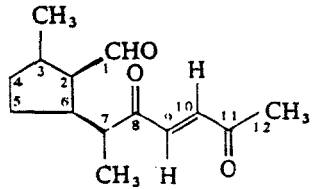


(III)

(Sugiyama and Kashima, 1970) were useful in structural establishment of Band D. In the NMR spectrum of *trans* III, the equivalent *trans* vinylic protons appear as a sharp singlet at δ 6.78 in $CDCl_3$, and the methyl ketones appear as a singlet at 2.38. The UV λ_{max}^{EtOH} of *trans* III is 228 nm (ε = 14,000). Corresponding values for *cis* III are δ 6.30, 2.29, and UV λ_{max}^{EtOH} 224 nm (ε = 8,600).

Presence of a methyl ketone in Band D was evidenced by the three-proton NMR absorption at δ 2.37, which corresponded to the methyl ketone absorptions of I and *trans* III (both 2.38), the prominent MS peak at *m/e* 43, and IR absorption at 1360 cm^{-1}. The sharp two-proton singlet at δ 6.89, the *trans* —CH=CH— IR absorption at 981 cm^{-1}, and the UV λ_{max}^{EtOH} 230 nm (ε = 11,200) all corresponded closely to the spectral data of *trans* III and indicated a symmetrical *trans* ene-dione system in Band D. While the aldehydic proton of the α,β unsaturated aldehyde of I appeared at δ 9.98 as a doublet (J = 8 Hz), the aldehydic proton of Band D appeared further upfield as a doublet at δ 9.79 (J = 3 Hz), suggesting a saturated aldehyde. The three-proton absorptions at δ 1.10 and 1.17 corresponded closely to the C-3 methyl of II (1.10) and were interpreted as methyls attached to tertiary carbon atoms. The eight additional protons detected in the NMR spectrum of Band D gave no clear splitting patterns and appeared to be aliphatic or alicyclic.

Incorporation of the methyl and saturated aldehyde moieties into Band D, while complying with the restrictions of only two olefinic protons and a molecular formula of $C_{14}H_{20}O_3$, was achieved by establishing a cyclopentanoid ring as in II. Although they possess similar functional groups, the retention time of Band D on OV-1 (Figure 1) was less than half that of I, sup-



(IV)

porting cyclization of Band D. On the basis of these data we propose structure IV, *gyrinidione*, as the compound represented by TLC Band D.

The broad filled-in doublet of the aldehydic proton absorption (C-1) can be explained by virtual coupling of the C-2, C-3, and C-6 protons (Silverstein and Bassler, 1967). The remaining methyl (δ 1.17) was assigned to C-7 since a methyl branch occurred at an analogous position in both gyrinidal and gyrinidone.

It becomes obvious that II and IV should be interconvertible by formation or opening of a ring involving a hemiacetal linkage between the aldehyde and the C-8 ketone. Confirmation of structure IV was achieved and a biosynthetic relationship to II was demonstrated by the conversion of IV to II. Gyrinidione dissolved in aqueous acetic acid (pH 3) for four days at room temperature, in darkness and under N_2, yielded a product (35% conversion) that had TLC, GLC, and mass spectral properties identical to II. The stereochemistry of IV at C-2 and C-6 was therefore assigned analogous configurations to those offered by Wheeler et al. (1972) for II.

TLC Band A (GLC Peak 5), isolated by preparative TLC from fresh defensive secretions, was readily interconvertible with I. Gyrinidal, upon 24 hours exposure to light while on silica gel thin-layer chromatogram, gave rise to Band A (yields >20%). Band A was converted to I in pyridine–acetic

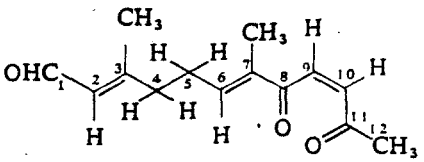
TABLE 3. NMR SPECTRUM OF TLC BAND A (ISOGYRINIDAL) FROM *Dineutus assimilis*

δ ($CDCl_3$)	Number of protons	Splitting pattern
1.83	3	d, J = 1 Hz
2.17	3	d, J = 1 Hz
2.23	3	s
2.2–2.6	4	m
5.87	1	d, J = 8 Hz
6.2–6.6	1	m
6.37	1	d, J = 12 Hz
6.58	1	d, J = 12 Hz
9.97	1	d, J = 8 Hz
	18	

anhydride (3:2) at 60°C for 0.5 hr (products identified by GLC–MS). Partial conversion of Band A to I occurred in both pyridine–acetic anhydride (3:2) at 22°C and in pyridine alone at 60°C.

The similar spectral properties of Band A and I suggested they were isomers. Band A demonstrated λ_{max}^{EtOH} 237 nm (ε = 20,500); a mass spectrum (M = 234) identical in all respects to that of I; and an IR spectrum (Figure 3) very similar to that reported by Schildknecht and Neumaier (1970) for I. Although the majority of the IR absorption bands of I and Band A were identical, a difference was detected in the region from 1000 to 650 cm^{-1} when the IR absorption of equivalent amounts of these compounds was recorded in CS_2 (Figure 3). While both compounds showed an absorption band at 975 cm^{-1} (*trans* —CH=CH— bending), I absorbed more strongly than did Band A. The characteristic *cis* absorption bands occurring at 745 and 770 cm^{-1} in the spectrum of Band A were lacking in the CS_2 spectrum of I. Similar absorptions are reported for the *cis* double bond of maleic acid (765, 785 cm^{-1}), *cis*-2,5-dimethoxy-2,5-dimethyl-2,5-dihydrofuran (770 cm^{-1}), and 15-*cis*-β-carotene (780 cm^{-1}).

The NMR absorptions of Band A (Table 3) showed the presence of 18 protons with a resonance pattern very similar to I. Despite the striking similarities of the NMR spectra of I and Band A, some differences in chemical shifts and splitting patterns of corresponding resonances gave reason to propose that Band A is the C-9 *cis* isomer of I, (E,E,Z)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal, *isogyrinidal* (V).



(V)

NMR assignments were made as follows: δ 1.83 (methyl of C-7), 2.17 (methyl of C-3), 2.23 (methyl of methyl ketone), 2.2–2.6 (methylene protons of C-4, C-5), 5.87 (vinyl proton of C-2), 6.2–6.6 (vinyl proton of C-6), 6.37, 6.58 (*cis* vinyl protons of C-9, C-10), 9.97 (aldehydic proton). The upfield shift of the methyl ketone absorption of V (δ 2.23) in relation to the methyl ketone of I and IV (δ 2.38) corresponds to the upfield shift of the methyl ketone absorption incurred in the transition of *trans* III (δ 2.38) to *cis* III (δ 2.29). An upfield shift in the center of the AB quartet pattern of the C-9, C-10 vinyl doublets of V (δ 6.45) was realized relative to I (δ 7.15). A similar upfield shift occurs in the vinyl proton absorption in the transition from *trans* III (δ 6.78) to *cis* III (δ 6.30). The reduction of the coupling constant of the C-9, C-10 vinylic protons from 16 Hz in I to 12 H_z in V also suggests a transition from

trans to *cis*. The other slight differences between the NMR spectra of V and I are consistent with this isomerization and suggest no further differences. Presence of the *cis* ene-dione system in V was confirmed by chelate formation with ferric chloride. When TLC chromatograms of gyrinid norsesquiterpenes were sprayed with 5% ethanolic ferric chloride, isogyrinidal formed a brick-red chelate while the other norsesquiterpenes did not.

GLC Quantification of Nonpolar Lipid Components

The relative composition of the nonpolar lipid portions of *D. assimilis* and *D. nigrior* pygidial secretions are presented in Table 4, and absolute quantities of compounds are shown in Table 5. Although the mean deviations show considerable individual variation, the average relative composition of the major norsesquiterpene components was surprisingly constant when gyrinid species and sexes were compared. Toxins constituted at least 98% of the detected compounds since Peaks 3, 5, 6, and 7 were all toxic to *P. promelas*.

The norsesquiterpene defensive titer of the two gyrinid species differed (Table 5). *D. assimilis* contained an average of 245 ± 73 μg norsesquiterpenes per individual while *D. nigrior*, the larger beetle, contained 144 ± 64 μg per individual (difference significant, $P = 0.01$). *D. assimilis* females contained a significantly higher defensive titer than did males ($P = 0.01$); however, no such difference was observed between the sexes of *D. nigrior*. As indicated by the large mean deviations, individual defensive titers were quite variable. The *D. assimilis* norsesquiterpene defensive titer ranged from 86 to 450 μg per individual and that of *D. nigrior* ranged from 51 to 317 μg per individual.

TABLE 4. RELATIVE COMPOSITION OF THE CHLOROFORM EXTRACTS OF PYGIDIAL SECRETIONS FROM INDIVIDUAL *Dineutus assimilis* AND *Dineutus nigrior* AS QUANTIFIED BY GLC

Species	Sex	Number of individuals examined	Relative percentage of GLC peaks[a]				
			1	3	5	6	7
D. assimilis	♂	18	1.3 ± 0.9[b]	36.9 ± 6.0	6.5 ± 1.9	6.6 ± 2.3	47.5 ± 18.0
D. assimilis	♀	8	0.8 ± 0.3	35.1 ± 5.3	7.6 ± 0.8	7.6 ± 3.4	48.0 ± 9.7
D. nigrior	♂	4	3.2 ± 3.1	36.2 ± 7.7	6.3 ± 0.5	6.8 ± 1.6	46.8 ± 4.4
D. nigrior	♀	9	1.0 ± 0.6	36.4 ± 4.8	4.4 ± 0.9	8.7 ± 4.0	48.1 ± 4.9

[a] GLC peaks numbered as in Figure 2, Peak 3 = gyrinidione, 5 = isogyrinidal, 6 = gyrinidone, 7 = gyrinidal; minor peaks not listed.

[b] Mean deviation.

TABLE 5. COMPOSITION OF THE CHLOROFORM EXTRACTS OF PYGIDIAL SECRETIONS FROM INDIVIDUAL *Dineutis assimilus* AND *Dineutus nigrior* AS QUANTIFIED BY GLC

Species	Sex	Number of individuals examined	Avg. body weight per individual (mg)	Average quantity of compound per individual (μg)					
				GLC peak[a]					Total compounds[b]
				1	3	5	6	7	
D. assimilis	♂	18	69.6±6.0[c]	3.3±3.0	80.9±22.7	14.3±5.9	17.0±9.5	99.7±29.2	217.1±58.8
D. assimilis	♀	8	74.7±5.0	3.3±1.1	110.1±24.9	27.5±8.3	27.6±13.5	136.5±30.1	308.9±73.2
D. nigrior	♂	4	93.0±4.5	3.2±1.8	58.2±24.6	10.6±5.7	10.8±6.1	71.3±25.8	158.4±61.1
D. nigrior	♀	9	92.9±4.0	1.8±1.9	48.9±26.7	6.3±3.4	13.5±9.4	61.1±24.5	138.8±60.0

[a] GLC peaks numbered as in Figure 2, Peak 3 = gyrinidione, 5 = isogyrinidal, 6 = gyrinidone, 7 = gyrinidal; minor peaks not listed.
[b] Summation of all GLC-detected compounds.
[c] Mean deviation.

Effects of Norsesquiterpenes and Steroids on Fish

Although a few differences were recognized, gyrinidione, gyrinidal, gyrinidone, DOC, and testosterone affected fish similarly when administered at 30 μg/ml water. Each compound initially caused considerable hyperactivity in fish; however, the hyperactivity elicited by the norsesquiterpenes was more violent and prolonged than that elicited by the steroids. In solutions of the steroids, fish rapidly became lethargic, lost their ability to orient into water currents, and soon turned on their sides showing little muscular activity aside from gill-pumping motions. Respiratory motions progressively diminished and ceased. Death soon followed and was often accompanied by muscular tremors. Norsesquiterpene-treated fish also rapidly became lethargic, but they maintained their ability to swim about and to orient into water currents comparatively longer than did those treated with equivalent dosages of steroids. Although the effect was delayed, norsesquiterpenes at 30 μg/ml also caused an equilibrium loss in fish, deep narcosis, and death.

Upon reaching a state of steroid narcosis where they could no longer right themselves, minnows placed into fresh water remained deeply anesthetized for approximately an hour but usually recovered fully. Minnows lightly anesthetized with norsesquiterpenes could likewise recover. A deeper state of steroid-induced narcosis was tolerated by the fish than that induced by the norsesquiterpenes.

Toxicity of Norsesquiterpenes and Steroids

Toxicities of gyrinidione, gyrinidal, gyrinidone, DOC, and testosterone are shown in Figure 4 as a function of external concentration. Log × log plots of minnow survival time *vs.* external concentration of toxin resulted in a linear response at the higher range of concentrations. As concentration decreased, a point of inflection was reached below which further decreases in concentration resulted in disproportionately longer survival times. Shortly below this inflection point minnows began to survive the treatments. The lowest concentration of toxin lethal to all fish contained therein is by definition the LC_{100} and is approximated by the concentration at which each curve in Figure 4 becomes perpendicular.

Although the three norsesquiterpenes killed fish with nearly equal rapidity at concentrations of 30 μg/ml and above, gyrinidione and gyrinidal were considerably more toxic than gyrinidone at concentrations below 20 μg/ml. The estimated LC_{100} of gyrinidone was 15 μg/ml while that of gyrinidione and gyrinidal was only 2 μg/ml. Estimated LC_{100} values for the steroids were 3 μg/ml for DOC and 8 μg/ml for testosterone. Toxicities of gyrinidione, gyrinidal, and DOC were very similar throughout the range of concentrations

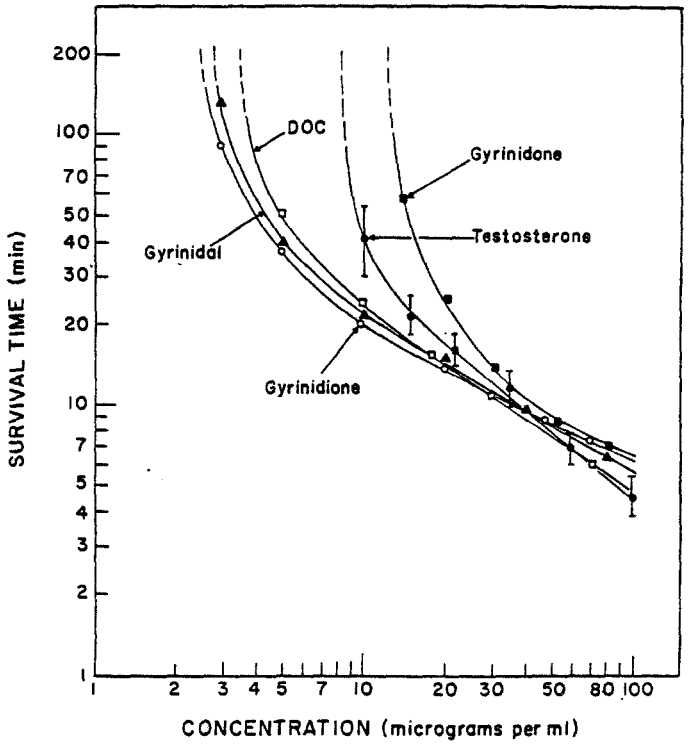


FIG. 4. Log × log plots of the survival time of *P. promelas* in 50-ml solutions of gyrinid norsesquiterpenes and several steroid standards. Standard deviations for testosterone are indicated by brackets and are representative of the deviations for the other compounds.

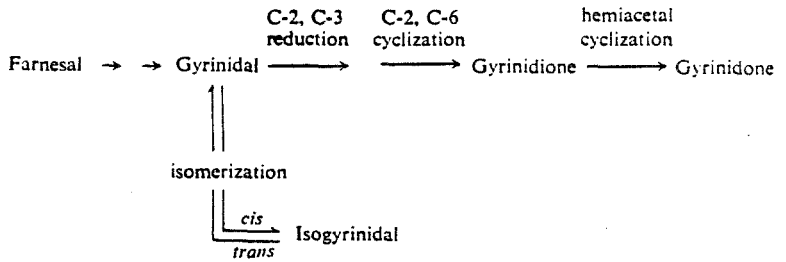
tested. Isogyrinidal was far less toxic than the other norsesquiterpenes requiring concentrations in the range of 70–90 μg/ml and exposures of over one hour to kill minnows.

In concentrations of norsesquiterpenes or steroids slightly lower than those that proved lethal, minnows were narcotized by both types of compounds, the latter inducing the deeper and more prolonged narcosis. Concentrations of these agents that were insufficient to cause such effects in minnows as equilibrium loss did cause them to become noticably lethargic. While remaining in sublethal concentrations of steroids or norsesquiterpenes minnows usually recovered completely from all effects within 3–5 hr, indicating that they could detoxify certain doses of these poisons. In general, if minnows survived for 3 hr in a given concentration of toxin administered in 50 ml water, they recovered from that treatment.

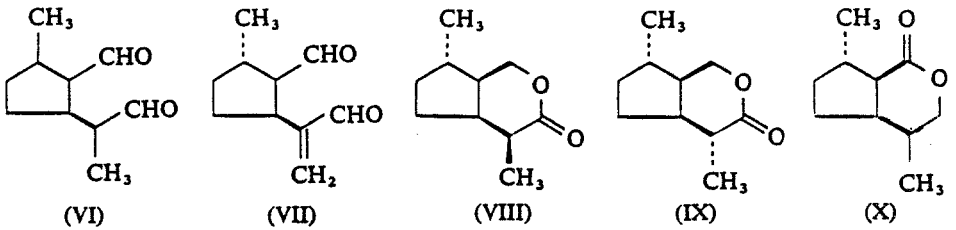
DISCUSSION

The pygidial glands of *D. assimilis* and *D. nigrior*, which occupy considerable space in the abdomens of these insects, secrete a complex mixture of components. Although at least 50% of the dry weight of pygidial secretions of these species was norsesquiterpenes, approximately 25% was characterized as polar-lipid and 25% as nonlipid. Paper chromatography of acid hydrolyzates of pygidial secretions demonstrated the presence of various amino acids, suggesting that some of the secretion is proteinacious. In water, gyrinid secretions form milky emulsions that can be broken by centrifugation or that settle out into a particulate layer upon storage. The non-norsesquiterpene portions of these secretions might serve important functions as adjuvants or spreaders of the norsesquiterpene toxins, or such components might themselves serve as defensive agents in a manner not reflected by the *P. promelas* bioassay.

In contrast to previous reports, *D. assimilis* and *D. nigrior* pygidial secretions contained more than one major norsesquiterpene component. Gyrinidal (48%) and gyrinidione (36%) were the major norsesquiterpene components of the secretions of these beetles but were found in combination with minor quantities of gyrinidone, isogyrinidal, and several unidentified trace components, some of which are isomers of the major components. Although we demonstrated that isogyrinidal slowly arises from gyrinidal upon exposure of the latter to light and that in acidic aqueous solution gyrinidione slowly gives rise to gyrinidone, chloroform extraction and GLC of gyrinid pygidial material immediately upon its excretion into water demonstrated the presence of both of these minor components. The average relative composition of norsesquiterpenes was identical (Table 4) in *D. assimilis* and *D. nigrior* and may reflect an established glandular equilibrium between norsesquiterpene components. One possible biosynthetic route by which these compounds may be related is represented by the following scheme.



A similar biosynthetic route starting from citral has been postulated and partially substantiated for the biosynthesis of the insect cyclopentanoid monoterpenes iridodial (VI), dolichodial (VII), iridomyrmecin (VIII), isoiridomyr-



mecin (IX) and the structurally related plant monoterpene nepetalactone (X) (Cavill and Robertson, 1965). The structural similarities of VI and VII to gyrinidione are striking, as are the structural similarities of VIII, IX, and X to gyrinidone.

The norsesquiterpene defensive toxins of *D. assimilis* and *D. nigrior* have limited chemical stability. Lengthy storage of these agents, their exposure to heat, air, pH changes, and especially light facilitate their degradation. Therefore, in establishing the glandular composition of these toxins, it is desirable to carry out the analyses as soon as possible after their collection. The presence of large amounts of gyrinidone and only minor quantities of gyrinidione and gyrinidal in stored aqueous suspensions of *D. assimilis* (Figure 1) was an artifact of the storage procedure. Future investigations on gyrinid defensive secretions should take the instability of the norsesquiterpenes into consideration.

Gyrinid pygidial secretions serve at least two defensive roles. As documented by Benfield (1972), pygidial secretions serve as potent feeding deterrents to predators. Although some predators initially ingested a beetle or two, they thereafter consistently rejected gyrinid beetles or food items to which the pygidial secretions were applied. As assayed by *P. promelas*, the norsesquiterpenes contained in the pygidial secretions are powerful toxins capable of serving a defensive role by poisoning predators. Under natural conditions aquatic organisms might contact enough beetle defensive agents by gill absorption to elicit behavioral responses; however, we doubt whether they would encounter sufficiently high external concentrations of these agents to be narcotized. It is more likely that predators would contact the defensive agents by ingesting whirligig beetles. Preliminary feeding experiments indicate that frogs (*Rana pipiens*) and fish (*Salmo gairdneri*) which are force-fed live whirligig beetles can contact enough defensive compounds to be stupefied or killed.

Although further feeding experiments are necessary to determine the oral toxicity of the norsesquiterpenes, an estimate of their toxicity to *P. promelas* when administered in external solution can be made from the data in Figure 4. For *P. promelas* in 50 ml of water the LC_{100} for gyrinidione and gyrinidal was slightly less than 3 μg/ml, with death occurring in approximately 2 hours. Since the 3 minnows in each beaker averaged 1.2 g, as much as 150 μg

of toxin could have been absorbed by the 3.6 g of fish or a reasonable estimate of the LD_{100} for gyrinidione and gyrinidal would be 42 μg/g fish. As calculated from Table 5, *D. assimilis* contains a combined average of approximately 210 μg of gyrinidione and gyrinidal per individual. Provided that absorption of these compounds from the digestive tract of a predator is reasonably rapid, it may take only a few consumed beetles to produce sufficient ill effects to communicate the message that their kind is an unsatisfactory food item.

One would expect that the effects of the beetle toxins would be dependent on the body weight of the predator. It follows that large fish and other large predators might be able to consume some whirligig beetles without suffering serious ill effects. The following observation related by Wilson (1923) supports such a contention. During the period of one summer, ponds containing immature bluegills, *Lepomis macrochirus*, were found to be highly populated by *D. assimilis* while an adjacent pond containing adult bluegills were kept free of these beetles. Examination of the stomach contents of the fish revealed that the young bluegills were consuming various aquatic beetles but no Gyrinidae. When some of the gyrinids were transferred to the pond containing adult bluegills, they were promptly eaten. Although this example demonstrates that some gyrinid adults are consumed by fish, only a few species are known to do so (Benfield, 1970). Among these are the bluegill fish and several species of bullheads. Extensive predation even by these species might be controlled by the defensive toxins.

Other fish species might just as well be able to consume gyrinids with slight ill effects, but they apparently do not. The repellent character of the pygidial secretions of gyrinids may largely preclude their being preyed upon by various predators large or small. Whether feeding patterns established when predators were small enough to be poisoned by gyrinid defensive toxins remain established in later life is a matter of speculation but may be a factor in gyrinid immunity from predation.

The observation that gyrinidal was repellent to fish (Meinwald et al., 1972) suggests that the toxic and repellent agents of gyrinid secretions may be one and the same. The distinction between the repellent and toxic nature of the norsesquiterpenes may be a matter of the dosage administered to the predator. Whether acting as repellents, toxins, or both, these agents undoubtedly play an important defensive role in the Gyrinidae, most likely in teaching predators to discriminate against whirligigs as an unsuitable food item. Examples of predators learning to avoid noxious prey, even when the effects of the defensive agents may be delayed, are discussed by Eisner (1970).

As assayed by fish, the physiological effects of the defensive norsesquiterpenes resemble those of the steroids. Both types of compounds have a protracted narcotizing action on fish that is lethal in high concentrations and reversible in low concentrations. DOC, testosterone, progesterone, and

various other steroid hormones common to vertebrates are recognized anesthetic agents that have a protracted narcotizing action on mammals as well as on fish (Selye, 1941*a,b,c*; Selye and Heard, 1943). The Dytiscidae have presumably exploited this anesthetic activity of steroids as their main defense against predation, although electrophysiological evidence that steroids affect the spontaneous and evoked neural discharges from the olfactory system of fish (Hara, 1967; Oshima and Gorbman, 1968) has led Clayton (1970) to speculate that the defensive action of steroids may also involve repellency. The pygidial norsesquiterpenes of the Gyrinidae apparently serve a defensive function similar to that of the defensive steroids of the Dytiscidae by also being narcotic to predators.

Since the physiological effects of the norsesquiterpenes are so similar to those of equivalent amounts of the anesthetic steroids, both types of agents may share a similar mode of action. Although steroid anesthesia has been extensively studied (see Kappas and Palmer, 1963 for a review) the mode of action of steroid anesthetics has not been elucidated. However, as characterized in the early reports on the subject (Selye, 1941*a,b,c*; Selye, 1942; Winter, 1941; Selye and Heard, 1943; Selye and Stone, 1944), steroid anesthesia is a nonspecific phenomenon, as numberous steroid compounds of diverse structure are anesthetic. In addition, anesthesia is achieved only when an organism is suddenly flooded by intravenous or intraperitoneal injection of these agents.

Norsesquiterpene narcosis also appears to be a nonspecific phenomenon since gyrinidione, gyrinidal, and gyrinidone represent some diversity of structure, and all are narcotic to fish. Studies of the absorption of norsesquiterpenes and steroids across *P. promelas* gill membranes (Miller and Mumma, 1974) demonstrate that equally rapid flooding is required for norsesquiterpene narcosis as for steroid narcosis.

One distinct difference was noticed between steroid and norsesquiterpene narcosis. Compared with the steroids, the dosage of norsesquiterpene required to be deeply anesthetic to fish lay closer to the dosage that proved lethal. Apparently the therapeutic index of the norsesquiterpenes is lower than that of the steroids.

Although numerous steroids demonstrate excellent anesthetic qualities, the medical use of most of them as anesthetics is barred by their hormonal side effects. Very few steroids have been found to possess both desirable anesthetic qualities and no hormonal activity. One such agent that has been developed and is being used as a basal anesthetic is 5β-pregnan-21-hydroxy-3,20-dione sodium succinate (Clarke et al., 1971). That the highly oxygenated norsesquiterpenes or their analogs may also have value as anesthetics for medical purposes is a possibility that warrants investigation. It seems unlikely that the pharmacological use of norsesquiterpenes would be limited by hormonal activity.

Various oxygenated terpene derivatives that are structurally related to the gyrinid norsesquiterpenes, such as VI–IX, have previously been isolated as defensive agents of arthropods. These compounds have usually been ascribed activity as general toxicants and in some cases insecticidal activity (Pavan, 1952). One compound that is structurally related to the gyrinid norsesquiterpenes and is also wellknown for its neurological activity is nepetalactone (X) the active component of catnip (*Nepeta cataria*) which is excitatory to felids. Whether neurological activity is a widespread attribute of oxygenated terpene derivatives remains to be seen.

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SEX PHEROMONE COMPONENTS OF THE REDBANDED LEAFROLLER, *Argyrotaenia velutinana* (LEPIDOPTERA: TORTRICIDAE)

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Abstract—Secondary pheromone components of the redbanded leafroller from female moth extracts have been identified as dodecyl acetate and *trans*-11-tetradecenyl acetate (t11-14 : Ac). The ratio of t11-14 : Ac to the primary pheromone component *cis*-11-tetradecenyl acetate was *ca.* 9 : 91 in abdominal tip extracts and 12 : 88 in female effluent collections, whereas the optimal ratio for attraction in field test was 6 : 94 in the spring flight and 6–10 : 94–90 in the summer flight. Dodecyl acetate was collected from calling females in ratios to the 14-carbon acetates as high as 5 : 4; in field tests these chemicals were the most attractive in ratios of 3 : 2 and 2 : 1.

Key Words—Tortricidae, redbanded leafroller, sex pheromone, sex attractant, synergist, inhibitor, *Argyrotaenia velutinana*.

INTRODUCTION

The primary sex pheromone component of the redbanded leafroller (RBLR), *Argyrotaenia velutinana* (Walker), was identified as *cis*-11-tetradecenyl acetate (c11-14 : Ac) (Roelofs and Arn, 1968). Subsequently, two other components have been implicated for increased male RBLR attractancy. *trans*-11-Tetradecenyl acetate (t11-14 : Ac) was found in field tests to be necessary for RBLR male attraction when present at low ratios (ca. 7 : 93) to the primary component, c11-14 : Ac (Klun et al., 1973), whereas higher ratios were found to inhibit male attraction (Roelofs and Comeau, 1968, 1971*a*). Dodecyl acetate (12 : Ac) was among the compounds found to increase trap catches of RBLR males in field screening tests (Roelofs and Comeau, 1968,

1971*a*); a number of 11- and 12-carbon acetates and some oxygen analogs of c11-14:Ac were also effective in increasing trap catches when present in 1:1 or higher ratios. This effect appeared to be rather general in its structural requirements and not critically dependent on release rates.

We have analyzed extracts of female RBLR sex pheromone gland extracts and effluvia from calling RBLR females for pheromone components that could be responsible for the activity described above. Further field tests were conducted to define the ratios of the three pheromone components needed for optimum male RBLR attractancy.

METHODS AND MATERIALS

Insects were reared continuously in the greenhouse on fava beans (Glass and Hervey, 1962) or on an artificial medium (Shorey and Hale, 1965). Field tests were conducted in apple orchards along Lake Ontario using Sectar® I (Zoecon Corporation, Palo Alto, California) traps, with test chemicals placed in polyethylene caps (OS-6 natural polyethylene closures, Scientific Products). Traps were hung at a height of 1.5 m, 1 trap per tree, with 5 replicates in a randomized complete block design. Data were submitted to an analysis of variance. In the Tables, means followed by the same letter are not significantly different at the 5% level as determined by Duncan's new multiple range test.

GLC was conducted utilizing a flame ionization detector and glass columns packed with the following: 3% methyl silicone (OV-1, 2 m × 4 mm) on 100–120 mesh Gas-Chrom Q; 3% cyclohexanedimethanol succinate (CHDMS, 2 m × 4 mm) on 100–120 mesh Gas-Chrom Q; and 3% phenyldiethanolamine succinate (PDEAS, 4 m × 2 mm) on 100–120 mesh Chromosorb W-AW-DMCS. Geometric isomers were analyzed on PDEAS, which had a detection limit of ca. 0.5% for *cis-trans* pairs. The mass spectra were obtained by Dr. L. Hendry, Pennsylvania State University. Electroantennograms (EAG) for assaying GLC collections were run as previously described (Roelofs and Comeau, 1971*b*). The c11-14:Ac used in the field studies was obtained from the Farchan Chemical Company and was purified to greater than 99.5% isomeric purity by thin-layer chromatography (TLC) on silver nitrate-impregnated silica gel-G. The t11-14:Ac was greater than 99.5% isomerically pure as obtained from the Farchan Chemical Company. Airborne collections were obtained by a method similar to that of Byrne et al. (1975) in which air flowing over calling RBLR females in a glass tube (1.2 m × 9 cm OD) passed through a Porapak-Q column (20 cm × 19 mm). The compounds trapped on the column were eluted with redistilled Skellysolve® B.

RESULTS AND DISCUSSION

Identification of Pheromone Components

Secondary component (general). Female RBLR abdominal tip extracts were analyzed by GLC for dodecyl acetate (12:Ac) and related compounds. GLC tracings from crude extract showed a peak at the retention time of dodecyl acetate on both OV-1 and CHDMS columns. This component was collected from CHDMS (175°C, standard 12:Ac eluted in 2.3 minutes) at 2–3 minutes, and then from OV-1 (175°C, standard 12:Ac eluted in 5.65 minutes) at 4.5–6.0 minutes. The purified component was identified as dodecyl acetate by the following criteria:

(1) Treatment with KOH caused a change in GLC retention times from that of 12:Ac on PDEAS (160°C, 4.2 minutes) and on OV-1 (175°C, 5.65 minutes) to the retention times corresponding to dodecyl alcohol on PDEAS (160°C, 4.4 minutes) and on OV-1 (175°C, 3.6 minutes). Treatment of the saponified product with acetyl chloride gave a product with retention times identical to the starting material and standard 12:Ac on both columns.

(2) The mass spectrum of the collected material is identical to that of standard 12:Ac.

The amount of dodecyl acetate extracted from female RBLR abdominal tips of varying ages was ca. 3% of the amount of the primary pheromone component, c11-14:Ac, which was present in amounts of ca. 30–80 ng/female. Airborne collections, however, showed that 12:Ac was released at much higher ratios than indicated from the abdominal tip extract analysis. One collection from 200 moths gave a ratio of 5:4 of 12:Ac/c11-14:Ac in the female effluent. This increased ratio in the effluent indicated that the females are either actively converting a precursor to 12:Ac at the time of calling or that this particular component is released from another glandular area that is not usually included in excised tips.

Secondary component (specific). Female RBLR abdominal tip extracts and effluent were analyzed for the presence of the primary pheromone component geometrical isomer, t11-14:Ac. A methylene chloride extract of 30 female abdominal tips was injected onto the CHDMS column (175°C) and the effluent collected at 5–6 minutes (retention time of 11-14:Acs was 5.25 minutes). The collected material was injected onto the OV-1 column (175°C) and collected at 11.5–13 minutes (11-14:Ac's retention time was 12.15 minutes). The collected material was chromatographed by TLC on silver nitrate-impregnated silica gel-G along with the standards c11-14:Ac and t11-14:Ac (R_f values of 0.26 and 0.39, respectively), with benzene as the developing solvent. The *trans* area was scraped from the plate, eluted with diethyl ether, and the extract injected onto the CHDMS column

(160°C). The major component was EAG active and had a retention time identical to t11-14:Ac (9.4 minutes), with no product visible at the retention time of c11-14:Ac (9.9 minutes). This indicated that a *trans* compound was present in the tip extract.

The *trans* compound extracted from the TLC plate was used for further structural characterization. The compound was injected onto PDEAS (160°C) and the effluent was collected at 9–11 minutes (retention time of t11-14:Ac was 9.55 minutes). Reinjection of an aliquot of the collected material on PDEAS (160°C) gave a peak at 9.55 minutes, which corresponded to the retention time of t11-14:Ac and not to c11-14:Ac (10.15 minutes). Further identification of the *trans* compound as t11-14:Ac was obtained as follows:

(1) Support for an acetate moiety was obtained by treating the collected material with KOH. The starting material elicited good EAG activity at the retention time of t11-14:Ac (12 minutes) when collected from OV-1 (175°C), but the product possessed no EAG activity at 12 minutes. Collection at the retention time of the corresponding alcohol, t11-14:OH (7.35 minutes), followed by treatment with acetyl chloride and reinjection onto OV-1 restored EAG activity at 12 minutes.

(2) Support for unsaturation at the 11- position was obtained by micro-ozonolysis (Beroza and Bierl, 1967). The material extracted from the TLC

TABLE 1. FIELD ATTRACTANCY OF RBLR MALES TO c11–14:Ac (7.6% *trans*-) AND VARYING AMOUNTS OF DODECYL ACETATE (12:Ac) (TEST CONDUCTED APRIL 23 TO MAY 14, 1973)

Treatment	Mean number/trap[b]
Attractant[a]	2.0 d
+ 1 μl 12:Ac	4.4 cd
+ 2 μl 12:Ac	10.2 bc
+ 5 μl 12:Ac	12.2 b
+10 μl 12:Ac	11.4 b
+15 μl 12:Ac	24.6 a
+20 μl 12:Ac	22.6 a
Unbaited	0.0 d

[a] 10 μl of a mixture of 92.4% c11–14:Ac and 7.6% t11–14:Ac in a polyethylene cap.

[b] Means followed by the same letters are not sigficantly different at the 5% level as determined by Duncan's new multiple range test.

TABLE 2. FIELD ATTRACTANCY OF RBLR MALES TO 15 μl DODECYL ACETATE AND 10 μl c11–14 : AC WITH VARYING AMOUNTS OF *trans*-ISOMER IN A POLYETHYLENE CUP (TEST CONDUCTED MAY 3–15, 1973)

Percent *trans*-	Mean number/trap[a]
2.0	6.0 d
4.4	14.2 bcd
6.1	29.4 a
7.6	20.4 b
9.7	14.4 bcd
11.6	13.6 bcd
14.3	14.6 bc
20.0	9.2 cd
Unbaited	0.4 e

[a] See footnote [b] to Table 1.

plate was collected from OV-1 (170°C) in a 2-minute collection centered around the retention time of t11-14 : Ac (8.4 minutes). Ozonolysis gave a product with the retention times of 11-acetoxyundecanal on CHDMS (170°C, 17.25 minutes) and on OV-1 (170°C, 6.05 minutes).

(3) Support for monounsaturation and a 14-carbon chain was obtained by finding that a mass spectrum of the TLC collected material is identical to that of t11-14 : Ac.

The above data confirm that t11-14 : Ac is produced by female RBLR moths. The amount of *trans* relative to the primary pheromone component c11-14 : Ac was then determined from abdominal tip extracts from various RBLR populations and from airborne collections. The *trans* : *cis* ratio obtained by averaging a total of 5 determinations using 3 separate female tip extracts of a greenhouse culture originating from the wild (Geneva, New York) within the year is ca. 9 : 91. A female tip extract of a culture reared in the greenhouse and originating from Vineland, Ontario, Canada, 25 years ago also showed a ratio of 8.7 : 91.3 (average of 3 determinations). A sample from an airborne collection of calling RBLR females originating within the year from the wild (Geneva, New York) and reared on an artificial medium showed a ratio of 12.3 : 87.7.

Field Tests

Field tests were conducted to determine the optimal quantities of each pheromone component needed in the polyethylene dispensers for maximum

TABLE 3. FIELD ATTRACTANCY OF RBLR MALES TO 15 μl DODECYL ACETATE AND 10 μl c11–14:Ac WITH VARYING AMOUNTS OF *trans*-ISOMER IN A POLYETHYLENE CAP (TEST CONDUCTED JULY 18 TO AUGUST 6, 1973)

Percent *trans*-	Mean number/trap[a]
2.0	7.0 de
3.0	8.0 cde
4.9	11.5 bcd
5.6	13.0 bcd
6.2	20.5 a
7.8	18.5 ab
10.2	20.5 a
11.5	15.5 bc
20.8	3.5 e
44.7	0.0 f
Unbaited	.0 f

[a] See footnote [b] to Table 1.

RBLR attraction. One field trial (Table 1) involved treatments containing a 7.6:92.4 mixture of t11-14:Ac/c11-14:Ac, similar to the ratios found in females and known to give good RBLR attractancy in the field (Klun et al., 1973), with varying amounts of 12:Ac. It was found that treatments with 15 or 20 μl of 12:Ac were the best.

Another test (Table 2) involved treatments containing 15 μl of 12:Ac and 10 μl of t/c11-14:Ac mixtures. This test, conducted during the spring flight, showed that 6.1% *trans*- was the most attractive, whereas, in a test conducted during the summer flight (Table 3), treatments containing 6.2, 7.8, and 10.2% *trans* were the best and are not significantly different from one another. Tests conducted with *cis*/*trans* mixtures without the 12:Ac synergist did not attract sufficient males for analysis.

In summary, we have shown that in addition to the primary pheromone component, c11-14:Ac, female RBLR moths also produce 12:Ac and t11-14:Ac. In New York, potent attractancy is obtained with polyethylene dispensers containing 15 μl 12:Ac and 10 μl of t11-14:Ac/c11-14:Ac in a 6:94 ratio.

Acknowledgments—We thank T. Baker for helping with the field tests, and F. Wadhams and K. Poole for maintaining the insect cultures. This research was supported by the Rockefeller Foundation and by NSF Grant No. GB-38020.

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SEX PHEROMONE COMPONENTS OF THE OMNIVOROUS LEAFROLLER MOTH, *Platynota stultana*[1]

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Abstract—A mixture of *trans*- and *cis*-11-tetradecenyl acetates have been found in omnivorous leafroller moth female tip extracts in a ratio of 88 : 12, respectively. In the field they are the most attractive to male omnivorous leafroller moths in a ratio of 94 : 6. Field attractancy can be increased by addition of small quantities (0.2–2.0%) of mixtures of *trans*- and *cis*-11-tetradecenyl alcohols, indicated to be present in female tip extracts in a ratio of 88 : 12, respectively.

Key Words—sex pheromones, sex attractants, omnivorous leafroller, *Platynota stultana*.

INTRODUCTION

The omnivorous leafroller moth (OLR), *Platynota stultana* (Walsingham) is a major tortricid vineyard pest in California, with potential for becoming a significant pest of other crops in the warmer climates. In the eastern United States, it is a problem primarily in greenhouses, feeding on a wide variety of plants. Clearly, the development of an effective synthetic sex pheromone could be very useful in integrated pest control programs, either as a monitoring tool for timing insecticide sprays or as an insect control tool (Tette, 1974).

A sex pheromone emitted by the female to attract males has been demonstrated for this insect (AliNiazee and Stafford, 1971). This paper reports the identification of the various pheromone components.

[1] This work was supported by the Rockefeller Foundation and by NSF Grant No. GB 38020.

METHODS AND MATERIALS

GLC columns (glass, 2 m × 4 mm or 4 m × 2 mm) were packed with 3% OV-1 (methyl silicone) on 100–120 mesh Gas-Chrom Q, 3% CHDMS (cyclohexanedimethanol succinate) on 100–120 mesh Gas-Chrom Q, or 3% PDEAS (phenyldiethanolamine succinate) on 100–120 mesh Chromosorb W-AW-DMCS; a hydrogen flame ionization detector was used. The mass spectrometer was a Hitachi RMU-6E interfaced with an Aerograph 1740–10 gas chromatograph (CHDMS column).

Moths were reared continuously in a greenhouse on fava beans (Glass and Hervey, 1962). Excised female tips were extracted with methylene chloride. Chemical analyses of tip extracts were carried out essentially as described elsewhere (Hill et al., 1974; Roelofs et al., 1971*b*). Solvents were distilled prior to use. Diethyl ether (dry, Mallinckrodt reagent) was used without distillation.

Electroantennograms (EAG) were carried out as previously described (Roelofs and Comeau, 1971*a*); they were used for assaying GLC collections of female tip extracts (Roelofs et al., 1971*a*) and for determining normalized response profiles of male antennae to a series of long-chain acetates, alcohols, and aldehydes (Hill et al., 1974).

RESULTS

Analysis of Female Tip Extracts

Aliquots (ca. 5 female equivalents) of female tip extracts in carbon disulfide were collected from OV-1 and CHDMS columns in one-minute fractions. Assay by EAG showed one major area of activity (10–12 minutes) from OV-1 (170°C) at the retention of *trans*-11-tetradecenyl acetate (t11-14:Ac; 11.05 minutes) (Figure 1), and one major area of activity (7–8 minutes) from CHDMS (170°C) also at the retention of t11-14:Ac (7.35 minutes). GLC tracings of female tip extracts on OV-1 showed 2 major peaks, A and B, with the retentions of *trans*-11-tetradecen-1-ol (t11-14:OH) and t11-14:Ac, respectively (Fig. 1). The ratio of A to B varied among all the extracts examined, with a relatively low ratio (1:14) observed with an extract of virgin females and higher ratios (1:1.4 to 1:0.7) observed with extracts of females from mixed populations in which some mating was known to have taken place. Although component A did not show EAG activity in the GLC collections, it was still considered a potential pheromone component, because (a) it was the only other major component found in the extract, and (b) minute quantities of secondary components eliciting reduced EAG activity are often overlooked.

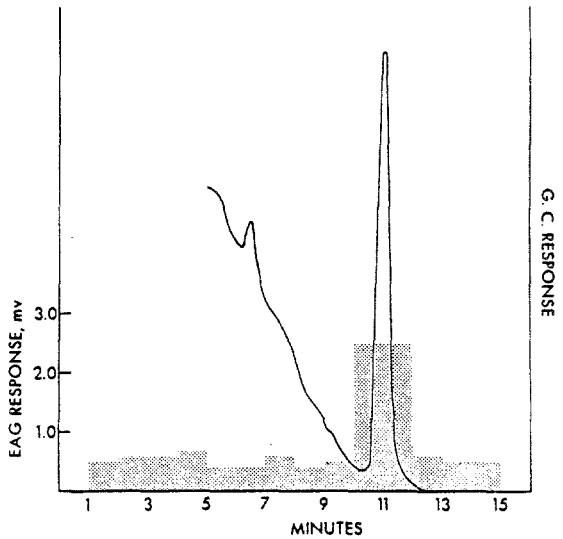

FIG. 1. OLR virgin female tip extract: EAG response of 1-minute collections and GLC tracing, using an OV-1 column at 170°C. The retention times for t11–14 : OH and t11–14 : Ac under these conditions were 6.55 and 11.05 minutes, respectively.

Chromatography of crude female tip extract was carried out on Florisil® using petroleum ether (30–60°C), 2, 5, 10, 25, and 50% diethyl ether in petroleum ether, and diethyl ether as eluants, followed by examination of the recovered materials using GLC on OV-1 and EAG assay of OV-1 collected samples. Component A was eluted with 50% diethyl ether (the alcohols fraction) and component B with 25% diethyl ether (the acetates fraction).

Analysis of A

A was collected from OV-1 for all analyses. Acetylation and subsequent saponification of A resulted in the GLC behavior on OV-1 expected for t11-14 : OH, including the appearance of EAG activity (10–11 minutes) after acetylation at the retention of t11-14 : Ac (10.9 minutes at 169°C, with t11-14 : OH at 6.35 minutes) and the disappearance of this activity after saponification. A apparently has no EAG activity at the nanogram level used in this test, although the 10 μg standard does elicit antennal responses (see section on EAG standards).

Microozonolysis of A and of t11-14 : OH produced fragments with similar retentions on OV-1 (6.55 and 6.5 minutes, respectively, at 165°C) confirming the presence of a double bond at the 11- position.

TLC of acetylated A on silver nitrate-impregnated silica gel-G resulted in recovery of an EAG-active material from the *trans* area. This EAG-active material was collected from CHDMS, 180°C at 5–6 minutes, which coincides with the retention time of t11-14 : Ac (5.7 minutes).

Examination of A on PDEAS (174°C) revealed three peaks with the retentions of tetradecyl alcohol (14 : OH; 12.5 minutes), t11-14 : OH (15.0 minutes), and *cis*-11-tetradecenyl alcohol (c11-14 : OH; 15.9 minutes) in the ratios of 6 : 83 : 11, respectively, or a *trans-cis* ratio of 88 : 12. The only other 14-carbon alcohol that can be converted to 11-hydroxyundecanal on ozonolysis is 12-methyl-11-tridecenyl alcohol (12Me-11-13 : OH); on PDEAS (175°C) the retention of 12Me-11-13 : OH was 0.5 minutes longer than that of t11-14 : OH.

These data confirm the presence of t11-14 : OH in female OLR tip extracts and strongly suggest the presence of c11-14 : OH and 14 : OH.

Analysis of B

Unless stated otherwise, B was collected from OV-1 for all analyses.

Saponification and reacetylation of B resulted in the GLC behavior on OV-1 expected for t11-14 : Ac, including the disappearance and subsequent reappearance of EAG activity (6–7 minutes at 180°C) at the retention of t11-14 : Ac (6.4 minutes at 180°C, with t11-14 : OH at 3.95 minutes).

The presence of only one double bond was confirmed by TLC of the acetoxymercuri-methoxy adduct of B on silica gel-G (Mangold and Kammereck, 1961). The crude extract as well as the acetate-containing fraction from Florisil chromatography were used in this procedure. EAG activity (8–9 minutes) at the GLC retention time of t11–14 : Ac (8.25 minutes) on OV-1 (180°C) was recovered only from the monoeneoates TLC area after regeneration of the free acetate.

TLC of B on silver nitrate-impregnated silica gel-G followed by recovery, collection from CHDMS (180°C), and subsequent EAG analysis resulted in recovery of EAG activity from both *trans* and *cis* areas, with most of it present in the *trans* area; all activities (5–6 minutes) coincided with the GLC retention of t11-14 : Ac (5.74 minutes). GLC of the recovered samples on PDEAS (170°C) revealed a material with the retention of t11-14 : Ac (9.3 minutes) from the *trans* TLC area and another with the retention of *cis*-11-tetradecenyl acetate (c11-14 : Ac; 9.8 minutes) from the *cis* TLC area.

On PDEAS (174°C), B was resolved into three peaks having the retentions of tetradecyl acetate (14 : Ac; 12.35 minutes), t11-14 : Ac (14.45 minutes), and c11-14 : Ac (15.3 minutes) in the ratios 10 : 80 : 11, respectively, or a *trans-cis* ratio of 88 : 12.

Location of the double bond at the 11- position was confirmed by micro-

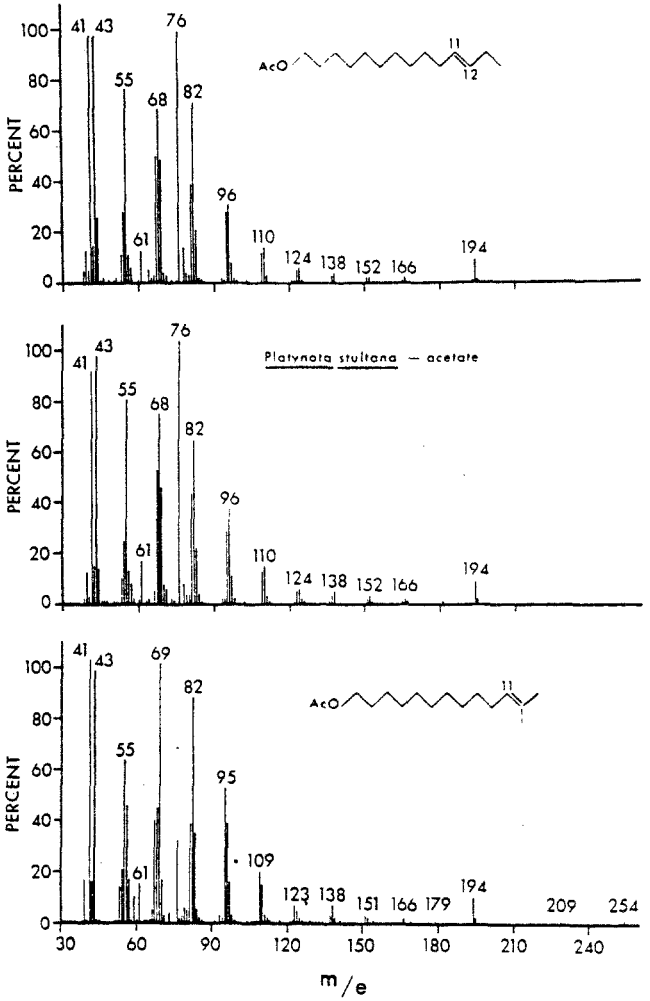

FIG. 2. Mass spectra of OLR acetates, t11–14 : Ac and 12Me-11–13 : Ac.

ozonolysis. An ozonolysis product of B and authentic 11-acetoxyundecanal produced from t11-14 : Ac had similar retentions on OV-1 (10.95 and 11.0 minutes, respectively, at 165°C) and on CHDMS (13.4 and 13.2 minutes, respectively, at 165°C).

A mass spectrum of B was identical to that of t11-14 : Ac, and differed somewhat from that of 12-methyl-11-tridecenyl acetate (12Me-11-13 : Ac; Figure 2), which was a plausible potential structure for B. In addition,

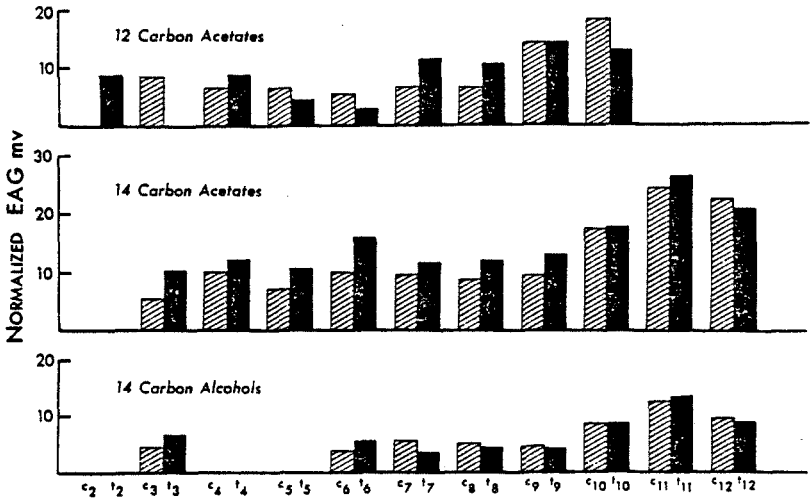

FIG. 3. Normalized EAG responses of male OLR antennae to 14-carbon alcohols and 12- and 14-carbon acetates using *cis*-6-tetradecenyl acetate as the standard. Normalization consisted of multiplying the average value of the EAG determinations (amplitude in mV) for each compound by 10 and dividing by the corresponding value of the standard.

12Me-11-13:Ac also had a retention time on PDEAS (170°C) that differed from that of the major component of B (9.8 and 9.25 minutes, respectively).

These data confirm the presence of t11-14:Ac and c11-14:Ac in the ratio of 88:12 in female OLR tip extracts, and strongly indicate the presence of 14:Ac.

Approximately 5 ng per female tip of peak B was found when assayed by GLC on OV-1.

EAGs of Standards

The normalized EAG responses on male OLR antennae of 14-carbon monounsaturated acetates and alcohols (Figure 3) show that t11-14:Ac elicits the greatest antennal response, with that from c11-14:Ac being almost as intense. While t11-14:OH and c11-14:OH elicit the greatest responses in the alcohol profile, these are only about half as intense as those from the corresponding acetates. None of the normalized responses for the 14-carbon aldehydes, 16-carbon acetates, alcohols, and aldehydes, and 12-carbon alcohols and aldehydes tested had values greater than 10. These data support the assignment of t11-14:Ac and c11-14:Ac as pheromone components.

DISCUSSION

The two compounds isolated from female OLR in an 88 : 12 ratio and identified as t11-14 : Ac and c11-14 : Ac, respectively, were shown in field tests to be the essential pheromone components of this species for attraction of males to females (Baker et al., 1975). These tests showed that t11-14 : Ac containing 4–9% c11-14 : Ac is a powerful attractant for OLR males, with optimum attractancy obtained with ca. 6% c11-14 : Ac. In most tests the synthetic lures were more than 5 times as attractive as virgin females. We have no explanation for the slight difference in the *trans-cis* ratio found in the female and the optimum ratio required for attraction of males in the field. Collection and analysis of the pheromone actually emitted by the female may resolve this discrepancy. Neither isomer by itself is attractive, and mixtures containing 20% or more of the *cis* isomer also are essentially non-attractive. The requirement of a specific geometric isomer ratio in a sex pheromone to obtain attraction of males has been reported for other tortricids, such as the redbanded leafroller moth *Argyrotaenia velutinana* (Walker) (Klun et al., 1973), the oriental fruit moth *Grapholitha molesta* (Busck) (Beroza et al., 1973; Roelofs and Cardé, 1974), the fruit tree leafroller moth *Archips argyrospilus* (Walker) (Roelofs et al., 1974), and the fruit tree tortrix moth *Archips podana* (Scopoli) (Persoons et al., 1974); for a pyralid, the European corn borer moth *Ostrinia nubilalis* (Hübner) (Klun et al., 1973; Kochansky et al., 1975); and for a gelechiid, the pink bollworm moth *Pectinophora gossypiella* (Saunders) (Hummel et al., 1973).

Field tests in which the 11-tetradecenyl acetates (11-14 : Acs) were mixed with the 11-tetradecenyl alcohols (11-14 : OHs) in approximately the same *trans-cis* ratios as the acetates (10–11% and 16–17% in two tests) showed that the alcohols increase trap catches when present as less than 3% of the mixture, optimally 0.2–2%, but decrease catches when present as greater than 20% of the mixture (Baker et al., 1975). Since females can contain the 11-14 : OHs in proportions that have been found to be inhibitory in field tests, it is possible that they function in several roles with this species. They could be biosynthetic precursors to the acetates, secondary pheromone components when released at a very low ratio with respect to the acetates, or inhibitors when released at higher ratios, possibly resulting from accumulation after mating. This last possibility would agree with the observation that the highest alcohols : acetates ratios were observed with extracts of females collected from mixed populations, while the lowest ratios were found in virgin female extracts.

Compounds found in female gland extracts which are closely related to the primary pheromone component and might even interact strongly with male antennae in EAG tests, are not necessarily pheromone components involved in attraction. It has been found that such compounds (a) can be inhibitory

to the attraction of males of the same species, e.g., 2-methyl-*cis*-7-octadecene is found in sex pheromone glands of the female gypsy moth *Porthetria dispar* (L.) (Bierl et al., 1970), but it inhibits the attraction of gypsy moth males to the synthetic pheromone 2-methyl-*cis*-7,8-epoxyoctadecane and to calling females (Cardé et al., 1973); or (b) can be inhibitory to the attraction of males of other species with no apparent effect on conspecific male attraction. An example of the latter is *cis*-9,*trans*-12-tetradecadien-1-ol, a compound emitted by females of the Indian meal moth *Plodia interpunctella* (Hübner). This compound has no apparent effect on conspecific male attraction to the pheromone *cis*-9,*trans*-12-tetradecadienyl acetate (c9,t12-14:Ac), but it does inhibit the response of male almond moths *Cadra cautella* (Walker) to c9,t12-14:Ac, which is a pheromone component common to both species (Sower et al., 1974).

Should synergism of the type described here for OLR, with compounds emitted at very low rates relative to the primary pheromone component, be a general phenomenon, it will add another factor to the task of defining these systems. Compounds found to be inactive or inhibitory for male attraction in the field at a level greater than 5% could be disregarded as pheromone components, however they could well be an integral part of the pheromone system at a very low level. These subtle components could provide the fine tuning used in species recognition, similar to individual recognition reported for higher animals (Müller-Schwarze et al., 1974; Thiessen et al., 1974).

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MUTUAL INHIBITION OF THE ATTRACTANT PHEROMONE RESPONSE BY TWO SPECIES OF *Ips* (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—The bark beetles, *Ips pini* and *I. paraconfusus*, are not cross-attractive in the field although they attack the same host material at the same time. Logs containing the pheromone-producing sex (males) of both species side by side attract significantly fewer beetles of each species than do males of either species alone. Ipsenol, a component of the male *I. paraconfusus* pheromone, duplicates the activity of male *I. paraconfusus* in inhibiting the response of *I. pini* to male *I. pini*. Linalool from male *I. pini* also reduces the catch of *I. paraconfusus* in response to male *I. paraconfusus*. Simultaneous production of a specific attractant pheromone and an interspecific chemical inhibitor favors exclusive use of the host substrate by the first arriving species.

Key Words—pheromone, inhibition, Scolytidae, *Ips*, *pini*, *paraconfusus*, ipsenol, linalool.

INTRODUCTION

The two bark beetle species, *Ips pini* (Say) and *I. paraconfusus* Lanier, are largely allopatric. They broadly overlap, however, in Jeffrey pine (*Pinus jeffreyi* Grev. and Balf.) over 5,000 feet elevation in the central Sierra Nevada mountains of California, and in ponderosa pine (*P. ponderosa* Laws.) in the southern Cascade mountain range. They occur in the same forest stands

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and infest the same portions of their common host, i.e., tops and limbs broken by wind or snow, logging slash, and large portions of the main bole. Both species attack this material at the same time of day and year. The two species are, however, seldom found in the same piece of host material and their galleries are not known to be intermixed.

It has been postulated that breeding isolation is maintained in sympatric species of *Ips* primarily by the specificity of their pheromones (Lanier, 1966; Wood, 1970; Lanier and Wood, 1975). The presence of only one species in any single piece of host material can similarly be attributed to the specificity of their pheromone systems. Earlier tests with these two species had shown that bolts containing either male *I. pini* or male *I. paraconfusus* do not attract the other species in the field (Lanier et al., 1972). In this paper we present experimental evidence suggesting that two mechanisms are involved in maintaining breeding isolation between *I. pini* and *I. paraconfusus* where they are sympatric: (1) specificity, in that attractant pheromones produced by one species are attractive only to that species, and (2) inhibition when the attraction response evoked by pheromones of one species is inhibited by volatile compounds produced by the other and vice versa.

METHODS AND MATERIALS

All field experiments but one were conducted during the summer and autumn of 1973 in the McCloud Flats area of the Shasta-Trinity National Forest, Siskiyou County, California. The area comprises approximately 50 square miles of ponderosa pine stands with abundant host material for *Ips*, originating from winter storm damage, logging activities, and chronic tree mortality caused by *Dendroctonus brevicomis* LeConte and *D. ponderosae* Hopkins. The one field test using linalool was conducted in the Sierra National Forest, Madera County, California, in a mixed conifer forest at 5,000 feet elevation where *I. paraconfusus* was known to be flying in the autumn of 1973.

Infested ponderosa pine was obtained from the McCloud Flats area and adult beetles were collected and sexed as they emerged from these logs in the laboratory (Browne, 1972).

Field evaluations followed procedures established over several years of work with species in this genus (Wood et al., 1968; Bedard and Browne, 1969; Lanier et al., 1972; Browne et al., 1973). Treatments, i.e., bolts containing males of either or both species, were placed into wire-mesh cylinders coated with Stikem Special® on pipe standards 4 feet above the ground. Each test consisted of several treatments set out at 50-meter intervals in a line. After each replication, i.e., one treatment in a given position for a 24-

hour period, the treatments were interchanged in a systematized way so that no treatment occupied the same site twice in any test. Beetles trapped during each replication were picked from the screens, placed in solvent, and later examined in the laboratory to check field identifications and to determine sex ratios.

Three separate field tests were conducted in which bolts of ponderosa pine containing both male *I. pini* and male *I. paraconfusus* side by side in the same bolt were compared with bolts containing each species separately and with bolts containing no beetles. Two densities of beetles were introduced into holes drilled in 25-cm bolts of ponderosa pine: 25 beetles of one species alone, or 25 plus 25 when the two species were combined; and 50 of one species alone, or 50 plus 50 when combined. Beetles were constrained in these holes by a fine metal screen, and each entire bolt was also wrapped with the same screen to prevent voluntary attacks by any of the wild population that may have evaded the sticky cylinder.

Materials assayed for biological activity were concentrates of volatiles from ponderosa pine logs infested with male beetles, synthetic ipsenol (2-methyl-6-methylene-7-octen-4-ol), and linalool (3,7-dimethyl-1,6-octadien-3-ol). Concentrates of volatile materials associated with male beetles boring in ponderosa pine were obtained by the method of Browne et al. (1973). In this procedure, a known number of males are inserted into holes drilled into the bark and phloem tissue of the host log, and the volatile attractants produced by these males boring for several days are condensed in a trap immersed in liquid nitrogen. The concentrate of condensed volatiles used for assay is obtained by ether extraction of the aqueous condensate followed by distillation of the ether. The concentrate can be diluted and eluted at a rate comparable to that of a stated number of males boring in a bolt per unit time, a rate expressed as beetle-minutes/minute (Browne et al., 1973).

Ipsenol was obtained from Chemical Samples Company, Cleveland, Ohio, at 95% purity and "research grade" linalool from Matheson Coleman and Bell. Both were further purified by gas-liquid chromatography in our laboratory to approximately 98% purity. Ipsenol is one of three synergistic terpene alcohols identified as the attractant pheromone produced by male *I. paraconfusus* (Silverstein et al., 1966), but apparently not by males of *I. pini* (Vité et al., 1972). Ipsenol was tested here following a preliminary field evaluation of potential attractant compounds for *I. pini* in 1972 (unpublished data). In that test, trap catches were reduced when ipsenol was eluted together with a bolt containing male *I. pini*, compared to catches on traps with male *I. pini* bolts alone. Linalool was tested following the concept of "differential diagnosis" (Vité and Renwick, 1970), since it is an isomer of ipsenol, does not occur in male *I. paraconfusus*, but is produced by male *I. pini* (Young et al., 1973*a*) and female *I. paraconfusus* (Young et al., 1973*a*), both of which

TABLE 1. EFFECT OF MALE *I. paraconfusus* AND MALE *I. pini* BORING SIDE BY SIDE IN THE SAME BOLT ON THE ATTRACTANT RESPONSE OF BOTH SPECIES: MCCLOUD FLATS, CALIFORNIA, 1973. MEAN NUMBER (RANGE IN PARENTHESES) AND SEX RATIO OF SPECIES TRAPPED ARE LISTED BY TREATMENT IN EACH TEST

	Treatment (males in bolt)	*I. pini*	♂:♀	*I. paraconfusus*	♂:♀	*E. lecontei*
Test 1[a]	25 ♂♂ *I. pini*	**21.0**[b1] (4–53)	1:2.0[c]	**0**	—	**0.5**[d] (0–1)
	25 ♂♂ *I. paraconfusus*	**0**	—	**2.8**[b4] (1–5)	1:0.9	**0.5** (0–2)
	25 ♂♂ *I. pini*+ 25 ♂♂ *I. paraconfusus*	**2.7**[b1] (0–10)	1:1.6	**0.2**[b4] (0–1)	—	**1.0** (0–4)
Test 2	25 ♂♂ *I. pini*	**27.8**[b2] (7–64)	1:1.2	**0**	—	**4.8** (0–12)
	25 ♂♂ *I. paraconfusus*	**0**	—	**4.0** (0–10)	1:1.4	**4.5** (1–9)
	25 ♂♂ *I. pini*+ 25 ♂♂ *I. paraconfusus*	**4.3**[b2] (1–8)	1:2.2	**0**	—	**6.0** (0–13)
Test 3	50 ♂♂ *I. pini*	**28.3**[b3] (5–64)	1:1.8	**0**	—	**5.6** (2–15)
	50 ♂♂ *I. paraconfusus*	**0**	—	**29.8**[b5] (5–51)	1:3.1	**4.5** (1–9)
	50 ♂♂ *I. pini*+ 50 ♂♂ *I. paraconfusus*	**11.3**[b3] (2–22)	1:1.7	**0.2**[b5] (0–1)	—	**6.6** (1–21)
Controls	Bolt containing no males (1 in each test)	**0**	—	**0**	—	**0.2** (0–1)

[a] Test 1: June 20–23, 1973. Tests 2 and 3: July 7–12, 1973. Six replicates of each treatment through time in each test.

[b] The probability (Student's *t* test) that means of trap catches assigned the same number would be obtained by chance is: (1) $P<0.05$; (2) $P<0.025$; (3) $P<0.2$ (NSD); (4) $P<0.005$; (5) $P<0.005$.

[c] Sex ratios of *I. pini* trapped within any test are not significantly different ($P>0.9$, χ^2 test).

[d] The probability (Student's *t* test) that means of trap catches of *E. lecontei* within each test would be obtained by chance is: Test 1, $P>0.5$; Test 2, $P>0.5$; Test 3, $P<0.4$.

reduce catches of *I. paraconfusus* on traps containing male *I. paraconfusus* bolts in the field (Table 1; Dahlsten, Wood and Bedard, unpublished).

Ipsenol and linalool were evaporated from 5-μl capillary tubes open at both ends (Drummond micro-caps®). The tubes were held in a vertical

position by taping them to the inside of a 35-mm film canister with holes drilled in its screw cap. The rate of evaporation of both ipsenol and linalool was approximately 1 mg over a 24-hour period, most of which probably evaporated during the daytime. This rate of evaporation was comparable to that used in earlier experiments with *I. paraconfusus* by Wood et al. (1968). The precise rate at which ipsenol is released by boring male *I. paraconfusus* is now being determined using the methods developed by Browne et al. (1973).

The response of beetles to the test materials in the laboratory was estimated by the number of beetles walking upwind to the source, in a multiple-choice, open-arena olfactometer (Wood et al., 1966). Beetles were assayed in groups of ten for a total of 50 to each stimulus. Those reaching the source after one or two attempts were scored as positive. Concentrates were eluted at a rate found to elicit a 70–80% response from females on the day of testing. Actual rates varied but were never above one beetle-minute/minute and were often much lower. The dosage level of ipsenol at 1×10^{-6} g/minute was used since this was the minimum level, at the time of testing, at which ipsenol alone elicited a measurable positive response from female *I. paraconfusus*.

RESULTS

Mutual Inhibition in the Field

In all three tests the bolts containing males of both species caught significantly fewer beetles of both species than did bolts containing males of either species alone (Table 1). Essentially no cross-attraction was found between the species as measured by trap catch on bolts infested with males of only one species.

Dissection of the bolts after Test 1 showed that there was no significant difference in the success of establishment of either species when alone, compared to both species when they were together in the same bolt. Only one or two individuals of either species failed to initiate boring activity in all cases. In addition, the activity of the males, as indicated by the extent of phloem excavation, did not differ noticeably between bolts containing both species together or separately. Thus it appeared that males of neither species were inhibiting the production of frass by the other species. This does not rule out the possibility, however, that production and/or composition of the pheromone present in the frass could be changed when males of both species are in close proximity.

The presence of male *I. pini* and male *I. paraconfusus* reduced the catch of *I. paraconfusus* over *I. paraconfusus* bolts alone by 97% on 25-male bolts

TABLE 2. INHIBITION OF THE ATTRACTANT RESPONSE OF FEMALE *I. paraconfusus* BY PHEROMONE CONCENTRATE FROM *I. pini* IN THE LABORATORY

Concentrate	Rate of delivery[a]	♀♀ *I. paraconfusus* (% response)
I. paraconfusus	0.1 bm/min	79[b]
I. paraconfusus+ *I. pini*	0.1 bm/min+ 0.5 bm/min	42
Controls (hexane only)	—	8

[a] Rates of delivery are expressed as beetle minutes/minute (bm/min).
[b] The probability (Student's *t* test) that these percent response figures would be obtained by chance is $P<0.001$.

and by 99% on 50-male bolts. In fact only two *I. paraconfusus* were caught on traps with bolts containing both species in all 18 replicates. At the same time, the presence of male *I. paraconfusus* in bolts containing 25 male *I. pini* reduced the catch of *I. pini* over traps containing only male *I. pini* bolts by 86%. This was a significant reduction in both tests. Although the catch was reduced on 50-male bolts by 60%, the difference was not significant. The sex ratios of *I. pini* caught on *I. pini* bolts alone and on the bolts containing both *I. pini* and *I. paraconfusus* were not significantly different, indicating that both sexes are equally affected by the treatment. Too few *I. paraconfusus* were caught by the bolts containing both species to make any comparison of sex ratios.

The predator *Enoclerus lecontei* (Wolcott) (Coleoptera: Cleridae) was the only associate insect caught in appreciable numbers. As the results indicate (Tables 1 and 4) *E. lecontei* responded equally to both species of *Ips* alone and to bolts containing both species together, and thus was apparently unaffected by the treatment.

Mutual Inhibition in the Laboratory

In the laboratory studies, concentrates from each species were effective in significantly reducing the response of females of the other species (and also males of *I. pini*) to volatiles produced by males of their own species (Tables 2 and 3). This response parallels that in the field although the reduction is not as great. The use of concentrates rather than active male beetles in the laboratory assay eliminates the possibility of visual and sound stimuli

TABLE 3. INHIBITION OF THE ATTRACTANT RESPONSE OF *I. pini* BY PHEROMONE CONCENTRATES OF *I. paraconfusus* AND BY IPSENOL IN THE LABORATORY

Concentrate	Rate of delivery[a]	*I. pini* (% response)	
		♀♀	♂♂
I. pini	0.5 bm/min	81[b1,3]	76[b2]
I. pini+ *I. paraconfusus*	0.5 bm/min+ 1.0 bm/min	28[b1,4]	20[b2]
I. pini+ ipsenol	0.5 bm/min 1×10^{-6} g/min	16[b3,4]	—
ipsenol	1×10^{-6} g/min	0	—
I. paraconfusus	1.0 bm/min	8	—
Controls (hexane only)	—	8	—

[a] Rates of delivery are expressed as beetle minutes/minute (bm/min) and as grams/minute (g/min).

[b] The probability (Student's *t* test) that percent response figures assigned the same number would be obtained by chance is: (1) $P<0.001$; (2) $P<0.001$; (3) $P<0.001$; (4) $P<0.2$ (NSD).

being involved from the males. However, further field tests are required in order to verify that the inhibition of attractant response observed in the field is a result of chemical stimuli.

Source of Inhibition—Effect of Ipsenol on I. pini

In four field tests, ipsenol significantly reduced the catch of *I. pini* on traps containing male *I. pini* (Table 4). The first of these tests demonstrated that ipsenol at the concentration used was as effective as actively boring male *I. paraconfusus* in inhibiting the attractant response of *I. pini*. The sex ratios of beetles caught on all three treatments were virtually identical, indicating a similar effect on both sexes.

Although beetle catch during the second test was low due to adverse weather conditions, the results still indicate a significant effect by ipsenol. Results of the third and fourth tests show that ipsenol will reduce trap catches of *I. pini* even at high population densities. When all tests are taken together, ipsenol reduced the catch of *I. pini* on traps containing bolts infested with 25 males of *I. pini* by 78% and with 50 males of *I. pini* by 71%.

In the laboratory, ipsenol delivered in place of *I. paraconfusus* concentrate also significantly reduced the response level of female *I. pini* to *I. pini* concentrate (Table 3). There was no significant response by female *I. pini* to

TABLE 4. EFFECT OF IPSENOL ON ATTRACTANT RESPONSE OF *I. pini* TO BOLTS INFESTED WITH MALE *I. pini*: MCCLOUD FLATS, CALIFORNIA, 1973. MEAN NUMBER (RANGE IN PARENTHESES) AND SEX RATIO OF SPECIES TRAPPED ARE LISTED BY TREATMENT IN EACH TEST

	Treatment (males in bolt)	*I. pini*	♂:♀	Replications	*E. lecontei*
Test 1[a]	50 ♂♂ *I. pini*	**4.50**[b1,2] (15–90)	1 : 1.8[c]	7	**5.7**[b7] (1–19)
	50 ♂♂ *I. pini*+ 50 ♂♂ *I. paraconfusus*	**2.8**[b1,3] (0–8)	1 : 1.8	6	**10.6**[b7] (3–41)
	50 ♂♂ *I. pini*+ ipsenol[d]	**4.0**[b2,3] (0–15)	1 : 1.9	5	**4.2**[b7] (0–10)
Test 2	25 ♂♂ *I. pini*	**3.3**[b4] (1–10)	—	12	—
	25 ♂♂ *I. pini*+ ipsenol	**0.75**[b4] (0–3)	—	12	
Test 3	50 ♂♂ *I. pini*	**192.6**[b5] (53–361)	—	8	—
	50 ♂♂ *I. pini*+ ipsenol	**54.6**[b5] (24–114)	—	8	—
Test 4	25 ♂♂ *I. pini*	**14.6**[b6] (2–48)	—	8	—
	25 ♂♂ *I. pini*+ ipsenol	**3.25**[b6]	—	8	—
Controls	Bolt with no beetles (1 in each test line)	**0**	—	—	—

[a] Test 1: July 7–12, 1973. Test 2: August 15–19, 1973. Tests 3 and 4: August 28–31, 1973.
[b] The probability (Student's *t* test) that means of trap catches assigned the same number would be obtained by chance is: (1) $P<0.005$; (2) $P<0.01$; (3) $P<0.2$ (NSD); (4) $P<0.025$; (5) $P<0.005$; (6) $P<0.05$; (7) $P>0.4$ (NSD).
[c] Sex ratios of beetles caught by the three treatments in test 1 are not significantly different ($P>0.9$, χ^2 test).
[d] Ipsenol evaporated at 1 mg/24 hr.

ipsenol delivered alone, or to *I. paraconfusus* concentrate. Thus, at these dosage levels in the olfactometer, ipsenol can duplicate the inhibitory effect of male *I. paraconfusus* concentrate toward *I. pini*.

TABLE 5. EFFECT OF LINALOOL ON ATTRACTANT RESPONSE OF *I. paraconfusus* TO BOLTS INFESTED WITH MALE *I. paraconfusus*: SIERRA NATIONAL FOREST, CALIFORNIA, 1973[a]. MEAN NUMBER (RANGE IN PARENTHESES) AND SEX RATIO (*I. paraconfusus*) OF SPECIES TRAPPED ARE LISTED BY TREATMENT

Treatment (males in bolt)	*I. paraconfusus*	♂:♀	*I. pini*
25 ♂♂ *I. paraconfusus*	**39.2**[b1,2] (0–183)	1:2.1[c]	**0.2**[b5] (0–2)
25 ♂♂ *I. paraconfusus* + 25 ♂♂ *I. pini*	**7.4**[b1,3] (0–15)	1:2.6	**0**
25 ♂♂ *I. paraconfusus* + linalool[d]	**10.5**[b2,3] (0–36)	1:2.1	**0**
25 ♂♂ *I. pini*	**0.25**[b4] (0–3)	—	**1.3**[b5,6] (0–7)
25 ♂♂ *I. pini* + linalool	**0.25**[b4] (0–3)	—	**0.4**[b6] (0–3)
Control (bolt containing no males)	**0.1**[b4] (0–1)	—	**0**

[a] Test run: August 7–16, 1973 with 12 replicates of each treatment.
[b] The probability (Student's *t* test) that means of trap catches would be obtained by chance is: (1) $P<0.05$; (2) $P<0.10$; (3) $P<0.20$ (NSD); (4) $P>0.50$ (NSD); (5) $P<0.10$; (6) $P<0.20$ (NSD).
[c] Sex ratios of *I. paraconfusus* trapped by the three treatments are not significantly different ($P>0.90$, χ^2 test).
[d] Linalool evaporated at 1 mg/24 hr.

Source of Inhibition—Effect of Linalool on I. paraconfusus

Bolts infested with males of both species reduced the catch of *I. paraconfusus* by 81% over the number caught on traps containing bolts infested with male *I. paraconfusus* alone (Table 5). However, the effect of linalool on the catch of *I. paraconfusus* was more variable and could only be demonstrated at a level of significance lower than that recorded for male *I. pini*. There was no significant difference between the number of *I. paraconfusus* responding to traps containing either male *I. pini* together with male *I. paraconfusus*, or linalool with male *I. paraconfusus*. However, the lack of any significant catch of *I. paraconfusus* on traps containing male *I. pini* bolts alone indicates that linalool is not the sole contributor from male *I. pini* that accounts for reduced catches of *I. paraconfusus* on traps containing male *I. pini*.

DISCUSSION

Inhibition of response to the sex pheromone of one species by another species, usually closely related, is a well established phenomenon in the Lepidoptera. When females of two species are confined together in a trap, catches are reduced over those when both are exposed separately (Ganyard and Brady, 1971; Haile et al., 1973). Berisford and Brady (1973) have also shown that combined extracts of two species of Olethreutidae catch fewer moths than do the individual extracts. In addition there are several examples showing that differences in the presence or concentration of one or more geometric isomers of the female sex pheromone can effect reproductive isolation between two species (Roelofs and Comeau, 1969, 1971; Roelofs et al., 1972; Kaae et al., 1973; Klun et al., 1973). The phenomenon of inhibition of attractant response by pheromone compounds has been demonstrated in two species of Coleoptera. Werner (1972) showed that pheromones from beetles of the genus *Dendroctonus* inhibit the response of *Ips grandicollis* Eichh. to host tree terpenes. Wood et al. (1967) demonstrated that the response of *I. latidens* LeC. to two of the three components of the pheromone of *I. paraconfusus* was inhibited by addition of the third component. The present study appears to be the first, however, which demonstrates *mutual* inhibition of attractant response to pheromones between closely related species of Coleoptera.

It has been hypothesized that the specificity of pheromones among sympatric species of bark beetles is probably an important mechanism in preventing interspecific mating and in minimizing competition for space and food (Wood, 1970; Lanier and Wood, 1975; Lanier and Burkholder, 1974). This would be especially true for sympatric species which breed in the same host tree species, such as *I. paraconfusus*, *I. latidens*, and *I. pini* in California, or *I. grandicollis*, *I. calligraphus* Germ., and *I. avulsus* Eichh. in the Southern United States. In each area of the country these sympatric species are in different species groups (Hopping, 1963) and are more distantly related than those species within species groups. Specificity has been demonstrated for several species which occur together in California (Vité and Gara, 1962; Wood, 1970; Lanier et al., 1972; Lanier and Wood, 1975) and other areas (summarized by Lanier and Wood, 1975). Within species groups, cross-attraction occurs between species and their distributions are generally allopatric (Lanier and Burkholder, 1974; Lanier and Wood, 1975).

The present study demonstrates, however, a mechanism other than the production of different attractant compounds or mixtures of compounds to account for and maintain species isolation: i.e., *I. paraconfusus* and *I. pini* boring in the same host log chemically inhibit the attractant response of one another. This mechanism is strongly supported by the field data. Pre-

liminary data also suggest that the source of this inhibition originates from the attractant pheromone system of the other species. Thus, active inhibition of the response of *I. pini* to boring males of *I. pini* can be mimicked by ipsenol, one component of the attractant pheromone system of *I. paraconfusus*. With *I. paraconfusus* inhibition, linalool, one component of the complex of volatiles produced by boring *I. pini* males, appears to mimic some of the inhibitory effect of *I. pini* males. To discuss the chemical mechanisms involved, however, we must know much more about the attractant pheromone systems of both species, especially *I. pini*, in which linalool has not otherwise been shown to have any biological activity. In addition, the more variable results recorded for inhibition caused by linalool might be clarified by testing different concentrations and by investigating the involvement of other compounds.

Studies in a number of *Ips* species (Renwick and Vité, 1972; Vité et al., 1972; Young et al., 1973*a*, *b*) have revealed no additional pheromone compounds to the three terpene alcohols identified by Silverstein et al. (1966) from *I. paraconfusus*. Several species utilize one or more of these compounds as attractants and one species, *I. calligraphus*, apparently requires host odors to increase activity (Renwick and Vité, 1972). A sequential isolation program (Silverstein et al., 1967) is clearly required to identify the compounds that elicit the inhibition of response demonstrated here.

In earlier work with *I. paraconfusus* (Silverstein et al., 1966), ipsenol was not attractive in the laboratory assay at a dosage level of 1×10^{-4} g. This is in contrast to the present study, where females responded to ipsenol at 1×10^{-6} g/minute. The apparent difference may be attributable to many factors, including varying response levels in different batches of the test insects, undetected trace amounts of active substances, and/or different methods used for evaporation of compounds.

Mutual pheromone inhibition imparts a clear adaptive advantage to the pioneer species by excluding a potential competitor for the supply of host substrate. For example, suitable host material, such as snow or wind breakage, occurs only periodically (although logging operations now ensure a more plentiful supply), and in many years this supply is undoubtedly scarce. In addition, the lack of cross-attraction between species helps to assure that the competitor will not arrive on the host at the same time. Thus, simultaneous production of a specific attractant pheromone and an interspecific inhibitor of the attractant response favors exclusive use of the host substrate by the first arrival.

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CHEMICAL ATTRACTANTS FOR THE SMALLER EUROPEAN ELM BARK BEETLE *Scolytus multistriatus* (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—The secondary attractant for the smaller European elm bark beetle *Scolytus multistriatus* is a mixture of three compounds: (-)4-methyl-3-heptanol (I); 2,4-dimethyl-5-ethyl-6,8-dioxabicyclo(3.2.1) octane (II); and (-)α-cubebene (III). The novel structure assigned to compound II was confirmed by synthesis. All three compounds were isolated from the volatile compounds collected on Porapak Q by aerating elm bolts infested with virgin female beetles. The GLC fractionations were monitored by two laboratory bioassays. Individually, each compound was inactive in the laboratory bioassays, but a mixture of all three showed activity nearly equivalent to that of the of the original Porapak extract. A mixture of synthetic I and II plus natural III (from cubeb oil) was highly attractive to beetles in preliminary field tests.

Key Words—aggregation pheromone, α-cubebene, 2,4-dimethyl-5-ethyl-6,8-dioxabicyclo(3.2.1)octane, Dutch elm disease, elm bark beetle, insect attractant, 4-methyl-3-heptanol, multilure, multistriatin, *Scolytus multistriatus*.

INTRODUCTION

The smaller European elm bark beetle *Scolytus multistriatus* (Marsham)

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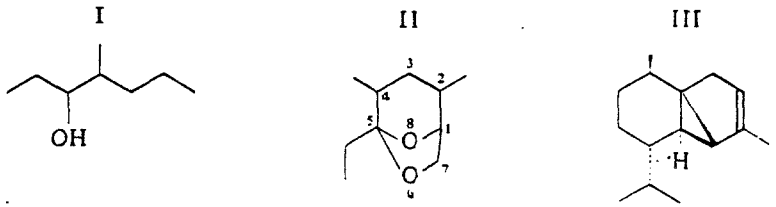
is the principle vector for the Dutch elm disease pathogen *Ceratocystis ulmi*. The disease has devastated elm populations in the northeastern United States and presently threatens American elms throughout their natural and cultivated range. The chemical elucidation of the aggregation pheromone for *S. multistriatus* has been motivated by the possibility that mortality traps baited with the attractant might be used for the survey and control of elm bark beetle populations as an integral part of future Dutch elm disease control programs.

Investigations by Martin (1936), Meyer and Norris (1967), and Peacock et al. (1971) have shown that adult elm bark beetles are weakly attracted to uninfested elm wood. The report by Peacock et al. (1971) also demonstrated that secondary mass attack by both sexes of beetles on potential breeding sites is directed by an aggregation pheromone produced by virgin females boring into the phloem-cambial region of weakened elm trees. The attractiveness of the virgin females is greatest during days 4 through 7 after initial infestation and is markedly reduced after mating. We report here that the chemical attractant is comprised of at least three compounds (I–III) that in



combination elicit beetle responses in both laboratory and field assays: 4-methyl-3-heptanol (I) and 2,4-dimethyl-5-ethyl-6,8-dioxabicyclo(3.2.1) octane (II) are beetle-produced pheromones, while α-cubebene (III) is a host-produced synergist.

ATTRACTANT SOURCE AND CHEMICAL ISOLATION

The total attractant source was a pentane extract of Porapak Q[5], a solid absorbent used to collect organic volatiles from aerated chambers containing virgin female-infested elm bolts.[5] A recent report by Byrne et al. (1975) demonstrated the general applicability of Porapak Q as a trapping agent for collection of insect pheromones by aeration. Initial attempts to isolate the pheromone utilized virgin female frass as a source (Peacock et al., 1973) since the frass of other scolytids is known to contain aggregation pheromones (Silverstein, 1970; Vité, 1970; Borden and Stokkink, 1971).

[5] Mention of a product does not imply endorsement by the Forest Service or the USDA.

TABLE 1. LABORATORY BIOASSAYS OF ATTRACTANT COMPONENTS

Test	Materials tested[a]	Dosage per replicate[b] (ng) Compounds I	II	III	Mean response (%) Bioassay 1[c]	Bioassay 2[d]
A	I	25	—	—	0	7.0
B	II	—	19	—	1.3	3.0
C	III	—	—	50	2.7	9.0
D	I+II	25	19	—	22.7	18
E	I+III	25	—	50	17.3	15
F	II+III	—	19	50	2.7	13
G	I+II+III	25	19	50	46.0	44
H	Porapak extract	25	19	50	45.3	54
I	*n*-Hexane[e]	—	—	—	0	0

[a] The Porapak extract used in this test was quantitatively analyzed by GLC for compounds I, II, and III. Test solutions A–H were prepared with *n*-hexane such that a 25-μl aliquot delivered the quantities indicated in the table.

[b] Each 25-μl aliquot of test solutions A–H represented 50 beetle hours (BH). The BH was the unit of biological quantitation. For example, a Porapak aeration extract of 7 days (168 hours) on 4000 ♀♀ represented: $168 \times 4000 = 6.7 \times 10^5$ BH.

[c] Peacock et al. (1973). Mean % responses of 3 replicates of 25 beetles each for tests A–F; 6 replicates of 25 for tests G and H.

[d] Moeck (1970). Mean % responses of 3 replicates of 25 for tests E and F; 4 replicates for tests A, B, C, and D; 7 replicates for test G; and 8 replicates for test H.

[e] Baker "Analyzed Reagent."

Subsequent laboratory and field comparisons of frass, frass extracts, and Porapak aeration extracts showed the Porapak extract to be a superior source of attractive material (Peacock et al., 1975).

Each infestation of 4,000–7,000 virgin females was aerated continuously for 7 days beginning at the 3rd day after introduction in order to coincide with the period of maximum pheromone production. The Porapak extracts were concentrated under a fractional distillation column (glass bead packing) and the concentrates were fractionated by preparative GLC. The fractionation schemes were monitored in the laboratory by the arrestant-excitant bioassay (Peacock et al., 1973) and by an attractant bioassay with an olfactometer described by Moeck (1970).

Biological activity of the original Porapak extract could be approximated only by recombination of 3 of the 7 initial GLC fractions (SE-30), each of which yielded 1 of the 3 active components after successive GLC fractionation on Carbowax 20M and Apiezon L columns. The activities of all possible combinations of compounds I, II, and III relative to the Porapak extract were determined by the bioassays mentioned above (Table 1). Thus, the laboratory

bioassays clearly indicate that none of the compounds is active individually, and that all 3 are necessary and sufficient to yield activity nearly equivalent to that of the Porapak extract.

BIOLOGICAL SOURCES AND RELEASE RATIOS

Virgin female *S. multistriatus* beetles retrieved from elm bolts within 3–5 days after infestation were mixed with powdered dry ice and mascerated with a mortar and pestle. A pentane extract of the mascerated beetle tissue was analyzed by GLC for compounds I, II, and III. Positive identifications for compounds I and II were obtained on two analytical columns (6.1-m Carbowax 20M, 6.1-m Apiezon L), but only a trace of III was observed. Similar analyses of a hexane-Waring blender extract of uninfested elm tissue (xylem and phloem strips) and a Porapak extract from the aeration of uninfested elm bolts yielded compound III, but no detectable quantities of I or II. On the basis of these results, we conclude that I and II are beetle-produced pheromone components and that III is a host-produced component.[6]

Quantitative GLC analyses of several Porapak extracts from aeration of virgin females on logs indicated that the release ratio of I to II was consistently 1 :1. The ratio of I or II to III, however, was variable between 1 :2 and 1 :10.

IDENTIFICATION AND SYNTHESIS

Compound I was identified as (-)4-methyl-3-heptanol ($[\alpha]_D^{26} - 15°$) by comparison of its MS, IR, and NMR spectra with those of a synthetic sample prepared by sodium borohydride reduction of 4-methyl-3-heptanone (Aldrich Chemical Co.). Since 4-methyl-3-heptanol contains two chiral centers, the synthetic material exists as two diastereomeric forms; however, only one form is produced by the female beetle. The synthetic diastereomers are separable by gas chromatography, with the natural alcohol corresponding to the diastereomer of shorter retention time on a Carbowax 20M column. Compound I was shown to be a single enantiomer by comparison of the ^1H and ^{19}F NMR spectra of the Mosher derivatives[7] of racemic I with those of natural I.

The novel structure assigned to compound II is consistent with the MS, IR, and NMR spectra. The carbon skeleton of II was determined by hydro-

[6] The possible role of microorganisms in the production of bark beetle attractants has not been investigated.

[7] The esters of R(+)α-methoxy-α-trifluoromethylphenylacetic acid (Dale et al., 1969).

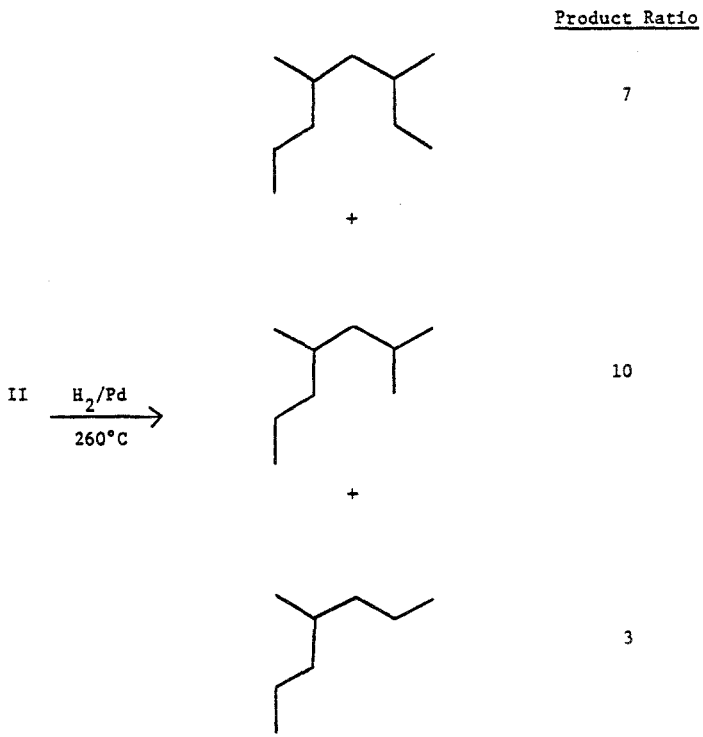
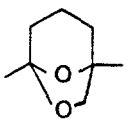
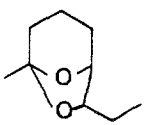


FIG. 1. Hydrogenolysis of α-multistriatin (II).

genolysis on palladium (Figure 1). The mass spectra of the hydrogenolysis products separated by gas chromatography were identical with those of authentic samples of 3,5-dimethyloctane, 2,4-dimethylheptane, and with the published mass spectrum of 4-methylheptane. The ring structure of II is significant since it is identical to the ring structures of two other scolytid aggregation pheromones, frontalin (Kinzer et al., 1969) and brevicomin (Silverstein et al., 1968).



Frontalin



Brevicomin

The spectra of a fourth compound isolated from the Porapak extract and inactive in the laboratory bioassay were also consistent with the ketal structure assigned to II. Structure II can theoretically exist as four diastereomers by inversions of stereochemistry at carbons 2 and 4 relative to carbon 1,

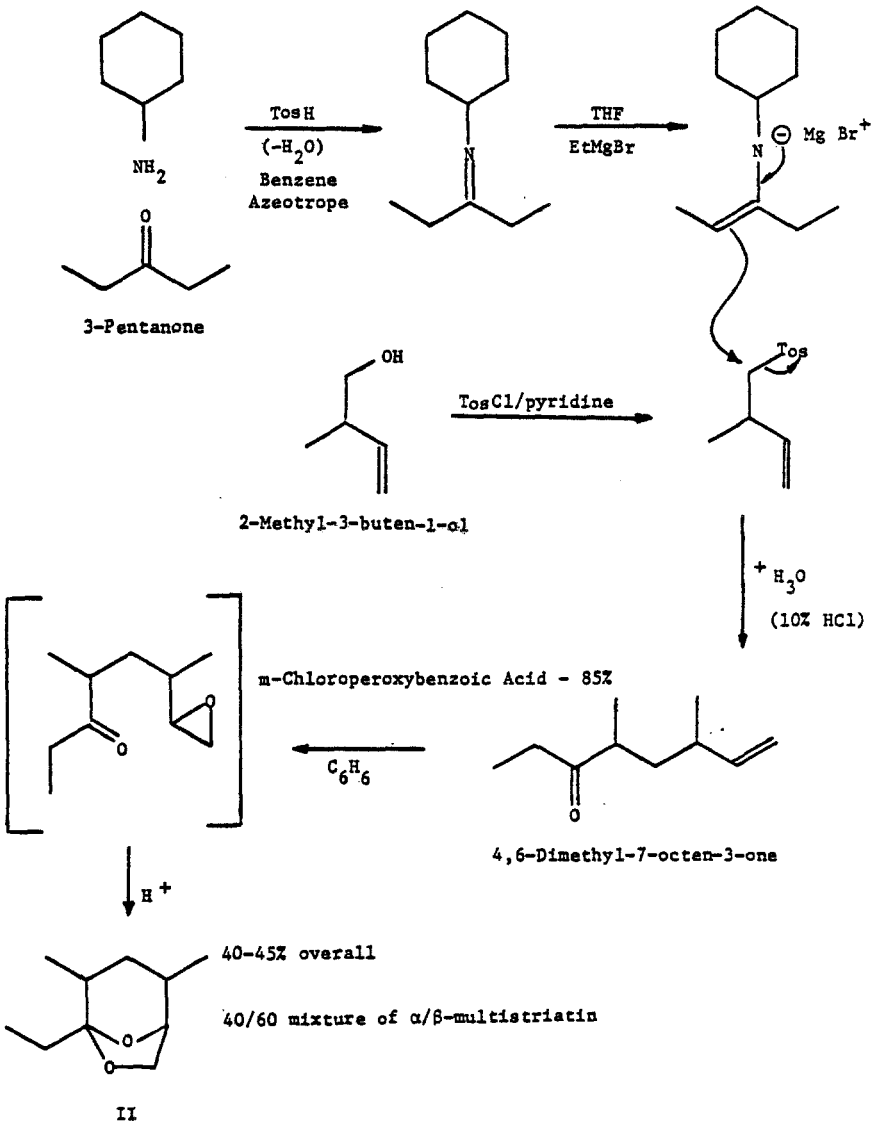


FIG. 2. Synthesis of α- and β-multistriatin.

thereby suggesting that the fourth compound is a diastereomer of II. The active and inactive ketals have been assigned the trivial names of α- and β-multistriatin, respectively.

The gross structure of α- and β-multistriatin was confirmed by synthesis (Figure 2). The final synthetic product was distilled through a short-path

microstill (bp 81–84°C, 22 mm Hg) in 40–45% overall yield from 2-methyl-3-buten-1-ol, and consisted of 90% total ketals with a 40/60 ratio of α/β multistriatin. The MS, IR, and NMR spectra of natural α- and β-multistriatin were congruent with those of the synthesized compounds. Results of investigations into the relative stereochemistry of α- and β-multistriatin will be reported in a later publication.

Compound III ($[\alpha]_D^{25} -24°$) was identified as (-)α-cubebene, a known sesquiterpene[8,9] whose structure and absolute stereochemistry have been rigorously determined (Ohta et al., 1966; Piers et al., 1971; Tanaka et al., 1972). The MS, IR, and NMR spectra of isolated III matched those of an authentic sample of (-)α-cubebene[10] and the specific rotation was in accord with the literature value ($[\alpha]_D^{30} -20.0°$) (Ohta et al., 1966; Vlakhov et al., 1967). In addition, GLC comparison by coinjection on 100-ft Carbowax and 50-ft Apiezon L SCOT columns further supported the assignment of III as α-cubebene. Quantities of (-)α-cubebene sufficient for field tests were obtained by distillation of 75 ml of cubeb oil[11] through a 40-plate spinning band fractional distillation column, with the purest fraction (1 ml, bp 30–32°C, 1.3 mm Hg) containing 90% (-)α-cubebene.

FIELD TEST OF SYNTHETIC ATTRACTANTS

The field tests were conducted in a residential section of Charlotte, North Carolina during April 30 and May 1, 1974. The test was designed to indicate the relative attractiveness of three pheromone preparations. These were:

(1) Porapak aeration extract (PE)

(2) Attractant mixture (AM).

(a) synthetic 4-methyl-3-heptanol (>99%), a 50/50 mixture of diastereomers.

(b) GLC-purified synthetic α-multistriatin (99%).

(c) (-)α-cubebene (90%), distilled from cubeb oil.

(3) "Multilure," crude attractant mixture (CAM).

(a) 4-methyl-3-heptanol (>99%) (Aldrich Chemical Co.), a 55/45 mixture of diastereomers.

[8] A major constituent of the essential oil of the fruits of cubeb (*Piper cubeba* L.) (Ohta et al., 1966); also isolated from Bulgarian peppermint oil (*Mentha piperita*) (Vlakhov et al., 1967) and several citrus oils (Veldhuis and Hunter, 1967).

[9] This compound was also isolated from 4–7-day virgin female frass.

[10] An authentic sample of α-cubebene was graciously supplied by Dr. Y. Hirose, Institute of Food Chemistry, Dojimanaka, Kita-ku, Osaka, Japan.

[11] Fritzsche Dodge & Olcott, Inc., New York, New York 10011.

TABLE 2. COMPARATIVE FIELD EVALUATION OF ATTRACTANT MIXTURES FOR *Scolytus multistriatus*[a]

Date	Paired comparisons					
	PE[b] vs. AM[c]		PE vs. multilure[c]		AM vs. multilure	
4/30	690	766	1164	843	929	1268
5/1	356	724	465	897	865	645
Total	1046	1490	1629	1740	1794	1913

Total beetles caught on: PE (20 traps); 2675
AM (20 traps); 3284
Multilure (20 traps); 3653

[a] Each entry is a total for the 10 traps used for each material indicated. Fresh screens were employed with the original vial for the second night's test.
[b] Dosage: Unanalyzed Porapak extract; 1×10^5 BH per vial (50 μl PE).
[c] Dosage: 1×10^5 BH corresponds to the following quantities of attractants per vial. 0.1 mg 4-methyl-3-heptanol (0.05 mg of active diastereomer). 0.05 mg α-multistriatin: 0.125 mg of α/β mixture in multilure. 0.2 mg (-)α-cubebene (90%): 2.0 mg of cubeb oil in multilure. The components were mixed prior to loading, and diluted with hexane so that 50 μl of solution was introduced per vial.

(b) crude synthetic multistriatin (90%), a 40/60 mixture of α- and β-multistriatin.

(c) cubeb oil, containing 10% (-)α-cubebene.

A paired comparison test was used to evaluate the relative attractiveness of the three attractant preparations. A total of 30 trapping sites were employed with 10 sites for each of the following paired comparisons: PE vs. AM, PE vs. multilure, AM vs. multilure. Each trap site consisted of two traps hung at one tree on opposite sides of the main bole. The traps were 31-cm-square hardware cloth (6-mm × 6-mm mesh) coated with Stikem Special®[12] baited at the center with 2.5-ml polyethylene snap-cap vials[13] containing one of the three attractant preparations. The vials were loaded and sealed with the attached cap 3 days prior to placement in the field. Positional effects were minimized by exchanging the positions of the traps at each site after the first day of trapping. Trap catches for the 48-hour test period are summarized in Table 2.

The following observations can be extracted from the field test data:

(1) The three attractant preparations appear to be equally attractive in the field.

[12] Michel and Pilton Co., Emeryville, California 94608.
[13] Bel Art Products, Pequannock, New Jersey 07440 (Catalogue No. F-17561).

(2) The inactive diastereomers of I and II do not inhibit responses of flying beetles.

(3) The remaining constituents of cubeb oil likewise do not deter attraction to the crude synthetic mixture.

CONCLUSIONS

Compounds I, II, and III appear to be the principal components of the secondary attractant for *Scolytus multistriatus*. Furthermore, multilure is a practical, inexpensive, and highly attractive mixture that may prove useful in large-scale trapping of beetles on a city-wide basis. A study is presently under way in Detroit, Michigan, to determine if Dutch elm disease incidence can be reduced by mass trapping of *S. multistriatus*.

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SUBSPECIES SPECIFICITY OF RESPONSE TO A MAMMALIAN SOCIAL ODOR

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Abstract—Two subspecies of deer, black-tailed deer (*Odocoileus hemionus columbianus*) and Rocky Mountain mule deer (*O. h. hemionus*) were exposed to the tarsal scents of both subspecies in a social setting. The scent was sprayed on one individual in a group. The responses, consisting of sniffing, licking, and following, were stronger to each subspecies' own tarsal scent. This discrimination may be important for sexual isolation in areas where the ranges of the two species overlap.

Key Words—deer, mammals, odor preference, odor specificity, olfactory discrimination, pheromone, sexual isolation, social odor, tarsal scent.

INTRODUCTION

Taxonomic specificity of social (including sexual) odors in mammals is considered important for the process of speciation and for maintaining the sexual isolation of species and perhaps also subspecies. Thus far, the specificity of responses to social scents in mammals has been demonstrated only in two groups of rodents, bank voles of the genus *Clethrionomys* (Godfrey, 1958; Rauschert, 1963) and deer mice, *Peromyscus* (Moore, 1965).

In *Clethrionomys*, a male typically "preferred" the odor of a female of his own species or subspecies to that of another. Rauschert (1963), in repeating Godfrey's experiments, found species specificity of the males' responses in three species. On the level of the subspecies, however, only one out of four subspecies tested showed a preference for the odor of its females. In *Peromyscus*, both sexes of *P. maniculatus* preferred the odor of the opposite sex of their own species to that of *P. polionotus*, while the latter species showed no such preference.

In the studies cited, the entire body odor was used as stimulus, and no attempts at localizing the source of the decisive odor component(s) were

made. We report a case of subspecies specificity of responses to an anatomically localized social scent in an ungulate species. Two subspecies, the black-tailed deer (*Odocoileus hemionus columbianus*) and the Rocky Mountain mule deer (*O. h. hemionus*) possess tarsal organs on the inside of their hocks. The tarsal organ consists of the tarsal gland in the skin, the skin surface covering it, the hair over the gland, and the scent contained on the skin and the hair.

Both subspecies communicate within an established group by means of the tarsal scent. They sniff the tarsal organs of group members regularly, in the black-tailed deer about once per hour per individual (Müller-Schwarze, 1971), with no further behavioral consequences. If, however, a new individual enters the group, most members of this group sniff its tarsal organs frequently as a prelude to pursuit and attack.

The odoriferous materials ("scent") that are present on the surface of the skin and the hair originate from glandular secretions, urine, and probably from interactions between them. The urine is deposited on the tarsal organs during "rub-urination," when the hocks are rubbed together while the animal urinates over them. This behavior occurs in both sexes and at all ages, but is particularly frequent in males during the rut.

O. h. columbianus occurs along the Pacific coast of North America from British Columbia to Northern California. *O. h. hemionus* occurs over the Rocky Mountains area from Alberta to Arizona and from Northeastern California to South Dakota. It is larger than *O. h. columbianus*, and more adapted to cold, having longer hair and more sluggish behavior. Black-tailed deer and mule deer are in contact along the common boundary of their ranges running from North to South. Often they are geographically isolated during the breeding season, because in fall black-tailed deer migrate West and mule deer go East. Hybridization has been described for Northern California (Shasta-Lassen region), Oregon (White River, east of Mount Hood), Washington (Yakima and Klickitat counties in the South, and Skagit and Whatcom counties in the North) and in the Pemberton-Boston bar area of British Columbia (Cowan, 1956).

According to our observations at close range, the basic motor patterns in social, agonistic, sexual, and maternal behavior are qualitatively identical in *O. h. hemionus* and *O. h. columbianus*, although interactions between individuals are more frequent and more intense in *O. h. columbianus*.

METHODS AND MATERIALS

Subjects and Maintenance

The black-tailed deer were taken as fawns several days old in California

TABLE 1. INDIVIDUAL DIFFERENCES OF RESPONSES IN THE MULE DEER GROUP (FREQUENCIES PER TEST SERIES)

Factor observed	♀1 (adult)	♂1 (yearling son of ♀1)	♀2 (adult)	♀3 (yearling daughter of ♀2)	Tests (*N*)
Frequency of sniffing sample (or tarsal area)					
Black-tailed deer tarsal odor	6	11	1	1	10
Mule deer tarsal odor	12	12	7	5	10
Untreated individuals (during all experiments)	7	20	3	7	20
Total	25	43	11	13	20
Frequency of licking sample (or tarsal area)					
Black-tailed deer tarsal odor	5	2	0	0	10
Mule deer tarsal odor	3	3	0	0	10
Untreated individuals (during all experiments)	2	4	0	4	20
Total	10	9	0	4	20
Frequency of nonresponse					
Black-tailed deer tarsal odor	2	20	11	8	10
Mule deer tarsal odor	4	12	9	9	10
Untreated individuals (during all experiments)	3	1	2	3	20
Total	9	33	22	20	20
% Response $\frac{\text{Sniffing}+\text{licking}}{\text{Sniffing}+\text{licking}+\text{nonresponse}}$	88	61	33	46	20

(Napa and Santa Cruz counties), and the mule deer were collected as fawns at the same ages in Eastern Utah. Both subspecies were hand reared in groups and kept in captivity. For the experiments on odor specificity, we used established groups of 4 animals from each subspecies. Such an established group is to be preferred for this type of experiment because experience shows that strange associations result in agonistic behavior that would obscure the tests. Throughout the experiments, each subspecies was kept in a separate pen, 70 × 30 M. They were fed alfalfa hay, fresh alfalfa, food pellets (50% alfalfa hay, plus barley, wheat bran, and minerals), salt, water, and occasionally, fresh apples. The group of black-tailed deer consisted of 3 adult females (1 was 5 years old and 2 were 4 years old) and one male (5 years old). The group of mule deer comprised 2 adult females (both 3 years old) and 1 male and 1 female yearling, offspring of the two does. As the mule deer females had been bred by a black-tailed deer, the 2 yearlings were hybrids. Their size, appearance, and sluggish behavior resembled closely that of mule deer. When tested for homogeneity, their responses to social odors showed no qualitative or quantitative differences to those of their mothers. These data are shown in Table 1. Therefore, they were used in the experiment, and their data were combined with those of their mothers. These 4 animals constituted all the tame mule deer available at the time.

Behavior Patterns

Three factors contributed to behavioral differences within the group of mule deer.

Firstly, the male was more active than the females. He not only sniffed and licked more often, but he also had the most encounters with no overt behavioral response (nonresponse).

Secondly, the yearlings showed a tendency to act as did their mothers: $♀_2$ and her daughter ($♀_3$) interacted little with other individuals, while $♀_1$ and her son ($♂_1$) were more active (Table 1).

Thirdly, social relationships between individuals were important: $♀_2$ and $♀_3$ repeatedly attacked the male upon his approach by striking him with their forelegs or by pushing him with their muzzles, thus terminating encounters.

Stimulus Preparation

The scent samples were prepared in the following way: Hair from the tarsal glands of male mule deer from northern Utah and from male black-tailed deer from central California were extracted with petroleum ether. Ten glands were used for each extract. The solvent was evaporated and the

extract distilled for 4 hours at 0.02 mm Hg. The distillate was frozen out in a cold trap (acetone and dry ice) and taken up again with petroleum ether. The concentration of the scent in the solvent was determined by analytical gas chromatography. The peak size of the main component (*cis*-4-hydroxy-dodec-6-enoic acid lactone in black-tailed deer; for mule deer, see footnote below) was measured and compared to a standard. Each sample contained 2 μg of the main component, with all other components in their naturally occurring proportions.

Stimulus Presentation

The sample was sprayed with a needleless syringe onto the outside of one hock of one adult female of each subspecies group, and the response of others in the group constituted evidence of stimulus. The individuals were allowed to interact freely and were observed for 20 minutes. All encounters were recorded, whether an interaction such as sniffing, licking, or following occurred or not. The tests were repeated ten times for each scent and each subspecies ($N = 40$). Each 20-minute period, starting with the application of the scent, is called a "test," while each single incident of licking is termed an "occurrence." The group members responded to the odor sample carried by one animal on its body by sniffing, licking, and following the odor carrier. We measured the frequency of sniffing, the duration of licking, and the distances followed per test. Proximity of one individual to another (50 cm or less between hock of one animal and nose of another) without reactions were also recorded (nonresponse).

RESULTS

Table 2 summarizes the results, including the responses of both sexes. It shows that the black-tailed deer responded more strongly to the tarsal odor of their own subspecies than to that of mule deer. The mule deer also responded more to their own subspecific tarsal odor, although only the frequency of sniffing and the response percentage reached a level of statistical significance (frequencies were treated by chi-square test, and durations by the Mann-Whitney test). The greatest differences were observed between the response levels of both subspecies, regardless of type of odor present (center column, Table 2). The mule deer are less active than black-tailed deer,

Note Added in Proof: One component of the tarsal scent of male mule deer apparently is the same lactone, as indicated by these preliminary findings: (a) closely matching retention times using two different GLC systems; (b) one sharp peak when coinjected; (c) identical odor. (Bro. Thos. McCullogh, CSC., Chemistry Dept., S.U.N.Y. Syracuse.)

TABLE 2. RESPONSES OF BLACK-TAILED AND MULE DEER TO TARSAL ODOR OF BOTH SUBSPECIES

	Responding subspecies						
	Black-tailed deer				Mule deer		
Response to:	Black-tailed deer	*P*	Mule deer	*P*	Black-tailed deer	*P*	Mule deer
Frequency of sniffing	58	<0.001	19	<0.001	19	<0.001	36
Duration of licking per test (seconds)	21.2	<0.025	4.8	<0.05	3.8	ns	5.0
Duration of licking per occurrence (seconds)	10.6	<0.05	4.4	<0.001	6.9	ns	8.3
Distances followed per occurrence (frequency of 0, 1–5, and over 5 M)[a]	3–8–8	0.066	6–7–5	<0.001	10–0–0	ns	8–3–0
Frequency of nonresponse	18	ns	19	ns	41	ns	30
% response $\frac{\text{all responses}}{\text{all encounters}} \cdot 100$	84.6	<0.05	72.5	<0.001	37.9	<0.01	60.0

[a] Repeated following during one test is counted separately; no following during a test counts as one occurrence of 0 meters.
Chi-square test was used for frequencies and the Mann-Whitney test for durations.

a fact observed by us in many different contexts over a period of three years (unpublished observations). They move less, both in pursuit of another individual or in avoidance of an approaching or sniffing animal. This is also demonstrated by a measure of activity (% response, Table 2) in which all responses (frequencies of sniffing, licking, and following, added together) are divided by all encounters (all responses plus nonresponses) and multiplied by 100. This quotient is the percentage of response. The values for the mule deer are lower than those for the black-tailed deer, and each subspecies shows more activity in response to its own tarsal odor.

DISCUSSION

Hybridization in the wild and in captivity can be facilitated by a number of factors, such as olfactory, visual, and auditory signals, density, and available choice of partners. In the specific area of olfactory communication, two facts must be considered. First, the tarsal odors may be very similar in composition. Second, familiarity with members of the other subspecies that live in the same area may help to overcome initial olfactory barriers. The first would be a largely genetically determined factor, while the latter is an individual learning process which can be reversed in captivity by prolonged separation. Even members of the same subspecies can be rendered incompatible by separation. When reentering its former group, a long-separated animal will be attacked, and this agonistic behavior is preceded by sniffing of its tarsal organs and of its anogenital area by members of the group. The described experiments show that tarsal odor can serve as a social and sexual barrier between two subspecies. However, when given no choice, both mule deer females were bred by a black-tailed deer buck.

The different intensities of activity in the two subspecies can be viewed as parts of differing ecological adaptations. The mule deer is more adapted to cold; larger size, thicker coat, and sluggish behavior are all components of its adaptation syndrome.

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HONEY BEE PHEROMONES: FIELD TESTS OF NATURAL AND ARTIFICIAL QUEEN SUBSTANCE*

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Abstract—Synthetic (*E*)-9-oxo-2-decenoic acid (9-ODA) was as attractive to drones as ether extracts of queen heads, suggesting that 9-ODA is the component of the sex pheromone that attracts drones from a distance. However, other substances produced in the heads of both virgin and mated queens cause drones to hover near a lure. Positive anemotaxis and short-range visual stimuli are also involved in the mate-finding process. "Keeper" substances ensure the gradual release of the pheromone. (*E*)-9-Hydroxy-2-decenoic acid (9-HDA) did not attract drones from a distance or affect their behavior near a lure.

Synthetic 9-ODA was slightly attractive to worker bees from a queenless swarm and 9-HDA was not attractive. However, ether extracts of whole queens or queen heads contain unidentified substances that are highly attractive. These are produced more abundantly by mated laying queens than by virgin queens.

Key Words—honey bee, queen, drone, worker, pheromones, sex attraction, swarm attraction, bioassay, (*E*)-9-oxo-2-decenoic acid, (*E*)-9-hydroxy-2-decenoic acid.

INTRODUCTION

The exocrine secretion of the mandibular glands of the queen bee of *Apis mellifera* (L.) functions as the sex attractant to males (drones) of this species (Gary, 1962). This secretion, which Butler (1954) called "Queen substance" (QS), is also attractive to worker bees both in the hive (Gary, 1961) and during swarming (Morse, 1963). QS is a multicomponent pheromone (Callow et al., 1964); at least two of its components have been reported to possess phero-

* Contribution Numbers 400 and 801 from Ottawa Research Station and Chemistry and Biology Institute, respectively.

monal activity: (*E*)-9-oxo-2-decenoic acid (9-ODA) and (*E*)-9-hydroxy-2-decenoic acid (9-HDA).

Gary (1962), Pain and Ruttner (1963), and Strang (1970) reported that drones on mating flights are attracted to the odor of synthetic 9-ODA. Since excessively large amounts of synthetic 9-ODA are required to match the attractiveness of QS, the authors suggested that QS contains other attractants or synergists. In contrast, Butler and Fairey (1964) demonstrated that 9-ODA is not complemented by other components of QS and that most, if not all, of the queen's scent that attracts drones is 9-ODA alone. Blum et al. (1971) showed that 9-HDA is not attractive to drones.

Pain (1961) and Simpson (1963) stated that synthetic 9-ODA is unattractive to queenless worker bees. However, Velthuis and van Es (1964) reported that worker bees from a queenless swarm are attracted by synthetic 9-ODA, but less so than by the total lipids extracted from the queen's head. In contrast, Butler and Simpson (1967) found under similar conditions that 9-ODA, especially when fortified with 9-HDA, can fully match the attractiveness of a live queen or a queen that has been crushed to release her pheromones. Moreover, as they would with a queen, the queenless workers form compact and stable clusters around a lure containing a mixture of synthetic 9-ODA and 9-HDA. Morse and Boch (1971) reported that 9-ODA alone or in combination with 9-HDA is less attractive to queenless swarms and that the clusters are less stabilized with these acids than with extracts of heads of queens. Butler and Simpson (1965) and Velthuis (1970) suggested that for full efficiency in attracting worker bees, QS of the mandibular glands must be complemented by other pheromones that the queen produces in glands on her abdomen.

The present study was undertaken to resolve some of the above-noted contradictions. In this paper, we report the results of quantitative bioassays, conducted under field conditions, comparing natural and synthetic pheromones. Live queens, extracts of whole queens, and queen parts were tested together with synthetic 9-ODA and 9-HDA for their attractiveness to airborne drones and swarming workers. An attempt was made to determine whether or not the activity of 9-ODA was complemented by 9-HDA or other components of the mandibular QS secretion. The activity of other exocrine secretions from the queen's thorax and abdomen also was investigated.

METHODS AND MATERIALS

Materials

(E)-*9-oxo-2-decenoic acid (9-ODA)*. Synthetic 9-ODA was obtained from Glaxo Laboratories, Greenford, Middlesex, England.

(E)-*9-hydroxy-2-decenoic acid* (*9-HDA*). Synthesis was effected by treating 1 g 9-ODA in 10 ml methanol, with 0.4 g sodium borohydride added in portions. After one hour, water was added and the aqueous layer, after acidification, was extracted with 3 portions of ether. The combined ether extracts were dried over anhydrous sodium sulfate, then evaporated to give a syrup. Thin-layer chromatography (TLC) analysis on silicagel with acetone–chloroform (1 :4 v/v) showed a major spot at R_f 0.24 and also showed the presence of some unreacted 9-ODA, so the reduction was repeated to afford 0.8 g (80% yield) of 9-HDA as a syrup. TLC analysis and also gas chromatography (GC) analysis of a portion methylated with diazomethane showed no trace of starting material.

The nuclear magnetic resonance (NMR) (Varian A60) and infrared (IR) (Beckman IR-20) spectra confirmed the expected structure of 9-HDA. NMR ($CDCl_3$) δ 1.18 (d,3,J = 6Hz, CH_3CH-), 1.40 (m,8,—$(CH_2)_4$—), 2.0–2.4 (d of m, 2, J = 6.5 Hz, —CH_2—CH=), 3.5–4.1 (m, 1, CH_3CH—), 5.82 (d,1, J = 16 Hz, —CH=CH—COOH), 7.10 (d of t, 1, J-16, 6.5 Hz, —CH_2—CH=CH—), 7.29 (s,1, -OH), and 11.4 (s,1, COOH). IR ($CHCl_3$) 3590 (alcohol), 2670 (acid hydroxyl), 1695 (carbonyl), 1650 (conjugated double bond), and 980 cm^{-1} ((*E*)-double bond).

Analytical Methods

Methylations were carried out using either diazomethane in ether or boron trifluoride in methanol (Shearer et al., 1970). The resulting methyl esters were analyzed using a Pye Model 104 gas chromatograph, with a flame ionization detector, and 1.5-m × 6.5-mm OD glass columns containing 4.5% DEGS or 10% EGS-SX on 80/100 Gas Chrom Q. Quantitation was achieved by preparing a series of standard solutions of synthetic 9-ODA ranging in concentrations from 5 to 2000 μg/ml. The amount of 9-ODA in a queen extract was calculated by interpolation from the two standards closest in concentration to those found in the extract.

Extraction

As each queen was removed from her colony, she was placed in a vial containing 1.5 ml anhydrous diethyl ether. Some queens were placed instead in empty vials, cooled to dry-ice temperature and subsequently decapitated, and the heads and bodies were put in separate vials of ether. The vials, each with one queen or part of a queen, were stoppered and stored at 4°C for up to 1 week.

To prepare the extract of a whole queen, the queen was removed from the vial, the head cut off and crushed. The body was washed with ether

and the crushed head extracted with these washings. After centrifugation, the supernatant was combined with the ether in which the queen had been preserved. The residue and the containers were washed and the washings combined with the extract. Part of the ether was evaporated under a slow stream of nitrogen and the extract made up to 1 ml with ether, or all ether was evaporated and the residue was taken up in 1 ml methanol.

Extracts from 20 or more queens were combined, and an aliquot was methylated and analyzed for 9-ODA. Based on this analysis, the volume of solution was adjusted for bioassay by evaporating the solvent under a stream of dry nitrogen until the 9-ODA concentration was 1 $\mu g/\mu l$. A solution of synthetic 9-ODA was prepared at the same concentration. Finally, both the extract and the synthetic solution were analyzed by GC to ensure that both contained the same amount of 9-ODA.

9-HDA from Queen Head Extracts

An ether extract of queen heads was subjected to TLC as described above and the spot at R_f 0.24 was scraped off and eluted from the silca gel with methanol–ether (1 : 10 v/v). The IR spectrum (KBr micro-pellet) of the eluate was congruent in all respects with that obtained from a sample of synthetic 9-HDA similarly treated. A portion of this eluate was methylated with diazomethane; subsequent GC analysis showed a single peak having the same retention time as the methyl ester of synthetic 9-HDA.

Bioassay with Drones

The biological evaluation of the sex attractants of the natural and artificial pheromones was made over a period of three summers on the grounds of the Central Experimental Farm, Ottawa, in an open field adjacent to an apiary. This site was chosen for convenience after it was observed that significant numbers of drones could be attracted to lures over most other areas of the 500-hectare farm. The test materials, dissolved in ether or methanol, were applied by microsyringe to porous polyethylene blocks ($20 \times 5 \times 5$ mm, 45-μl pore size, Bel-Art Products, Pequannock, New Jersey) that had been preextracted with ether. After the solvent had evaporated, the plastic lure was attached to a fine nylon thread and tethered 2 m below a white helium-filled balloon (diameter 1 m). The balloon was elevated so that the lure was 5–10 m above the ground, and the number of drones approaching the lure was recorded for the next 3 minutes. The balloon was lowered between trials for a period of 5–15 minutes. The counts of drones approaching the lure were made by assistants who were not aware of the test sequence. The numbers recorded may include multiple visits since it was not feasible to eliminate

drones that were attracted repeatedly during the brief observation period.

In some experiments, live queens were used. These were placed individually in small tubular plastic screened cages (3-mm mesh, 11-mm inside diameter, 60-mm long) and elevated by the balloons. Lures with extracts or synthetic substances, for comparison, were displayed in similar cages and elevated to the same heights. After each bioassay, the cages were thoroughly cleaned with methanol and air dried.

For the comparative bioassay of relative drone attraction, a sequential display of candidate substances or queens was chosen, although it had the shortcoming of being affected by fluctuations of drone flight intensity. To minimize this effect, drone attractancy tests were undertaken when conditions for drone flight and counting were optimal (i.e., on sunny, relatively calm days, between 1300 and 1700 hrs EDT, from May through September, and when temperatures were between 20° and 30°C), and when a minimum of 50 drones were initially attracted in a 3-minute period to a lure containing 100 μg synthetic 9-ODA. A random-sequence design was employed which permitted statistical comparisons between test materials.

In preliminary tests, a simultaneous display of two attractants was attempted in the same immediate area (10–75 m apart). It was observed that adjacent bioassays interfered with one another in that a strongly attractive pheromone source made it difficult for drones to find a relatively weak source, or that during temporary windshifts a downward source became more attractive.

During the initial experiments, the numbers of drones approaching a cage in a 3-minute test period frequently exceeded the numbers that observers could count reliably. Thereafter, where possible, smaller amounts of pheromone were used. The lures were treated with synthetic 9-ODA or pheromone extract equivalent to about one tenth of a queen or less. Also, by moving colonies away from nearby apiaries, from which drones had come, the number of drones flying in the experimental area was reduced. The observers could then count the drones that approached a lure, and in some instances could also observe their individual behavior near the lure.

Butler (1967) and Strang (1970) reported that drones could be trained to aggregate in large numbers in a particular area by regularly exposing excessive amounts (milligrams) of 9-ODA. After such conditioning, the drones were often attracted to unscented lures, even when 9-ODA was no longer exposed in the area. To avoid a similar situation at Ottawa, care was taken to use the minimum amounts of attractant and not to expose any test substance or queen for longer than necessary. Blank lures (solvent only) were repeatedly elevated after tests in which large numbers of drones had been attracted, until they were no longer unduly excited and attracted by sight alone, to ascertain that they had not been conditioned to the location.

Bioassays with Workers

Natural and synthetic queen pheromones were also evaluated on worker bees. An artificial swarm of 8,000–10,000 bees was allowed to cluster around its caged queen on a wooden cross-stake 1.2 m high. After the swarm had remained clustered for 1–2 days, the queen was removed. Typically, within 10–20 minutes, the swarm became restless and many workers flew off in search of their queen. These workers were then offered a choice of two lures, each pinned on one end of the cross arm of a stake which was 3 m from the swarm cluster. The number of workers touching or alighting on each lure within a 10-minute period was recorded and indicated to which lure the workers were more attracted. All bees that alighted on a lure were driven off or, if possible, quickly captured by their wings with fine forceps and placed in a small cage. The stakes with the lures were turned 180° every 2 minutes and the cross arms were adjusted so that they were always perpendicular to the wind direction.

RESULTS AND DISCUSSION

A preparation of 9-HDA has been reported by Eiter (1962), who lists a melting point of 43–45°C for his product. We repeated his preparation and also obtained a product with a melting point of 43–45°C. The IR spectrum did not confirm the presence of more than a trace of the hydroxyl band nor did it correspond to the IR reported by Eiter. Repeated crystallizations with ether–petroleum ether raised the melting point to 53–54°C, and this product proved to be identical to the starting material, 9-ODA. Thus, in agreement with Callow et al. (1964), it appears that Eiter's procedure does not give complete reduction of 9-ODA to 9-HDA, and the validity of any tests carried out using his product is questionable.

Field Tests with Drones

A. Live queens vs. synthetic 9-ODA. The attraction of drones to live queens was compared to lures containing 100 μg synthetic 9-ODA applied in 25 μl methanol. The queens tested were 20 laying queens from strong colonies, and 20 virgin queens reared in small colonies that occupied only three to five combs. Each queen was removed from her hive, placed alone in a clean cage, and elevated on the balloon. A test of a queen was preceded and/or followed by tests of lures with 9-ODA or blanks with methanol only.

6 (30%) laying queens and 18 (90%) virgin queens attracted fewer drones than a lure with synthetic 9-ODA. 5 (25%) laying queens attracted twice as

TABLE 1. ATTRACTIVENESS OF LIVE QUEENS COMPARED TO A LURE CONTAINING 100 μg (*E*)-9-OXO-2-DECENOIC ACID (9-ODA)

Attractiveness relative to 9-ODA (standard = 1.0)	Virgin queens	Laying queens
≤0.4	9	3
0.5–1.0	9	3
1.0–1.5	1	6
1.5–1.9	1	3
≥2.0	0	5

many or more drones than 100 μg 9-ODA. Table 1 suggests that the virgin queens released less sex pheromone than the laying queens. The virgin queens were nubile and between 4 and 18 days old. The laying queens were between 1 and 4 months old.

Our results seem inconsistent with the findings of Gary (1970), who reported that caged, live virgin queens, ca. 1–2 weeks old, were significantly more attractive to flying drones than a wide range (10 μg–10 mg) of synthetic 9-ODA displayed in similar cages and elevated to the same height. Gary (1963) photographed the drones that hovered near the cages and thus was able to record not only the number of drones actually attracted but also the duration of their visits. We counted the drones only as they approached the cages from a distance downwind but were unable to determine the duration of their visits. The apparent discrepancy between our data and those of Gary (1970) may lie in the fact that the drones hovered longer near cages with live queens than with lures.

B. Live queens vs. extracts of queens. The attractiveness of 40 queens was determined in tests similar to those described above. 11 queens attracted a smaller number of drones than a lure with 100 μg 9-ODA, whereas 7 queens attracted more than twice as many. Aliquots (0.2 queen equivalents) of the combined extracts of the 11 less attractive and 7 highly attractive queens were tested alternately for drone attraction. The highly attractive queens gave extracts that attracted 1.5 times as many drones (totals 785 vs. 515) as the extracts from the less attractive queens. Although the attractiveness differed by a greater factor in the live queens than in the extracts, the results of this experiment suggest that the rate of pheromone release in a live queen is proportional to the amount in the glandular reservoirs.

C. Contents of 9-ODA and 9-HDA in extracts of queens. Certain queens used in Experiment A were killed and extracted immediately after the drone attraction tests. The queens selected for extraction were the 9 virgin queens

TABLE 2. NUMBER OF DRONES ATTRACTED BY LIVE QUEENS AND BY 100 μg SYNTHETIC 9-ODA (QUEEN EXTRACTS WERE ANALYZED FOR 9-ODA AND 9-HDA)

Colony designation[a]	Drones attracted to		Queen attractiveness[b]	Extract contents of	
	Live queen	9-ODA		9-ODA (μg)	9-HDA (peak height)
433	10	105	0.1	75	36
484	55	260	0.21	32	53
448	70	300	0.23	125	41
472	25	110	0.23	25	28
543	100	350	0.29	50	85
586	125	390	0.32	57	34
454	67	195	0.34	37	7
404	53	200	0.38	52	8
419	130	345	0.38	22	8
Avg	71	251	0.28	53	33
C4	48	240	0.2	120	39
12	35	95	0.37	75	14
7	65	175	0.38	110	22
8	360	180	2.0	228	75
5	330	160	2.1	315	53
6	310	150	2.1	322	68
13	175	85	2.1	325	26
B4	330	100	3.3	225	41
Avg	207	148	1.4	215	42

[a] Hives with numbers above 400 contained small colonies and virgin queens. Hives with numbers below 100 contained strong colonies and laying queens.
[b] Queen attractiveness equals ratio of the number of drones attracted to a live queen vs. 9-ODA.

and the 3 laying queens which had an attractiveness of 0.4 or less as compared to that of 100 μg 9-ODA. The 5 laying queens that had more than twice the attractiveness of the standard 9-ODA lure were also extracted. Table 2 indicates that the attractiveness of a live queen generally was related to the amount of 9-ODA later found in the extract, but it was not related to the amount of 9-HDA.

D. Whole queen extract vs. synthetic 9-ODA. In preliminary tests, synthetic 9-ODA was more attractive to drones than the natural queen pheromone. In 12 trials, synthetic 9-ODA (100 μg per lure) attracted a total of ca. 2400 drones compared to ca. 2000 for the whole queen extract. The lures with extract contained exactly 100 μg 9-ODA as determined by GC. This unexpected result of the preliminary tests might be explained if the whole queen extracts contained substances that partially masked the pheromone.

TABLE 3. NUMBER OF DRONES ATTRACTED TO WHOLE QUEEN EXTRACTS AND SYNTHETIC 9-ODA[a]

Trial	Natural 9-ODA[b]			Synthetic 9-ODA			Blank (methanol)
	20 μg	10 μg	5 μg	20 μg	10 μg	5 μg	
1	156	—	—	133	—	117	—
2	—	97	—	—	168	—	0
3	—	120	86	—	235	67	1
4	—	105	84	—	242	126	—
5	—	38	25	—	76	64	—
6	—	0	3	—	12	11	—
7	—	44	117	—	101	122	0,0
8	—	214	142	—	—	111	3
9	—	77	33	—	108	94	1
10	—	46	34	—	80	9	4
11	—	10	4	—	55	3	12,3
Avg	156	75	59	133	119	72	3

[a] Each lure contained the indicated amount of 9-ODA.
[b] Each lure was treated with a volume of queen extract known to contain the indicated amount of 9-ODA.

Such substances might have been extracted from the dead queen bodies or might have consisted of impurities contained in the solvents used.

Therefore, in a new series of tests, fresh extracts and solutions were prepared and precautions were taken to either avoid or equalize possible contaminations by solvent impurities. Table 3 shows that 10 μg synthetic 9-ODA was significantly more attractive than the extract which matched it for content of 9-ODA. The difference between lures with 5 μg natural or artificial 9-ODA was not statistically significant.

Higher-boiling lipid components of the extract may function as "keepers" and thus effect a reduction in the evaporation rate of the pheromone. This suggestion was supported by the observation that lures with queen extracts remained attractive to drones for longer periods (days) than lures with equivalent amounts of synthetic 9-ODA. A few drops of paraffin oil added to a solution of synthetic 9-ODA extended its activity on the lures to the same duration as that of the extracts. Hence, we are inclined to believe that the queen pheromone contains a "keeper" substance. Butler (1969) similarly suggested that the fatty acids contained in the queen's mandibular gland secretion may act as "fixatives" for the pheromone. Goodrich (1970) showed that a pheromone may be solubilized and retained by cuticular or other lipids of an insect, being protected from oxidation and released gradually.

Drones appear to be extremely sensitive to the concentration of 9-ODA. Low concentrations induce them to fly upwind following the scent gradient, and at a certain higher concentration they begin to orient optically. Drones frequently seem to become suddenly attracted to each other at some distance downwind from lures containing excessive amounts (>100 μg) of 9-ODA. Under these circumstances they no longer approach the lure but follow each other, flying in an erratic fashion reminiscent of a pinwheel, until they lose the scent gradient. Thereafter, they resume normal queen-seeking behavior. However, it is conceivable that this behavior may be caused partially by contamination of drones with queen pheromone, either by contacting the lures occasionally or by hovering near them.

Our observers were usually not required to note differences in the behavior of the drones near a lure. Only in tests 10 and 11 (Table 3) was special attention given to the possibility that drones might behave differently near a lure with either extract or synthetic 9-ODA. We observed that drones approaching a lure with natural pheromone usually hovered for a second or two beneath and downwind from the lure, or they flew in narrow loops, repeatedly passing close to the lure as if examining it before flying away. Fewer drones, on the other hand, were seen to approach and hover near a lure with synthetic 9-ODA; they often flew rapidly upwind, homing in on the lure, but then flew by it in a rather sweeping motion.

Butler (1971) similarly observed differences in the thoroughness with which drones examined different queens, and he demonstrated that they examined a queen's body more intensively when she had an unidentified pheromone in addition to 9-ODA than when she had 9-ODA alone, but the total number of drones attracted from a distance did not differ. Pain and Ruttner (1963) observed that the swarms of drones near a lure with an ether extract of a queen's mandibular glands were bigger and better "stabilized" than swarms near lures with artificial 9-ODA.

The polyethylene lures did not closely resemble the visual appearance of a queen. This may explain why drones after approaching a lure with extract did not attempt to copulate with it. It is very likely that drones have an acute ability to discriminate visually between objects that do or do not resemble a receptive queen. The visual cues for releasing copulatory behavior have been recently investigated by Gary and Marston (1971).

E. Extract of queen heads and bodies. In this experiment, we sought to determine the source of the substance attractive to drones. Table 4 shows that drones were attracted in approximately equal numbers to a total extract and to the extract of heads alone. Only a few drones were attracted to the washings of the decapitated queen bodies.

Our results confirm those obtained by Gary (1962) and Butler and Fairey (1964), showing that the pheromone responsible for attracting drones is

TABLE 4. NUMBER OF DRONES ATTRACTED BY EXTRACTS OF QUEEN HEADS, WASHINGS OF HEADLESS BODIES, AND MIXTURES OF THE TWO (TOTAL EXTRACT)[a]

Trial	Head extract	Body washings	Total extracts	Blank (methanol)
1	73	4	—	0
2	126	18	—	—
3	151	—	164	5
4	89	—	80	3
5	45	2	—	0

[a] Each lure contained 0.1 queen equivalent.

contained predominately in the queen's head. Since the headless bodies of queens were slightly attractive, it appears that a small amount of pheromone was dispersed over the surfaces of the queen's thorax and abdomen. Morse et al. (1962) observed that some queens whose mandibular glands had been extirpated early in life, were still capable of mating, which indicates that in addition to QS in the mandibular glands, a queen may also produce attractants of lesser potency in other glands in the head or elsewhere on her body.

Since we have observed no differences in the intensity with which drones examined lures with the different extracts, we conclude that the substances which cause drones to hover and closely examine a queen are produced or accumulated mainly in the queen's head, perhaps by the mandibular glands as suggested by Gary (1962) and Pain and Ruttner (1963). Our results show that other exocrine glands on the queen's thorax and abdomen stimulate a minimal, if any, drone response.

F. Total extracts of virgin and laying queens. Renner and Baumann (1964) and Hammann (1957) observed that virgin queens, 1–2 weeks old, had a clearly perceptible odor that might have a function during the mating process. We applied extracts of virgin queens, 4–18 days old, and laying queens, up to 1 year old, to lures. Only the ether extracts of virgin queens had a strong odor that was reminiscent of either the scent of Sweet Alyssum (*Lobularia maritima*), floral honey, dilute phenylacetic acid, or, sometimes, chocolate vanilla. When various areas of the virgin queens' bodies were wiped with small pieces of filter paper, highly odoriferous "wipes" were obtained from the abdominal tergites, which indicated that the odor is produced possibly by the glands described by Renner and Baumann (1964). Some of this odor was also obtained from the abdominal sternites. Although the abdominal "wipes" contained a strong scent, insufficient material was obtained for chemical analysis. Moreover, the production of this material seems to be

TABLE 5. NUMBER OF DRONES ATTRACTED BY EXTRACTS OF A WHOLE VIRGIN QUEEN (7 DAYS OLD) AND A MATED, LAYING QUEEN (1 YEAR OLD)

Trial	Virgin queen extract	Laying queen extract
	0.1 queen equivalent	**0.1 queen equivalent**
1	17	66
2	36	88
3	49	65
4	25	28
5	22	48
Avg	30	59
	0.2 queen equivalent	**0.1 queen equivalent**
6	64	49
7	85	92
8	105	116
Avg	85	86
	20 μg 9-ODA[a]	**20 μg 9-ODA[a]**
9	231	160
10	172	116
11	96	173
Avg	166	150

[a] The extracts were analyzed for 9-ODA and then fortified with enough synthetic 9-ODA to make all extracts contain 20 μg.

restricted to nubile queens; "wipes" of newly emerged and very young laying queens had none of the perfume described above.

Drone attraction bioassays of the extracts (Table 5) gave results similar to those in Experiments A and B, and showed that an extract from a laying queen was twice as attractive as that of a virgin queen (trials 1–5). Subsequent GLC analyses showed that the laying queen extract contained 285 μg 9-ODA, whereas the virgin queen extract contained 150 μg 9-ODA. In trials 6–8, the lures were again treated with 0.1 queen equivalents of the laying queen, but the virgin queen extract was now doubled to 0.2 queen equivalents per lure. Drone attraction was then no longer significantly different to both lures. The behavior of the drones when near the lures at close range appeared similar.

In trials 9–11, both extracts were fortified with synthetic 9-ODA so as to obtain the same concentrations of 9-ODA. Thus 1.85 mg and 1.71 mg 9-ODA were added per ml of extract of virgin and laying queens, respectively. The resulting solutions, each containing 2 mg 9-ODA per ml, were then di-

TABLE 6. NUMBER OF WORKERS ATTRACTED BY WHOLE QUEEN EXTRACTS, SYNTHETIC 9-ODA, AND 9-HDA

	Natural 9-ODA[b]		Synthetic 9-ODA		
Trial[a]	10 μg	20 μg	10 μg	20 μg	Blank (methanol)
1	38	—	2	—	—
2	17	—	—	0	—
3	—	46	—	3	—
4	—	9	0	—	—
5	—	—	22	—	1
6	—	—	—	32	0
			Mixture synthetic 9-ODA+9-HDA[c]		
7	—	52	5		—
8	86	—	9		—

[a] Four or more tests per trial.
[b] Each lure was treated with a volume of queen extract known to contain the indicated amount of 9-ODA.
[c] 10 μg each.

luted to 10 volumes with ether, and 100 μl (= 20 μg 9-ODA) were applied to the lures. Drone attraction tests showed no significant difference.

The results indicate that drone attraction to extracts of queens is mainly, if not entirely, a function of the contents of 9-ODA. The role of the perfume of nubile queens is unknown.

G. Synthetic 9-ODA, 9-HDA, and mixtures of both. Lures with 20 μg synthetic 9-ODA, 20 μg synthetic 9-HDA, 20 μg 9-ODA plus 20 μg 9-HDA, and methanol (blank) attracted on an average 166, 3, 170, and 4 drones, respectively. These results show that 9-HDA is not attractive to drones and does not have a synergistic effect with 9-ODA.

Field Tests with Worker Bees

In contrast to the results obtained in tests with drones, worker bees from queenless swarms were significantly more attracted to extracts of queens than to synthetic 9-ODA. In two-choice preference tests, the synthetic 9-ODA alone or in combination with 9-HDA showed little or no attractiveness for workers if a queen extract was simultaneously available to them (Table 6). In four more trials, 20 μg 9-ODA on a lure was compared with another lure bearing 20 μg 9-ODA plus 20 μg 9-HDA; 44 and 43 workers were attracted, respectively.

TABLE 7. NUMBER OF WORKERS ATTRACTED BY WHOLE EXTRACTS OF A VIRGIN AND LAYING QUEEN

Trial[a]	Virgin queen extract (0.1 queen equivalent)	Laying queen extract (0.1 queen equivalent)
1	9	75
2	5	80
	20 μg 9-ODA[b]	20 μg 9-ODA[b]
3	27	106

[a] Four tests per trial.
[b] The extracts were analyzed for 9-ODA and then fortified with enough synthetic 9-ODA to make both extracts contain 20 μg.

Virgin and laying queen extracts previously tested on drones (Experiment F) also were tested on workers of queenless swarms. The laying queen extract was much more attractive to workers than the extract of a virgin queen (Table 7). Even when both extracts were equalized in terms of their 9-ODA contents, the laying queen extract attracted 106, while the virgin queen extract attracted 27 workers (trial 3).

Queen head extracts and body washings previously used in drone attraction tests (Experiment E) were tested also on swarming worker bees using 0.1 queen equivalents. Table 8 indicates that the substances attractive to the workers are contained predominantly in the queen's head.

It appears, therefore, that worker bees are less attracted by 9-ODA or 9-ODA plus 9-HDA than by another pheromone produced more abund-

TABLE 8. NUMBER OF WORKERS ATTRACTED BY EXTRACTS OF HEADS AND HEADLESS BODIES OF LAYING QUEENS

Trial[a]	Head extract	Body extract	Head + body extracts
1	65	—	86
2	13	—	9
3	20	1	—
4	21	1	—

[a] 0.1 Queen equivalent per test and four tests per trial.

antly in laying queens than in virgin queens. Preliminary attempts to isolate this pheromone showed that it may be extracted with aqueous sodium carbonate solutions.

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COLLECTION ON PORAPAK Q OF THE AGGREGATION PHEROMONE OF *Scolytus multistriatus* (COLEOPTERA: SCOLYTIDAE)[1,2]

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Abstract—The attractive volatiles in the air around the virgin female of the smaller European elm bark beetle, *Scolytus multistriatus* (Marsham), tunneling in elm logs can be collected by passing the air through a column of Porapak Q. These volatiles can be removed from the Porapak by Soxhlet extraction with hexane, yielding an extract that is attractive to in-flight beetles in the field. GLC analyses of this extract and an extract of virgin female frass indicate that the aeration extract contains active chemicals that are not in the extract of frass.

Key Words—aggregation pheromone, *Scolytus multistriatus*, Porapak, Ulmus, elm bark beetle, volatile attractants.

INTRODUCTION

Virgin females of the smaller European elm bark beetle, *Scolytus multistriatus* (Marsham), produce an aggregating pheromone as they tunnel in declining American elm trees (*Ulmus americana* L.) (Peacock et al., 1971).

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[2] The use of a trade name in this paper is for the information and convenience of the reader. It does not constitute an official endorsement or approval by the U.S. Department of Agriculture.

Furthermore, frass produced by virgin female beetles and extracts of this frass, elicit a response from walking male beetles in a laboratory olfactometer (Peacock et al., 1973). We concluded that responses of walking beetles may be related to the field responses of beetles in flight and that virgin female frass may be a source of both host- and insect-derived aggregation attractants.

However, virgin female frass or frass extracts have failed to attract in-flight beetles in several field tests. This could indicate that (1) frass does not contain all the chemical components of the pheromone bouquet required for beetle attraction in the field, or (2) one or all of the components are lost between frass production in the laboratory and testing of frass in the field. Whatever the reason, we now feel that frass is not a good source of the total pheromone bouquet for *S. multistriatus*, despite the fact that pheromones have been isolated from the frass of several other scolytid beetles (Silverstein, 1970; Vité, 1970; Wood, 1970; and Borden and Stokkink, 1971).

The failure of frass or frass extracts to attract in-flight beetles in field tests prompted us to investigate another method for acquiring the pheromone: collection and concentration of the volatiles from the air around virgin female beetles tunneling in elm logs. Initially, we considered collecting the volatiles by freeze-trapping, since this method has been useful in pheromone research on other insects (Jones et al., 1965; Vité et al., 1963; Yamamoto, 1963). The "total condensation" technique of Browne et al. (1974) seemed to be especially promising. However, the relatively large quantity of water collected by this method complicates subsequent isolation of the small amounts of pheromone. Furthermore, freeze-trapping requires considerable attention. Consequently, we investigated trapping of the *S. multistriatus* pheromone on an absorption column as a means of avoiding these problems.

Silverstein and Rodin (1966) studied the collection of volatiles on absorbent columns and concluded that columns containing Chromosorb® A coated with a liquid substrate could be used to trap and recover small amounts of model organic compounds from an airstream. In a more recent study, Byrne et al. (1975) have shown that a wide variety of organic compounds (including known pheromones) can be absorbed from an airstream onto Porapak® Q (ethylvinylbenzene-divinylbenzene copolymer) and can then be extracted from the Porapak in satisfactory yields. Rudinsky et al. (1973) reported the use of Porapak Q for collecting small quantities of the anti-aggregative pheromone of *Dendroctonus pseudotsugae* Hopkins.

We investigated the use of the Porapak extraction system for collecting *S. multistriatus* pheromone and obtained an extract that, like a frass extract, elicits a response from walking male and female beetles in the laboratory. Unlike the frass extract, the extract containing the volatiles collected on Porapak attracts large numbers of in-flight beetles of both sexes in the field.

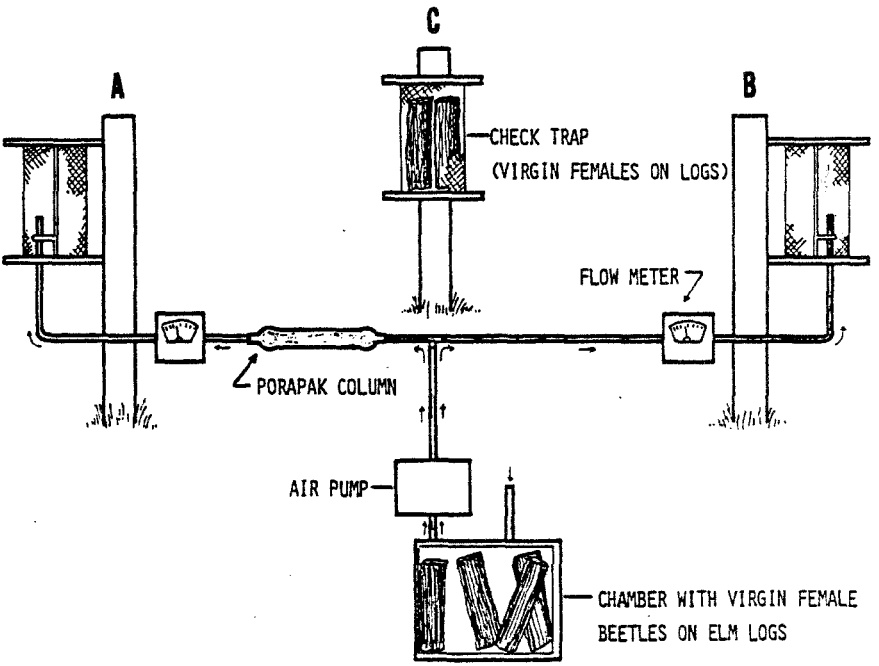

FIG. 1. Diagram of field-trapping apparatus used to evaluate Porapak Q for collection of beetle and host-produced volatiles. Arrows denote direction of air flow.

This paper is a report on (1) experiments that confirm the suitability of Porapak for collecting *S. multistriatus* pheromone, (2) laboratory and field bioassays of attractant extracts, and (3) preliminary chemical studies of active extract fractions.

METHODS AND MATERIALS

Preliminary Field Evaluation of Porapak Q for Pheromone Collection

We first evaluated Porapak Q for collecting the attractant volatiles, produced by tunneling virgin female beetles, in a field test with the apparatus diagrammed in Figure 1. Three traps, similar to those used in our initial studies of the *S. multistriatus* pheromone (Peacock et al., 1971), were positioned 17 m apart in a straight line. Attractant odors, produced by 160 virgin *S. multistriatus* females boring in 4 elm bolts 10–12 cm in diameter and 24–28 cm in length, were pumped from the chamber containing the bolts to traps A and B. A glass column containing 20 g conditioned[6] Porapak Q (50 to

[6] The Porapak was conditioned prior to use by the method described by Byrne et al. (1975).

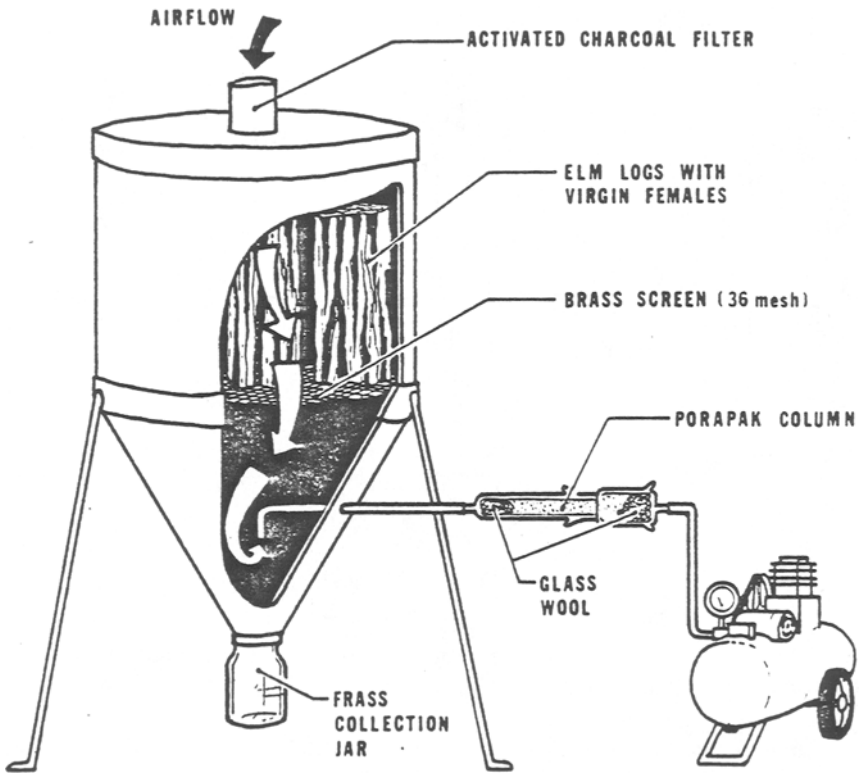

FIG. 2. Apparatus used for simultaneous collection of frass and of host- and beetle-produced odors in air around females tunneling in logs.

80 mesh) (Waters Associates, Inc., Framingham, Massachusetts) was inserted into the air line leading to the treatment trap (A).

The air-flow rate to both traps A and B was maintained at 5 liters/min with needle valves and flow meters positioned in the lines just ahead of the traps. The air lines were switched daily from one trap to the other to average out position effects. A check trap (C) contained virgin females tunneling in elm bolts (40 females confined on each of 4 bolts). The duration of the experiment was 9 days. Significantly fewer beetles captured at trap A compared to the number captured at traps B and C would indicate absorption of the pheromone on the Porapak column.

Laboratory Collection of Attractant Volatiles

The apparatus diagrammed in Figure 2 was used in the mass production and simultaneous collection of frass and volatiles produced by virgin female

beetles on elm bolts. Virgin female beetles (6,000 to 8,000) were released on 15 to 20 elm bolts (50–56 cm in length and 5–13 cm in diameter) that had been "aged" 7 days at 25°C, 50% RH. Charcoal-filtered air was drawn through the chamber containing beetles on logs and then through a glass column containing 20 g conditioned Porapak Q (50 to 80 mesh) at a flow rate of 6–8 liters/min.

Collection of frass and volatiles commenced 72 hours after infestation and continued for 168 hours. This collection period corresponds to the period (1) when virgin females are most attractive in the field (Peacock et al., 1971), and (2) when frass collected from females is most active in laboratory olfactometers (Peacock et al., 1973). The same Porapak was used without changing throughout the entire 168 hours.

After this 7-day collection period, the frass and the Porapak Q containing attractant volatiles were stored separately in glass bottles at −20°C until subsequent bioassay or extraction with organic solvents. In these studies, the amount of frass and volatiles collected during aeration was expressed quantitatively in terms of beetle-hours. For example, 100 virgin females tunneling in logs for 10 hours will produce 1,000 beetle-hour equivalents of frass or volatiles.

Extraction of Attractant Volatiles from Frass and from Porapak Aeration Column

The frass produced by virgin females during the 4th through 10th days after infestation of logs was combined and used to prepare crude extracts for laboratory and field studies. Fifty-gram lots of frass were extracted with hexane in a Waring blender (6 times with 300-ml portions of fresh hexane), then with benzene, and finally with methanol. Each extract was concentrated by fractional distillation before laboratory or field assays. Since bioassays indicated that the benzene and methanol extracts and the extracted frass residue were inactive, we used only hexane for the large-scale extraction of frass.

Two methods were investigated for the removal and collection of volatiles from Porapak columns. In one method, the Porapak column containing trapped volatiles was backflushed with N_2 at 150°C for 6 hours and the eluted volatiles were collected in two 3-mm U-tubes placed in series. The first tube was chilled with dry ice/acetone, and the second with liquid air. In the second method, the Porapak was Soxhlet-extracted with pentane for 24 hours; the extract was then concentrated by fractional distillation (Byrne et al. 1975).

The volatiles obtained by both methods were compared on the basis of gas-liquid chromatography (GLC) profiles and bioassays. This comparison

indicated that both techniques yielded comparable quantities of biologically active material. However, we considered Soxhlet extraction to be the more convenient method for large-scale processing of Porapak traps.

Laboratory and Field Bioassays of Frass, Frass Extract, and Porapak Extract

Laboratory bioassays were conducted with an olfactometer and by methods previously reported (Peacock et al., 1973).

Field bioassays were conducted in Delaware, Ohio, in 3 plots containing 10–18-cm diameter elms (*U. americana*). Each plot had a check trap (containing 40 virgin female beetles confined on each of 4 elm bolts) and 5 traps for presentation of attractant material. Traps were spaced 7 m apart in straight lines perpendicular to the prevailing wind in each plot.

Frass was presented in a glass petri dish on the bottom shelf of the trap. Extracts were delivered from glass tubes (2-mm ID × 50-mm length) positioned in a Styrofoam® block attached to an upright steel rod in the center of the trap. One end of the tube was sealed; delivery of the attractant was by evaporation. The tubes were charged by injecting a dilute hexane solution of the crude extract into the bottom of the tube; the hexane evaporated within a short time after injection of the sample.

Partial Purification of the Crude Frass and Porapak Extracts

After laboratory or field bioassays confirmed the attractiveness of the Porapak and frass extracts, we initiated studies to isolate and identify the attractant chemicals. The active hexane extract of frass was subjected to short-path vacuum distillation onto a cold finger cooled with dry ice, with a cold trap cooled by liquid N_2 between the distillation apparatus and the vacuum pump. This technique yielded a pot residue, washings from the cold trap, a distillate, and cold-trap material, each of which was subjected to laboratory bioassay.

The active distillate was fractionated by GLC on a column packed with 5% SE-30 on Chromosorb W. GLC fractions were collected using the method of Brownlee and Silverstein (1968). The GLC chromatogram was divided into three regions: A, B, and C. Regions A and B were eluted consecutively from the GLC column; C was obtained by backflushing residual material from the column.

The Porapak aeration extract was fractionated by GLC without prior purification. GLC fractions from an SE-30 column were collected as two regions that corresponded to regions A and B of the frass extract chromatogram. The Porapak extract did not contain any material in the region corresponding to fraction C from the frass extract.

Initial biological activity of all the fractions at each stage of the isolation was assessed with walking male beetles in the laboratory olfactometer.

RESULTS AND DISCUSSION

Preliminary Field Evaluation of Porapak Q for Collection of Pheromone

Preliminary tests of the field-trapping system diagrammed in Figure 1 suggested that at least some of the volatile components necessary to attract in-flight *S. multistriatus* were removed from the airstream by the Porapak Q. During the trapping period, only 1 beetle was attracted to the Porapak-filtered air emitted from treatment trap A, compared with 48 beetles that were attracted to the unfiltered air emitted from trap B. Fifty-six beetles were attracted to the check trap.

From these data, we concluded that Porapak might be suitable for the collection of the attractant volatiles produced by tunneling female beetles. We subsequently constructed an apparatus (Figure 2) for the simultaneous collection of the frass and volatiles surrounding tunneling females.

Laboratory Bioassays of Frass, Frass Extract, and Porapak Aeration Extract

Laboratory olfactometer studies indicated that both sexes of walking beetles were stimulated (excitant-turning reaction, Peacock et al., 1973) by virgin female frass, frass extract, and the Porapak aeration extract (Table 1). The responses of both males and females to 10 mg of frass were unlike those we recorded in our earlier studies (Peacock et al., 1973), when 10 mg of

TABLE 1. RESPONSE OF WALKING MALE AND FEMALE *S. multistriatus* IN LABORATORY OLFACTOMETER TO VIRGIN FEMALE FRASS, FRASS EXTRACT, AND PORAPAK AERATION EXTRACT (MEAN OF 3 TRIALS FOR EACH SEX WITH 25 BEETLES PER TRIAL)

Material tested	Amount tested	Beetle-hour equivalent[b]	Mean response (%)	
			Males	Females
Virgin female frass[a]	10 mg	240	42.7	32.0
Frass extract	5 μl	240	54.8	44.0
Porapak aeration extract	25 μl	50	45.3	40.0

[a] Sample from combined frass collected for 4 hours on 6th day after infestation of virgin females.

[b] In each case, represents optimal test level, based on prior dose-response studies.

TABLE 2. FIELD RESPONSE OF IN-FLIGHT BEETLES TO NATURAL ATTRACTANTS IN THE FIELD

Material tested	Amount tested[a]	Number of beetles captured during day										Total
		1	2	3	4	5	6	7	8	9	10	
Virgin female frass	625 mg	0	0	1	0	1	0	1	0	2	0	5
Frass extract	25 μl	0	1	0	2	0	0	1	1	1	0	6
Porapak aeration extract	25 μl	0	12	22	30	21	10	38	24	11	9	177
Virgin females on logs[b]	—	3	12	39	54	29	3	21	39	36	16	252

[a] Amount in each case equivalent to 15,000 beetle-hours.
[b] Forty females on each of 4 logs.

frass evoked a 70% response from males and a 4% response from females. On the contrary, both males and females responded to frass in the present studies, although females were somewhat less responsive (32% mean response for females compared to 42.7% response for males). On several occasions we have found that beetle responses to frass in the laboratory are inconsistent and variable; this could account for the lower mean response for males and the higher response for females (Table 1) as compared to the responses recorded in our earlier tests.

Both males and females responded to the frass extract and the Porapak aeration extract; male responses were about 5–10% higher than females in each case. The frass extract was about 12% more stimulating than was frass itself for both males and females. This increased activity of the frass extract may be due to the presence in the extract of additional pheromone liberated during pulverization of the frass in the extraction procedure.

The Porapak aeration extract was more stimulating to both males and females than either frass or the frass extract, considering that only 50 beetle-hour equivalents of the aeration extract elicited a response nearly equal to the response to 240 beetle-hour equivalents of frass or frass extract. Females were again less responsive than males. The greater activity of the Porapak aeration extract may indicate that this extract contains more pheromone or a more complete pheromone blend than does frass or the frass extract.

Field Bioassays of Frass, Frass Extract, and Porapak Aeration Extract

The attraction of frass, frass extract, and Porapak aeration extract in the field are compared in Table 2. The attraction of 177 beetles to the crude Porapak aeration extract represented our first success at attracting wild beetles

to any source other than elm logs or beetle–log combinations. This experiment confirmed the suitability of Porapak Q for the collection of the *S. multistriatus*-aggregating attractant.

The Porapak aeration extract was less attractive than the female beetle–elm log combination in the check trap (177 beetles trapped vs. 252). An insufficient dosage of crude extract or use of an inadequate delivery system could account for this difference. Preliminary dose-response studies were inconclusive, as were studies to improve the glass-tube delivery system.

The sex ratio of beetles attracted to the Porapak aeration extract was approximately 1 : 1, which is similar to the sex ratio of beetles emerging from naturally infested wood (Wallace, 1940; Bartels and Lanier, 1974), and to the ratio of beetles captured at the check trap.

In contrast to the Porapak extract, virgin female frass and the pentane extract of frass were virtually unattractive in the field (Table 2). These results are not consistent with laboratory results (Table 1), in which frass and the crude extract of frass stimulate walking male and female beetles. This difference in behavior of walking beetles in the laboratory and flying beetles in the field may be due to the fact that frass contains sufficient active components to stimulate beetles at close range (10–15 mm between the test material and walking beetles in the laboratory olfactometer), but does not contain the additional components of the pheromone bouquet needed to attract flying beetles at longer ranges.

Partial Purification of Crude Frass and Porapak Extracts

The hexane extract of frass and the Porapak aeration extract were both subjected to GLC fractionation. The frass extract was used despite its inactivity in the field, because the positive responses of walking male and female beetles in the laboratory indicated that the extract might be a source of short-range attractants. The first step in fractionation of the frass extract was a short-path vacuum distillation of the concentrated extract. Laboratory bioassays on the resulting fractions demonstrated that approximately 75% of the biological activity of the frass extract was recovered in the distillate (41.2% mean response). The pot residue and washings from the cold trap were completely inactive.

The active distillate from the frass extract and the active Porapak aeration extract were fractionated by GLC on an SE-30 column; the resulting fractions were then bioassayed. Most of the activity of the frass distillate was apparently due to compounds found in the region of the gas chromatogram represented by fraction B (Table 3). In fact, at the 2,400 beetle-hour test level, the mean response (64.0%) of male beetles to fraction B exceeded the response (41.2%) of males to the distillate. Fraction A from the frass

TABLE 3. LABORATORY BIOASSAYS COMPARING WALKING MALE BEETLE RESPONSE TO GLC (SE-30 COLUMN) FRACTIONS OF SHORT-PATH DISTILLATE FROM FRASS AND TO FRACTIONS FROM PORAPAK AERATION EXTRACT (MEAN OF 3 TRIALS WITH 25 BEETLES EACH)

Material tested[a] (GLC fraction)	Mean response (%)	
	Frass short-path distillate[b]	Crude Porapak extract[c,d]
A	0.0	61.2
B	64.0	38.8
C	12.0	—

[a] All fractions tested at optimal levels, based on prior bioassays.
[b] Mean response to total distillate tested at 2400 beetle-hour level was 41.2%.
[c] Mean response to total extract tested at 250 beetle-hour level was 72.0%.
[d] No material present in fraction C from Porapak extract.

distillate was completely inactive; the low response to fraction C was probably insignificant.

Both GLC fractions (A and B) from the Porapak aeration extract stimulated walking male beetles (no material was present in fraction C resulting from the backflushing of the SE-30 column). Fraction A from GLC of the Porapak extract was nearly as active as the total extract itself (61.2% mean response compared with 72.0% mean response to the total extract), quite unlike the inactive fraction A from the frass extract. These data suggest that fraction A from the Porapak extract may contain the chemicals responsible for long-range attraction of in-flight beetles; the absence of these chemicals in frass and frass extracts probably accounts for their unattractiveness in the field.

Male beetle responses to fraction B from the Porapak extract and also to fraction B from the distillate of the frass extract indicate chemical similarities between these two fractions. The active chemicals in these fractions could be the short-range attractants to which we attributed the activity of frass and frass extracts in the laboratory olfactometer.

Our present studies demonstrate the feasibility of the Porapak aeration-trapping technique for the collection of the *S. multistriatus* pheromone. The most important finding of this study is that an extract containing the volatiles collected on Porapak will attract flying beetles in the field.

These field data and data from laboratory bioassays on GLC fractions lead us to conclude that the Porapak extract is a good source of the pheromone blend produced by virgin female *S. multistriatus* tunneling in elm logs. We also conclude that virgin female frass is unattractive in the field because it lacks the long-range attractants required for attraction of beetles in flight.

Compared with other methods of pheromone collection (for example, freeze-trapping), the Porapak technique is uncomplicated and inexpensive from both chemical and entomological standpoints. Our findings with *S. multistriatus* indicate that the Porapak aeration-trapping method offers a promising means to collect and concentrate airborne pheromones of other insect species. Application of this methodology to *S. multistriatus* resulted in the identification of the chemical attractants (Pearce et al., 1975).

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ALLELOPATHIC POTENTIAL OF WESTERN BRACKEN

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Abstract—In laboratory studies, water-soluble extracts from senescent western bracken (*Pteridium aquilinum* [L.] Kuhn var. *pubescens* Underw.) fronds reduced germination of western thimbleberry (*Rubus parviflorus* Nutt.) and delayed germination of salmonberry (*R. spectabilis* Pursh.), but did not affect Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco). In greenhouse studies, unincorporated western bracken litter reduced emergence of all three species but did not influence root and shoot length or dry weight. However, bracken litter incorporated into the soil reduced both shoot length and dry weight of western thimbleberry seedlings. Allelopathic interactions may explain the relative absence of woody shrubs such as western thimbleberry and salmonberry from sites dominated by western bracken.

Key Words—allelopathy, phytotoxicity, *Pteridium aquilinum* var. *pubescens*, *Pseudotsuga menziesii*, *Rubus spectabilis*, *Rubus parviflorus*.

INTRODUCTION

Bracken (*Pteridium aquilinum* [L.] Kuhn.) has an almost worldwide distribution. The Pacific coast variety, western bracken (*P. aquilinum* var. *pubescens* Underw.), occurs in moist woods or dry open slopes from Alaska to Mexico (Abrams, 1955). In the Coast Ranges of Oregon and Washington, it attains maximum development on disturbed sites, particularly following repeated fires (Isaac, 1940). Communities dominated by western bracken frequently have fewer herbaceous and woody species than adjacent sites without bracken (Figure 1). This dominance by bracken over other vegetation has been attributed to competition, smothering by senescent fronds, or feeding activities

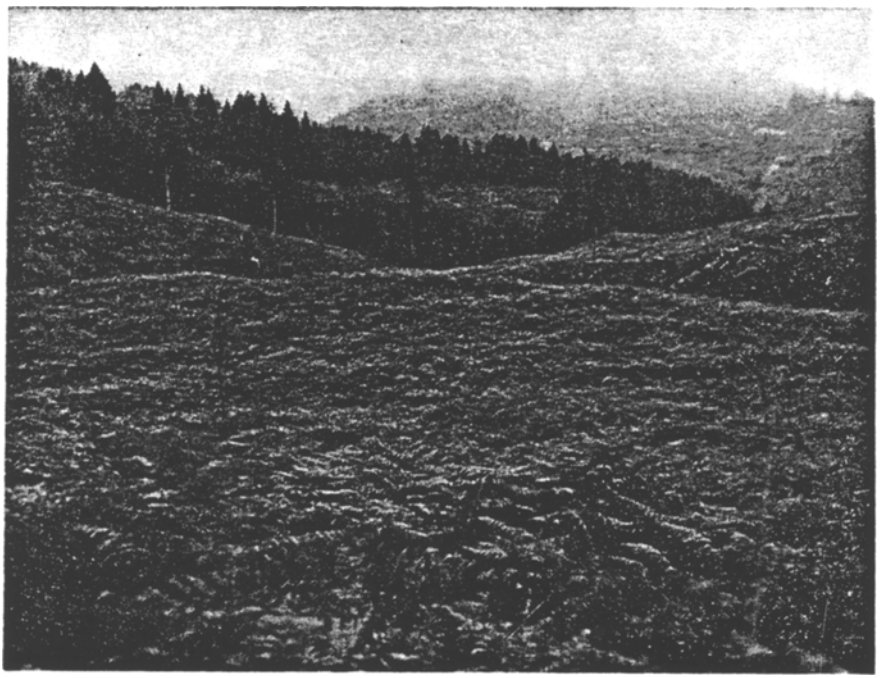

FIG. 1. A 10-year-old cutting in the Oregon Coast Ranges dominated by 2-meter-high western bracken.

of animals associated with western bracken habitats (Dimock 1964; Worthington 1955; Staebler *et al.*, 1954). However, recent studies suggest that bracken fronds contain water-soluble phytotoxins capable of reducing germination and growth of certain herbaceous species (del Moral and Cates, 1971; Gliessman and Muller, 1972). These phytotoxins are either absent or less effective in green fronds; they become more effective as fronds die at the end of each growing season.

Allelopathy is the inhibition of germination, growth, or metabolism of one plant because of the release of organic chemicals by a different plant. In mild, moist environments such as found in the Coast Ranges of Oregon and Washington, conditions are conducive to rapid decomposition of metabolites, and allelopathy may be overlooked as a mechanism influencing plant growth (del Moral and Cates, 1971). However, previous studies show that inhibitors produced in dead western bracken fronds collected in similar environments are water soluble and soil active (Gliessman and Muller, 1972). Further, the inhibitory effect of the first rain after frond senescence is most

important, but subsequent rains contain enough toxic leachates to act as a reinforcement.

Phytotoxic effects of western bracken leachates have been demonstrated with herbaceous species (del Moral and Cates, 1971; Gliessman and Muller, 1972); previous bioassays with conifers were inconclusive (del Moral and Cates, 1971). However, such allelopathic effects may explain the relative absence of conifers and other woody plants in western bracken communities. Laboratory and greenhouse studies were initiated to determine if this allelopathic potential exists for bracken in relation to a conifer, Douglas fir (*Pseudotsuga menqiesii* [Mirb.] Franco), and two common coastal shrubs, salmonberry (*Rubus spectabilis* Pursh) and western thimbleberry (*R. parviflorus* Nutt.).

METHODS AND MATERIALS

During September 1972, standing dead western bracken fronds were collected before the first fall rains in a 10-year-old cutover area on forest land in the Coast Ranges of Oregon. The frond material was broken by hand into small pieces and stored dry for two months. Douglas fir, salmonberry, and western thimbleberry seeds were collected from various locations in the Coast Ranges and stored dry in a refrigerator until the start of the experiment.

Laboratory Study

The objective of the laboratory study was to verify that water-soluble compounds from the senescent fronds were involved in inhibition. Under natural conditions, seeds would be exposed to western bracken leachates during imbibition, stratification, and germination. To more nearly duplicate this condition, seeds of the three species were exposed throughout the study to either distilled water or extracts from bracken fronds.

Five replications of 50 seeds each for each species were presoaked for 24 hours in distilled water (untreated) or western bracken extract (extract). The extract was prepared by soaking 100 g of broken dry fronds in 1.5 liters of distilled water for 2 hours and then filtering the solution to remove solids. Seeds were then stratified in vermiculite moistened with distilled water or extract at 3°C for 30 days (Douglas fir), 90 days (western thimbleberry), or 120 days (salmonberry). Starting dates for presoaking were staggered to allow termination of stratification on the same date for all three species.

Stratified seeds were germinated on sterile pumice in petri dishes in a growth chamber at temperatures of 25°C and 15°C for 12 hours each and a

photoperiod of 16 hours of light and 8 hours of darkness. At the start of germination, 25 ml of distilled water or freshly prepared bracken extract were added to the control and treatment dishes, respectively, and replenished as needed during the study. The number of seedlings with a radicle extruding at least 2 mm was recorded daily for 30 days.

Greenhouse Study

The objective of the greenhouse study was to determine if the inhibitors were soil active. For each species, there were 10 replications of 50 seeds each, presoaked for 24 hours in distilled water, and then stratified as described above. After stratification, seeds were sown in individual 15-cm-deep pots containing (1) 1500 g of air-dried soil (untreated), (2) 1500 g of soil with seeds covered by 15 g of western bracken frond material (unincorporated), or (3) 1500 g of soil containing 15 g of frond material incorporated into the soil (incorporated). The sandy-clay-loam A horizon of a coastal Oregon forest soil was used for all treatments after it had been passed through a 2-mm

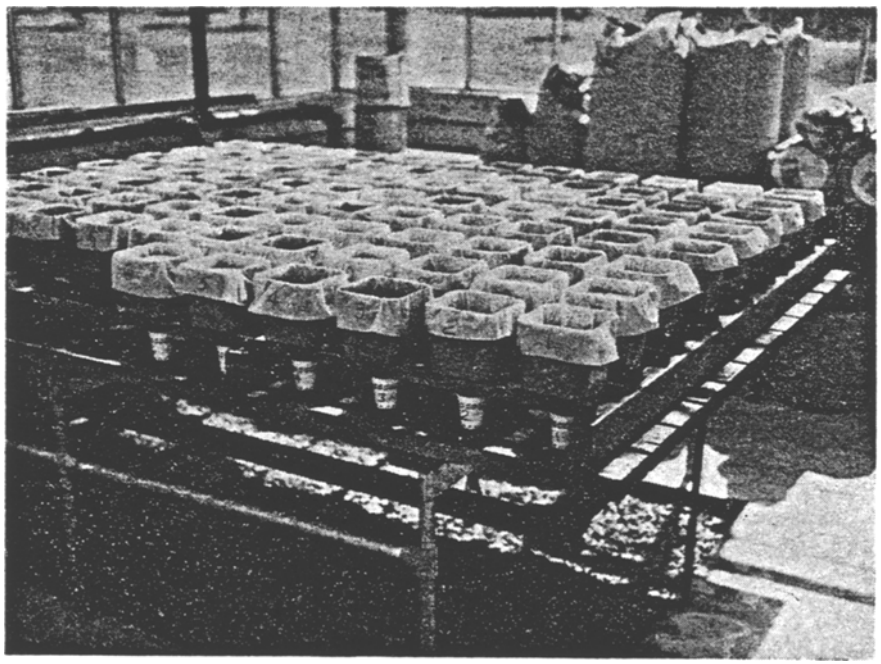

FIG. 2. Seedlings were grown in pots on a greenhouse bench. Paper cups were used to collect excess water which was added back to the pots.

screen (U.S. Standard Soil Sieve #10) to remove roots and large soil aggregates. Soil was collected from a 2-year-old clearcut free from bracken fern but dominated by salmonberry, western thimbleberry, and Douglas fir seedlings. Seeds in untreated or incorporated treatments were covered with sterile pumice to a depth equivalent to that of the unincorporated litter to minimize variation caused by depth of emergence.

Pots were placed on a greenhouse bench and watered periodically with tap water (Figure 2). Drainage water was collected in a paper cup below the drain hole in each pot and added back to the pots when water holding capacity permitted. Cumulative emergence was then recorded for 30 days. At the end of this period, all but the five largest seedlings were clipped from each pot. New germinants were recorded and removed thereafter. At the end of 110 days of test, the five original seedlings were harvested from each pot for determination of length of longest root, length of shoot, and root and shoot dry weights after 48 hours of drying at 75°C.

Experimental data were analyzed by analysis of variance for both studies. Where analysis showed significant treatment effects, treatment means from the greenhouse study were compared by a Duncan's multiple range test (Li, 1965) at the 5% level.

RESULTS

Laboratory Study

Water-soluble extracts of senescent western bracken fronds affected germination of the three species differently (Table 1). Western thimbleberry seeds were most sensitive and showed a 26% reduction in total germination compared with seeds germinated in distilled water. In contrast, Douglas fir seeds were not affected, and salmonberry seeds showed an intermediate response. Bracken extracts reduced the speed of salmonberry germination, but the difference in cumulative germination at the end of the test was not statistically significant. However, prolonging the time required for germination can reduce the probability of seedling establishment during the dry summer season typical of coastal Oregon and Washington.

The western bracken extract was dark brown in color and undoubtedly contained a number of organic compounds in solution. The osmotic effect of this extract may be capable of inhibiting seedling germination. However, del Moral and Cates (1971) were able to demonstrate growth inhibition of three species in more concentrated extracts that had been corrected for osmotic potentials. These extracts generally had potentials greater than -2 atm. Although osmotic potentials were not measured, the more dilute solutions used in the present experiment probably did not have significant osmotic effects on germination.

TABLE 1. CUMULATIVE GERMINATION OF DOUGLAS FIR, SALMONBERRY AND WESTERN THIMBLEBERRY IN DISTILLED WATER AND WESTERN BRACKEN EXTRACT

Species	Treatment	Cumulative germination (%) after[a] 10 days	20 days	30 days
Douglas fir	Water	7.6a	61.6a	70.0a
	Extract	6.8a	61.8a	71.6a
Salmonberry	Water	16.8	25.6	29.6b
	Extract	3.2	12.8	17.6b
Western thimbleberry	Water	70.8	73.6	76.8
	Extract	38.4	45.6	50.4

[a] Within the same column, numbers followed by the same letter are not statistically different at the 5% level of probability according to analysis of variance.

Greenhouse Study

Because of the placement of seeds in relation to the source of phytotoxin, leaching from unincorporated western bracken litter should affect germination more readily than incorporated litter. After germination, however, seedling roots would be in close association with bracken litter in the soil, and

TABLE 2. EMERGENCE, ROOT AND SHOOT LENGTH, AND ROOT AND SHOOT DRY WEIGHT IN UNTREATED SOIL AND IN SOIL WITH UNINCORPORATED OR INCORPORATED WESTERN BRACKEN LITTER

Species	Treatment	Emergence[a] (%)	Length (mm)[a] Root	Shoot	Dry weight (g)[a] Root	Shoot
Douglas fir	Untreated	64.8a	198a	69a	0.067a	0.103a
	Unincorporated	58.0	187a	81a	0.056a	0.105a
	Incorporated	68.8a	220a	77a	0.063a	0.093a
Salmonberry	Untreated	13.8b	153b	63b	0.025b	0.088b
	Unincorporated	2.0	103b	46b	0.015b	0.063b
	Incorporated	16.8b	146b	39b	0.015b	0.048b
Western thimbleberry	Untreated	64.8	242c	105c	0.089c	0.272c
	Unincorporated	28.6	230c	114c	0.085cd	0.289c
	Incorporated	75.2	232c	69	0.055d	0.144

[a] Within the same column, numbers followed by the same letter are not statistically significant at the 5% level of probability according to Duncan's multiple range test.

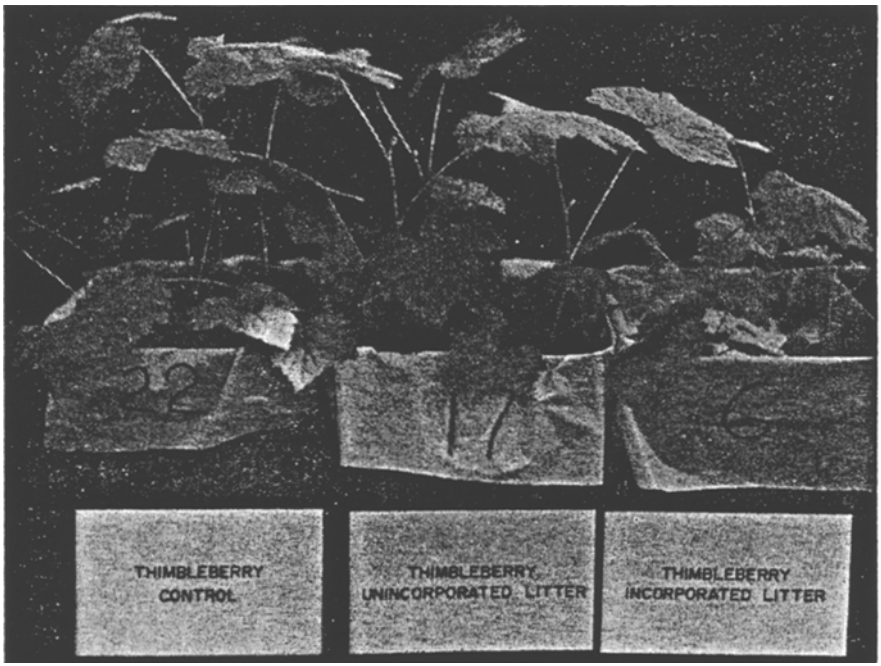

FIG. 3. Incorporated western bracken litter reduced shoot growth of western thimbleberry seedlings compared to unincorporated litter or untreated.

incorporated litter should affect seedling growth. In fact, unincorporated litter did affect the emergence of all three species, while incorporated litter slightly increased emergence of the most sensitive species, western thimbleberry (Table 2). However, incorporated litter reduced root dry weight and shoot length and dry weight of western thimbleberry seedlings (Figure 3). Root and shoot growth of the more resistant species, Douglas-fir and salmonberry, were not affected.

DISCUSSION

del Moral and Cates (1971) found a slight reduction in Douglas fir radicle length in the presence of bracken litter extracts during the first 7 days after germination. Other studies also note reduced height growth of 2-year-old Douglas fir seedlings planted on sites dominated by western bracken (Dimock 1964; Worthington 1955; Staebler *et al.*, 1954). However, differences in shoot or root growth were not evident in 110-day-old seedlings in the present study. Perhaps the reduced height growth of Douglas fir observed in previous field

studies is because of reduced light levels under dense stands of bracken or due to competition for moisture and soil nutrients.

Data from laboratory and greenhouse experiments suggest that dead western bracken fronds contain water-soluble phytotoxins capable of reducing establishment and growth of seedlings of woody plants. This allelopathic potential may be because of the presence of cinnamic or benzoic acid derivatives found in other species of bracken fern (Bohm and Tryon, 1967; Gliessman and Muller, 1972). Toxic effects were evident in a forest soil, indicating that inhibitors can be transferred to other plants by leachates washed into the soil by rain. Simulated rainfall studies by Gliessman and Muller (1972) support this conclusion. For example, they found soil-active concentrations of phytotoxins in leachates obtained after five rains of ¼ in each. The inhibitory effect of the first rain was most important, but subsequent rainfall could act as a reinforcement. Gliessman and Muller (1972) advance the hypothesis that phytotoxins of bracken are ecologically effective in areas of high rainfall. This may explain the relative absence of salmonberry and western thimbleberry on bracken-dominated sites in the Oregon Coast Ranges where annual rainfall is 60–80 in.

Seed dissemination of most species occurs prior to bracken senescence in the fall with germination during early fall or the following spring. Therefore, seeds would be subjected to bracken leachates throughout the period of imbibition and stratification. In dense bracken, the accumulation of dead fronds may cover the soil to a depth of several inches. After compaction by rain and snow, this material can form a significant barrier to seedling emergence. The combination of reduced germination caused by phytotoxins and reduced seedling emergence caused by accumulated litter may prevent ecesis of many species in established western bracken communities.

Western thimbleberry seeds were most sensitive to western bracken leachates; Douglas fir was least sensitive and salmonberry intermediate in response. Field observations suggest that Douglas fir establishment is often slowed or retarded on bracken-dominated sites (Dimock 1964; Worthington 1955; Staebler *et al.*, 1954). Salmonberry and western thimbleberry, while common throughout the Coast Ranges, rarely occur in dense bracken communities. Although the data from this study are not conclusive, the sensitivity of the two shrub species to bracken extracts is suggestive of allelopathic interaction. The question of Douglas fir establishment deserves further study. Other factors such as shading, competition for soil moisture and nutrients, and mechanical injury from smothering by dead fronds are undoubtedly important in limiting ecesis of species in dense bracken. Unfortunately, the relative importance of these factors is unknown; but future studies should consider allelopathy as well as more traditional competitive interactions.

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ALLELOPATHIC EFFECTS OF HACKBERRY IN A BOTTOMLAND FOREST COMMUNITY

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Abstract—Hackberry was previously found to produce toxins that inhibit the growth of associated herbaceous species in a grassland community. Nevertheless, it was hypothesized that bare areas under hackberry trees in a bottomland forest community may not be caused by allelopathy, since inhibitory compounds may be leached or metabolized faster than in a grassland community. Investigations indicated that the relatively bare areas under hackberry were not due to competition for minerals, light, or water, or to differences in texture or pH. The percent of soil moisture was always higher under hackberry trees than under bur oak trees where herbaceous species were prominent. Decaying hackberry leaves, leaf leachate, and soil collected from under hackberry trees significantly reduce seed germination and seedling growth of test species. Ferulic, caffeic, gentisic, and *p*-coumaric acids, and scopolin, and scopoletin were identified as phenolic phytotoxins produced in hackberry leaves. Thus it appears that the reduced growth of herbaceous vegetation associated with hackberry trees in both grassland and forest communities is due primarily to allelopathy, with the initial inhibition being accentuated by competition.

Key Words—allelopathy, *Celtis laevigata*, forest community, phenolic, ferulic acid, caffeic acid, gentistic acid, *p*-coumaric acid, scopolin, scopoletin, phytotoxins.

INTRODUCTION

A previous study of *Celtis laevigata* (hackberry) demonstrated that it exerted allelopathic effects against all important tall grass species in a grassland research plot containing hackberry trees (Lodhi and Rice, 1971).

Because of the possible ecological significance of the inhibitors of seed

germination and seedling growth (Lodhi and Rice, 1971), additional work was initiated with *Celtis laevigata* in a bottomland forest where these inhibitors possibly can be leached and metabolized much faster and may lose their allelopathic activity. Bare areas frequently occur under and around hackberry, although several herbaceous species may grow profusely under adjacent tree species that cause shade that is just as dense. This paper reports on experiments designed to determine whether the relatively bare areas under the hackberry in a forest community are chiefly because of competition for minerals, water, or light; or because of chemicals produced by the hackberry. The nomenclature used here follows Waterfall (1966).

LOCATION AND DESCRIPTION OF STUDY AREA

A bottomland plot was established in Oliver Wildlife Preserve located on the University of Oklahoma campus in Norman (Sec. 7, T8NR2W in Cleveland County). The bottomland plot is on a level flood plain of the South Canadian River. The soil is a sandy clay loam. The vegetation consisted of a floodplain forest dominated by *Fraxinus pennsylvanica* (green ash), *Quercus macrocarpa* (bur oak), and hackberry, with several minor tree species.

The growth of herbaceous species was observed to be considerably better under bur oak than hackberry. Light intensities were measured under several hackberry and bur oak trees. Readings were taken twice a month in June and July of 1969. Ten readings were taken with a Weston light meter under each species at each sampling time. An average range of 600–700 ft-c light intensity was obtained under both hackberry and oak trees. No differences were obtained that could explain the differences in growth of herbaceous species under test and control trees.

To describe quantitatively the zone of reduced growth associated with hackberry trees in the Oliver Preserve, 30 randomly located quadrats, 0.25 m^2 in area, were clipped under hackberry trees and 30 under bur oaks in July. Species were separated, oven-dried, and weighed. To obtain quantitative data for *Bromus japonicus* (brome grass), 30 quadrats were clipped for this species in late May and June 1971, because this species is a winter annual. Oven-dry weights of all species sampled were significantly lower under hackberry trees than under bur oaks (Table 1).

EXPERIMENTATION AND RESULTS

Physical and Chemical Analyses of Soil

Soil moisture, pH, texture, and several selected mineral analyses were

TABLE 1. RESULTS OF FIELD CLIPPING OF SPECIES ASSOCIATED WITH HACKBERRY AND BUR OAK IN OLIVER PRESERVE

Species	Mean oven dry weights in g/0.25 m²	
	Hackberry	Bur oak
Elymus virginicus	1.72±0.34	3.65±0.49[a]
Solidago gigantea	8.80±0.66	14.14±0.42[a]
Ambrosia trifida	1.75±0.23	3.76±0.55[a]
Other species	1.47	4.28
Mean total weight	13.72	24.80
Bromus japonicus (May)	1.78±0.20	3.68±0.31[a]
Bromus japonicus (June)	2.21±0.78	5.32±0.45[a]

[a] Dry weight significantly different from that under hackberry at 0.05 level.

made to see if the differences in the vegetation under the hackberry trees were due primarily to physical or chemical properties of the soil.

Soil moisture was determined during the summer of 1969, by taking soil samples at the 0–15-cm and 15–30-cm levels. Ten samples were taken at each level under hackberry trees and ten at each level under bur oak trees at each sampling time. All samples were weighed, oven-dried for 48 hours 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. The percent of soil moisture was always significantly higher under hackberry trees than under bur oak trees (Table 2).

TABLE 2. COMPARISON OF SOIL MOISTURE UNDER HACKBERRY TREES AND UNDER BUR OAK TREES

Time of soil collection	Level of the soil (cm)	Under hackberry	Under bur oak
June, 1969	0–15	22.70±0.75	19.80±0.60[a]
	15–30	20.60±0.44	16.90±0.80[a]
July, 1969	0–15	22.62±0.74	19.82±0.61[a]
	15–30	20.38±0.44	16.57±0.79[a]
August, 1969	0–15	22.20±0.73	19.91±0.60[a]
	15–30	19.90±0.45	16.24±0.77[a]

[a] Percent moisture significantly different from amount under hackberry at 0.05 level.

For physical and chemical soil analyses, 10 soil samples minus litter were collected at the 0–30-cm level under hackberry and 10 under bur oak trees. Visible pieces of organic matter were removed by hand, after which the soil was passed through a 2-mm sieve. The pH was determined by the glass electrode method of Piper (1942), and a mechanical analysis with a modified Bouyoucus hydrometer method (Bouyoucos, 1963; Piper, 1942). After the pH and texture were determined, the samples were ground in a soil mill to pass a 0.5-mm sieve. Total phosphorus was determined by the method of Shelton and Harper (1941), total carbon by the Walkey and Black method (Piper, 1942), and total nitrogen by the macro-Kjeldahl method of Bremner (1965). Iron, zinc, manganese, and copper were determined by using a Perkin-Elmer Model 303 atomic absorption spectrophotometer after extraction according to the instructions in the analytical manual supplied with the instrument (Perkin-Elmer Corporation, 1968). All calculations were based on the oven-dry weight of the soil. No significant differences were found in the pH, texture, organic carbon, or amounts of any of the mineral elements under hackberry as compared with control soil (data available upon request). These studies showed that the failure of the herbaceous species to grow well under hackberry was not due to any of the factors discussed above.

Experiments were subsequently initiated to determine if hackberry trees produce chemicals inhibitory to select herbaceous species from the bottomland forest community.

Effects of Decaying Hackberry Leaves on Germination and Seedling Growth

Thirty seeds of brome grass were planted in each of ten 10-cm glazed pots containing 1 g air-dried hackberry leaf powder per 454 g of a 3:2 soil and sand mixture, and because of poor germination, a large number of *Elymus virginicus* seeds were planted in each of 10 pots containing a similar mixture. The hackberry leaves employed were harvested and air dried in October, 1969. In the control pots, one gram of peat moss per 454 g of the soil-sand mixture was used, and 10 pots were planted as described above with each species.

After two weeks, the plants were thinned to the four largest seedlings per pot. The plants were grown for two additional weeks, then harvested and oven-dried for 48 hr at 36°C. Seedling growth of both test species was significantly reduced by decaying leaf material, and seed germination of brome grass was inhibited slightly, indicating an allelopathic effect (Table 3).

Effects of Leaf Leachate on Germination and Seedling Growth

A fine mist of cistern water was spread over freshly collected leafy hack-

TABLE 3. EFFECTS OF DECAYING HACKBERRY LEAVES ON GERMINATION AND SEEDLING GROWTH

Species and experiment no.	Mean oven-dry weight of seedling (mg)		Germination (% control)
	Control	Test	
Elymus virginicus			
1	157±7.23	100±7.76[a]	—
2	127±7.64	100±8.59[a]	—
Bromus japonicus			
1	131±9.30	101±6.70[a]	88
2	141±8.51	97±7.73[a]	94

[a] Dry weight significantly different from control at 0.05 level.

berry branches, and the leachate collected in this manner was used to water 10 pots of each test species in a 3:2 soil–sand mixture. Planting was done as described above. Ten control pots of each species were treated in the same manner, except they were watered with equal amounts of cistern water that was not passed over hackberry branches. After two weeks the plants were thinned to the four largest seedlings per pot. Seedlings were allowed to grow for two additional weeks, harvested, oven-dried for 48 hr and weighed.

The oven-dried weight was reduced significantly in each species by the leachate, and the germination of brome grass seeds was reduced slightly in one experiment (Table 4).

TABLE 4. EFFECT OF LEAF LEACHATE ON GERMINATION AND SEEDLING GROWTH

Species and experiment no.	Mean oven-dry weight of seedlings (mg)		Germination (% control)
	Control	Test	
Elymus virginicus			
1	158±7.94	103±7.00[a]	—
2	131±7.53	100±8.95[a]	—
Bromus japonicus			
1	126±9.32	101±6.36[a]	93
2	141±8.09	98±7.50[a]	99

[a] Dry weight significantly different from control at 0.05 level.

TABLE 5. EFFECT OF FIELD SOIL FROM UNDER HACKBERRY TREES ON GERMINATION AND SEEDLING GROWTH

Species and date soil taken	Mean oven-dry weight of seedlings (mg)		Germination (% control)
	Control	Test	
Elymus virginicus			
July, 1969	128±6.32	127±6.78	—
Jan., 1970	151±7.76	99±7.56[a]	—
Bromus japonicus			
July, 1969	144±8.67	141±8.32	106
Jan., 1970	149±8.56	102±7.56[a]	54
June, 1971	158±6.39	121±8.20[a]	61

[a] Dry weight significantly different from control at 0.05 level.

Effects of Field Soils on Germination and Seedling Growth

To determine if the phytotoxins of hackberry are stable in the soil under field conditions, soil collections were made in July, 1969, January, 1970, and June, 1971 under hackberry (test) and oak trees (control) in the Oliver Preserve. Collections were made with a sharp-nose shovel, and the soil was transferred directly into the pots in order to disturb the profile as little as possible. Seeds of test species were placed in pots, as explained before. Ten test pots and ten control pots were planted with each species. After two weeks the plants were thinned to the four largest seedlings per pot. These were allowed to grow for two additional weeks, harvested, oven-dried for 48 hr and weighed.

The July, 1969 soil did not significantly affect germination or seedling growth (Table 5). The January, 1970 soil, however, reduced germination of brome grass appreciably and significantly inhibited seedling growth of both test species (Table 5). Apparently the toxic compounds are more active in soil in late fall and winter after the accumulation of hackberry leaves and other plant parts, as reported by Lodhi and Rice (1971). The inhibitors were possibly either leached from the soil by the early summer rains of 1969, or were oxidized because of exceptionally hot weather in late July of 1969. To check these possibilities, a soil collection was made in June, 1971 and was treated in the same manner as the previous collections but with only one test species, brome grass. Interestingly, seed germination was lowered appreciably and seedling growth was significantly reduced (Table 5). Therefore, it appears that the phytotoxins of hackberry are stable in the soil under field conditions unless some exceptional weather conditions occur.

Identification of Phytotoxins from Hackberry Extracts

The two procedures used to isolate the compounds from hackberry leaves were those of Rice (1965) and Guenzi and McCalla (1966). The identifications were based on the methods of Rice (1965).

Ten percent aqueous extracts of hackberry leaves were acidified to pH 2.5 using 2 N HCl, and extracted with two half volumes of diethyl ether. Ether and water fractions were evaporated to dryness and were taken up in 5 ml 95% ethanol and 10 ml distilled water, respectively. These fractions were chromatographed in two dimensions on Whatman 3 MM paper with *n*-butanol–acetic acid–water (63:10:27 v/v/v), BAW, followed by 6% aqueous acetic acid, (6% AA). The chromatograms were inspected with short (2537 Å) and long (3360 Å) ultraviolet light. Compounds were marked under UV light and subsequently eluted with 95% ethanol. The eluates were reduced to dryness in vacuo, taken up in 3 ml 95% ethanol, and chromatographed in one dimension on Whatman No. 1 paper in three different solvent systems: BAW, 6% AA, and isopropanol–butanol–water (140:20:60 v/v/v) IBW. The R_fs in various solvent systems, colors in UV light, colors in various reagents (Rice, 1965), and maximum absorption peaks in 95% ethanol before and immediately after the addition of 2 drops of 2 N NaOH per cuvette, indicated the presence of scopolin and scopoletin in the extracts (Tables 6 and 7).

Following Guenzi and McCalla (1966), 10 g plant material were ground to pass a 10-mesh screen and hydrolyzed with 150 ml 2 N NaOH in an autoclave for 45 minutes. The extract was filtered and acidified to pH 2.0 with HCl and extracted with two half volumes of diethyl ether. The ether extract was shaken with two half-volumes of 5% $NaHCO_3$ and the ether portion was discarded. The alkaline portion was acidified again to pH 2.0 and reextracted with two half-volumes of ether. The ether fraction was evaporated to dryness and the residue was taken up in 5 ml 95% ethanol. Acid hydrolysis was carried out on a similar amount of ground material by refluxing with 150 ml 2 N HCl for 30 minutes. Ether extractions were carried out as previously described.

Ferulic, caffeic, and *p*-coumaric acids were identified from alkaline hydrolysis (Tables 6 and 7). Only one compound, gentisic acid, was identified from acid hydrolysis.

The biological activity of all the compounds identified was determined. Ethanolic eluates of all the compounds identified and of a similar sized area from a blank chromatogram were evaporated to dryness and were taken up in 2 ml of phosphate buffer, pH 5.65. These buffer solutions were added to petri plates containing 50 seeds each of *Amaranthus palmeri* or brome grass on filter paper. The amounts of the inhibitors applied were not known. The

TABLE 6. CHROMATOGRAPHY OF PHYTOTOXINS FROM *Celtis laevigata*

Compound	R_fs on Whatman No. 1[a]			UV fluorescence[c]		Reagent colors[b,c]		
	BAW	6%	IBW	long	short	Sulfan. acid	$FeCl_3$–$K_3Fe(CN)_6$	*p*-nit.
Scopolin	0.53	0.80	0.52	b bl	b bl	none	none	none
Suspected scopolin	0.53	0.79	0.53	b bl	b bl	none	none	none
Scopoletin	0.80	0.46	0.83	b bl	b bl	f br rose	bl	bl black
Suspected scopoletin	0.81	0.46	0.83	b bl	b bl	f br rose	bl	bl black
Ferulic acid	0.88	0.40	0.77	b bl	b bl	f tan	bl	f br black
Suspected ferulic acid	0.87	0.39	0.76	b bl	b bl	f tan	bl	f br black
p-Coumaric acid	0.90	0.46	0.70	0.86	pur abs	pur abs	or red	bl	br black
Suspected *p*-coumaric acid	0.89	0.47	0.71	0.85	pur abs	pur abs	or red	bl	br black
Caffeic acid	0.80	0.32	0.66	0.71	bl	bl	none	bl	f br black
Suspected caffeic acid	0.81	0.32	0.66	0.71	bl	bl	none	bl	f br black
Gentisic acid	0.85		0.65	0.65	bl	bl	f tan	bl	f br black
Suspected gentisic acid	0.85		0.64	0.65	bl	bl	f tan	bl	f br black

[a] See text for solvent systems.
[b] Diazotized sulfanilic acid, ferric chloride–potassium ferricyanide, and diazotized *p*-nitraniline.
[c] b = bright; bl = blue; br = brown; f = faint; abs = absorption; or = orange; pur = purple.

TABLE 7. MAXIMUM ABSORPTION SPECTRA (IN 95% ETHANOL) OF INHIBITORS FROM HACKBERRY LEAVES AND EFFECT OF INHIBITORS ON GERMINATION

Compound	Maximum absorption (nm)	Maximum absorption with NaOH	Germination (% control)	
			Amaranthus palmeri	Brome grass
Scopolin	326	345		
Suspected scopolin	325	346	33	27
Scopoletin	344	392		
Suspected scopoletin	344	390	26	31
Ferulic acid	285	343		
Suspected ferulic acid	282	340	33	48
p-Coumaric acid	283	330		
Suspected *p*-coumaric acid	285	332	49	39
Caffeic acid	288	265		
Suspected caffeic acid	286	264	24	27
Gentisic acid	330	295		
Suspected gentisic acid	328	293	47	37

eluate from the blank paper was used as the control. Germination was determined after 5 days, and the results expressed as a percent of control germination (Table 7).

Field soils from under hackberry trees were collected and extracted according to Wang *et al.* (1967), and then were treated for isolation and identifications following the procedure of Rice (1965). The papers were examined under UV light and the visible spots were eluted with 95% ethanol. These eluates were then tested for biological activity by the *A. palmeri* germination bioassay and were found to be very toxic to this species. However, I was not able to identify any of these toxins and suspected that they consisted of phenolics bound to other compounds. Therefore, a hydrolytic method modified from Guenzi and McCalla (1966) was used to extract these compounds from the soil. Ferulic, caffeic, and *p*-coumaric acids were identified from this method. All compounds identified from the January soil collection were present in concentrations of 800–1100 μg/g of soil.

DISCUSSION

The reduced growth of test species under hackberry trees was apparently not primarily because of physical factors, deficient soil moisture, or mineral deficiencies. Light intensity, pH, soil texture, organic carbon, and amounts

of mineral elements measured were not significantly different under hackberry than under bur oak trees. Soil moisture was always significantly higher under hackberry trees than under control trees. Decaying hackberry leaves, leaf leachate, and soil from under hackberry trees were all found to inhibit seed germination and seedling growth of herbaceous species that grow considerably better when away from hackberry trees than when under them. Apparently the allelopathic effects of hackberry trees in a forest community are just as effective as in a grassland community (Lodhi and Rice, 1971). Thus, the original hypothesis that hackberry may not be allelopathic in a forest community because of a rapid removal of toxins was not supported.

The phytotoxins identified from hackberry leaves were scopolin, scopoletin, and ferulic, caffeic, *p*-coumaric, and gentisic acids. Scopolin and scopoletin were found in aqueous extracts of leaves, whereas all others were found only after acid or alkaline hydrolysis. Lodhi (1975) reported that ferulic, caffeic, and *p*-coumaric acids were identified from the soil under hackberry trees only after alkaline hydrolysis. Guenzi and McCalla (1966) found ferulic and *p*-coumaric acids in the residues of corn, oats, sorghum, and wheat and that *p*-coumaric acid can be released in amounts sufficient to inhibit plant growth. Wang *et al.* (1967) sampled soil from several croplands and found *p*-coumaric acid and ferulic acid plus several other phenolic acids.

The concentration of phenolic acids quantified in many soils was found to suppress the growth of several young crop plants when applied to plants growing in nutrient culture solution. Rasmussen and Rice (1971) isolated ferulic and *p*-coumaric acids from *Sporobolus pyramidatus* and found allelopathic effects on associated species resulting in either reduced growth or elimination from the stand. Langdale and Giddens (1967) reported that small quantities of ferulic and *p*-coumaric acids are effective in inhibiting IAA activity in *Avena* coleoptiles. Zenk and Muller (1963) found that *p*-coumaric and ferulic acids increase IAA decarboxylation, resulting in reduced growth. Olmsted and Rice (1970) found that *p*-coumaric acid was significantly inhibitory to the growth of 12-day-old seedlings of *Amaranthus retroflexus*. Del Moral and Muller (1970) found that *p*-coumaric, ferulic, and caffeic acids from *Eucalyptus camaldulensis* were toxic to the germination of test seeds. Hennequin and Juste (1967) found that caffeic, ferulic, and *p*-coumaric acids have phytotoxic effects on seed germination and seedling growth. Rice (1965) found that *Ambrosia psilostachya* produced a glucose ester of caffeic acid that is inhibitory to nitrogen-fixing and nitrifying bacteria, and Rice (1968, 1971) reported that *A. psilostachya* and its leaf leachate caused a significant reduction of nodulation in three legume species. Neill and Rice (1971) reported that the root exudate, leaf leachate, and decaying leaves of *A. psilostachya* inhibited many of the early invaders of abandoned fields.

Al-Naib and Rice (1971) found that *Platanus occidentalis* inhibited seed

germination and seedling growth of many associated species, and scopolin, scopoletin, and other phenolic compounds were isolated from *P. occidentalis* leaves and mature fruits. Einhellig *et al.* (1970) found that growth of tobacco, sunflower, and pigweed was inhibited by a 5×10^{-4} M scopoletin concentration. Net photosynthesis in tobacco plants treated with a 10^{-3} M concentration of scopoletin was depressed to 34% of that of the controls. Lodhi and Nickell (1973) found that osmotically inactive water extracts of hackberry significantly reduced the shoot growth and the rate of photosynthesis, but significantly increased the rate of dark respiration of *Andropogon gerardi*, *A. scoparius*, and brome grass (species associated with hackberry). Lodhi (1975), Rice (1971), Al-Naib and Rice (1971), and Wilson and Rice (1968) suggested that the additive effect of a combination of inhibitors may be more detrimental than each compound separately.

Thus, it appears that allelopathy expressed by hackberry in a grassland community (Lodhi, 1975; Lodhi and Rice, 1971) or in a bottomland forest community may be important ecologically in helping determine the patterning of herbaceous vegetation.

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PHYTOTOXIC SUBSTANCES IN TWELVE SUBTROPICAL GRASSES[1]

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Abstract—Aqueous extracts of 12 subtropical grasses inhibited seed germination and radicle growth of *Lactuca sativa* var. Great Lakes at osmotic concentrations as low as 10 milliosmol. *Acroceras macrum*, *Chloris gayana*, *Digitaria decumbens*, and *Panicum maximum* exhibited the highest inhibition, while *Cortaderia selloana* revealed the least. Toxic spots were found on chromatograms of the ether fraction of aqueous extracts. *Cynodon dactylon*, *Setaria sphacelata*, and *Tripsacum laxum* showed more than six toxic spots, while *Andropogon nodosum*, *Bracharia mutica*, and *Chloris gayana* gave less than three toxic spots. The phytotoxins ferulic, syringic, *p*-coumaric, vanillic, *p*-hydroxybenzoic, and *o*-hydroxyphenylacetic acids were identified. These compounds are differentially distributed in the 12 grasses studied. Additionally, most of these compounds were also found in the associated soils; the control (nonherb-growth) soil provided the toxic compounds in significantly less amount than did the grass soils.

Key Words—allelopathy, phytotoxins, subtropical grasses, ferulic acid, syringic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxybenzoic acid, *o*-hydroxyphenylacetic acid.

INTRODUCTION

It has been demonstrated by many investigators that one plant may release toxic substances that suppress the growth of another plant (Molisch, 1937; Börner, 1960; Muller, 1966; Rice, 1967; Muller and Chou, 1972). Most phytotoxic substances have been found in shrubs or woody plants, and a few

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in herbaceous plants. In recent years, Tamés *et al.* (1973) isolated five growth inhibitors from the tubers of *Cyperus esculentus* var. *aureus*. Naqvi (1969) identified four allelopathic compounds in the straw of *Lolium multiflorum*. Chou and Chung (1974) found seven phytotoxins in a dominant species, *Miscanthus floridulus*, and in its associated soils. Additionally, Rice and his associates have demonstrated the toxic nature of many grass species (Rice, 1964, 1968, 1971, 1972; Rasmussen and Rice, 1971; Rice and Pancholy, 1973).

In Taiwan many herbaceous species are more widespread and they frequently form relatively pure stands. The elucidation of the mechanism of dominance by grass vegetation has not been clearly demonstrated, and it has always been thought that this mechanism might be due to either competition for physical factors, such as light, soil moisture, and nutrients, or the exudation of some growth inhibiting substances by the grasses. The purpose of this study was, therefore, aimed particularly toward an evaluation of the phytotoxic nature and the distribution of phytotoxins in 12 subtropical grasses.

METHODS AND MATERIALS

Materials

Twelve subtropical grasses were collected from the Taiwan Sugar Corporation Farm Animals Breeding Station at Chunan, Taiwan. The grasses were *Acroceras macrum*, *Andropogon nodosum*, *Brachiaria mutica*, *Chloris gayana*, *Cortaderia selloana*, *Cynodon dactylon*, *Digitaria decumbens*, *Eragrostis curvula*, *Panicum maximum*, *Plicatulum paspalum*, *Setaria sphacelata*, and *Tripsacum laxum*. Several of these species have been introduced for forage. Leaves of grasses were harvested when the grass was about 50 cm high. The leaves were brought back to the laboratory and air-dried at room temperature. Soil samples were collected from the top 10-cm layer of the earth in areas beneath the aforementioned grasses. Each soil was air-dried, screened with a 2-mm sieve, and carefully examined to remove all visible root fragments from the soils.

Bioassay of Aqueous Extracts of Grasses

Leaves of each grass were chopped into small pieces of about 2.5 cm length. To 25 g of chopped leaves, 250 ml of distilled water was added and shaken for 2 hr. Each aqueous extract was obtained by suction filtration and further centrifuged at 5000 rpm if necessary to clear the filtrate. The osmotic concentration of each extract was determined cryoscopically with an osmometer, Fiske model G-66, and was further diluted to 100, 50, 25, and 10 milliosmol. Lettuce seeds (*Lactuca sativa* var. Great Lakes) were used

to determine the phytotoxicity of the solutions by using a bioassay method called the "sponge bioassay" described by Muller (1966). Distilled water served as the control. Tests and controls were triplicated. After 72-hr incubation at 25°C, the results were taken by measuring the length of radicle growth in millimeters. The bioassay data were analyzed statistically by means of analysis of variance, and the least significant difference (LSD) at 5% and 1% level of confidence were obtained.

Isolation of Phytotoxins

Since most phytotoxic substances in grasses were thought to be phenolic in nature, an attempt was made to isolate toxic phenolic compounds from grasses. Each aqueous grass extract obtained by the aforementioned techniques was further concentrated in vacuo to a volume of 10 ml. It was then extracted with 100 ml of ethyl ether three times. The ether fractions were combined and allowed to evaporate to dryness at room temperature. The residue of ether fractions was finally dissolved in 2.5 ml of 99.5% ethanol. About 500 μl (equivalent to 5 g of original leaves) of the ethanolic solution was streaked uniformly on a sheet of 10×57 cm Whatman 3 MM chromatographic paper which was then developed with 2% glacial acetic acid. After the papers were developed, they were air dried to get rid of any residue of acetic acid. The separated compounds absorb and fluoresce under short wave UV light differently. Each zone appearing to contain a compound, or more than one, was cut out, cut into a 5-mm square, and extracted with 50 ml of 99.5% ethanol. The eluate was evaporated to 1 ml at 50°C. This eluate was rechromatographed with *n*-butanol–acetic acid–water (4:1:5, v/v/v, called BAW). The elution processes were repeated twice to obtain a purified compound.

Chromatographic Bioassay

The phytotoxic qualities of compounds isolated from the ether fraction of aqueous extract of each grass were determined by chromatographic bioassay described by McPherson *et al.* (1971), with lettuce as the bioassay species. Washed paper strips, spotted with an ethanol solution of the ether fraction and another unspotted control strip, were developed simultaneously with 2% glacial acetic acid. Toxicities were revealed by bioassaying relevant chromatographic spots or R_f segments of a whole chromatogram.

Identification of Toxic Compounds

The isolated toxic spots on the chromatogram were rechromatographed

by using two developing solvents as described above. After developing, the spots were detected under shortwave UV light and the chromatograms sprayed with three reagents: (1) DPNA: diazotized *p*-nitroaniline followed by 10% sodium carbonate (Hais and Macek, 1963), (2) DQC: 2,6-dichloroquinone chlorimide followed by saturated sodium borate (Vásquez et al., 1968), and (3) 0.3% ethanolic ninhydrin solution. The synthetic chemicals were chromatographed simultaneously, using the same techniques. Phytotoxic phenolics appeared as absorbing or fluorescing spots under the UV light, and appeared in distinguishable colors after spraying with DPNA and DQC, while nitrogen-containing compounds appeared in various colors after spraying with ninhydrin. Quantitative comparison of all toxic phenolics was made by grading arbitrarily to three classes based on the average size of spot from three to four replications.

RESULTS

Phytotoxicity of Aqueous Extracts

Aqueous extracts of each grass were bioassayed using the sponge bioassay. It was found that at the osmotic concentration of 100 milliosmol (about 25 g leaves in 250 ml distilled water), all extracts revealed a significant inhibition of the growth of lettuce radicles and the degree of inhibition could be classified into three types. The first type involved complete inhibition of seed germination by the extracts, resulting in a swelling and blackening of the seeds. When these seeds were washed with distilled water and reincubated for another 72 hr, they did not germinate. *Acroceras macrum*, *Chloris gayana*, *Digitaria decumbens*, and *Panicum maximum* belonged to this type.

In the second type of inhibition seed germination was inhibited, but the seeds were able to germinate after washing and reincubation. *Andropogon nodosum*, *Brachiaria mutica*, *Cynodon dactylon*, *Eragrostis curvula*, *Plicatulum paspalum*, *Setaria sphacelata*, and *Tripsacum laxum* belonged to this type. In the third type of inhibition seed germination was not inhibited, but radicle growth was suppressed, resulting in the radicle turning dark brown and being twisted in shape. The bioassay results of extracts at 10, 25, and 50 milliosmol (Table 1) revealed that inhibition was decreased with dilution. At 10 milliosmol, the inhibition ranged from 60% to 85%. *Acroceras macrum* and *Digitaria decumbens* showed 80–85% inhibition, while *Brachiaria mutica* revealed 60% inhibition. Comparing the inhibition exhibited by the grass with that by a mannitol solution, the former provided inhibition significantly higher than the latter (Chou and Young, 1974). The results indicate that the grass extract does contain significant inhibitory substances.

TABLE 1. EFFECTS OF AQUEOUS EXTRACTS OF GRASS LEAVES AT FOUR OSMOTIC CONCENTRATIONS ON THE RADICLE GROWTH OF LETTUCE
(Data represent the percentage of growth, using distilled water as the control)

	Osmotic concentration (milliosmol)			
Species	10	25	50	100
Acroceras macrum	18	14	5	0
Andropogon nodosum	31	27	15	4
Brachiaria mutica	40	29	15	9
Chloris gayana	26	14	10	4
Cortaderia selloana	33	19	13	13
Cynodon dactylon	33	17	13	7
Digitaria decumbens	14	7	5	4
Eragrostis curvula	28	13	7	4
Panicum maximum	22	10	5	0
Plicatulum paspalum	26	14	13	4
Setaria sphacelata	25	20	12	5
Tripsacum laxum	36	28	10	4
LSD between osmotic concentrations		5 % = 0.8		
		1 % = 1.2		
LSD between species		5 % = 1.6		
		1 % = 2.0		

Chromatographic Bioassay

Using the chromatographic bioassay techniques as described earlier, the water-borne phytotoxic substances on paper chromatograms can be detected. It was assumed that when a segment inhibited the experimental planting, as compared to control, by more than 35% (being significant below 1% level of confidence), this could be regarded as a toxic spot. With the developing solvent of 2% glacial acetic acid, most of the inhibitory spots were located at R_f values of 0.64–0.97, although a few toxic spots were found at low R_f values. Particularly, segment 9 with R_f 0.80–0.87 gave 100% inhibition. The number of toxic spots found on the chromatograms varied among the different species. *Cynodon dactylon*, *Setaria sphacelata*, and *Tripsacum laxum* revealed more than six spots, while *Andropogon nodosum*, *Brachiaria mutica*, and *Chloris gayana* gave less than three toxic spots. The rest of the plants had between three and six toxic spots.

TABLE 2. R_f VALUES AND COLOR RESPONSES OF INDIVIDUAL ISOLATES AND SYNTHETIC CHEMICALS[a]

Compound	R_f value		Color reaction			
	2% Ac	BAW	sUV	DPNA	DQC	ninhydrin
Isolate 1	0.27	0.92	bl–fl	bl	bl	—
Ferulic acid	0.35	0.89	bl–fl	bl	bl	—
Isolate 2	0.44	0.91	ab	dk–bl	bl to yel	—
trans-p-Coumaric acid	0.45	0.91	ab	dk–bl	bl to yel	—
Isolate 3	0.51	0.85	ab	bl	bl	—
Syringic acid	0.48	0.87	ab	bl	bl	—
Isolate 4	0.55	0.90	ab	vi	sk–bl	—
Vanillic acid	0.56	0.91	ab	vi	sk–bl	—
Isolate 5	0.62	0.93	ab	red	bl–gr	—
p-Hydroxybenzoic acid	0.62	0.93	ab	red	bl–gr	—
Isolate 6	0.74	0.90	ab	dk–bl	bl to yel	—
cis-p-Coumaric acid	0.75	0.91	ab	dk–bl	bl to yel	—
Isolate 7	0.84	0.93	—	vi	bl	—
o-Hydroxyphenylacetic acid	0.83	0.93	—	vi	bl	—
Unknown A	0.89		ab	red	—	vi
Unknown B	0.92		—	—	—	vi

[a] BAW = Butanol–acetic acid–water (4:1:5, v/v/v); 2% Ac = 2% glacial acetic acid; sUV = shortwave UV light; DPNA = diazotized *p*-nitroaniline; DQC = 2,6-dichloroquinone chlorimide; ab = absorption; bl = blue; dk = dark; fl = fluorescence; sk = sky; vi = violet; yel = yellow; gr = gray.

Identity and Distribution of Toxic Phenolics in Grasses and Soils

Since toxic spots were found on the chromatograms, the identification of the responsible toxins in the grasses and related soils was performed. The eluate of each toxic spot on chromatograms was rechromatographed and identified. These isolated substances were identified by comparison with synthetic chemicals (Table 2). They were ferulic, syringic, *cis*- and *trans-p*-coumaric, *p*-hydroxybenzoic, vanillic, and *o*-hydroxyphenylacetic acids and two unknown toxic substances, which appeared to be amino acids or other nitrogen-containing substances. A quantitative comparison of the toxins based on the size of the spots on chromatograms was attempted among these species (Table 3). Comparing these results with those of the chromatographic bioassay, most of grasses, such as *Acroceras*, *Andropogon*, *Brachiaria*, *Panicum*, and *Setaria*, are in good correlation. However, *Chloris* was altogether different.

Phytotoxic substances may be released from the plant to the environ-

TABLE 3. THE DYNAMICS AND DISTRIBUTION OF PHYTOTOXINS IN VARIOUS GRASSES[a]

Species	Compound							
	FA	*p*CA	SA	VA	*p*HBA	*o*HPAA	Unknown A	Unknown B
Acroceras macrum	−	+	−	++	+	++++	−	+
Andropogon nodosum	−	−	+	+++	−	+	−	+
Brachiaria mutica	−	−	−	++	++	+++	−	+
Chloris gayana	++	+++	+++	+++	+	++	−	+
Cortaderia selloana	−	++	+	+++	+	+++	−	+
Cynodon dactylon	−	++	+++	+++	+	+++	−	+
Digitaria decumbens	+++	−	+++	++	−	+++	−	+
Eragrostis curvula	−	−	−	++	−	−	++	+
Panicum maximum	−	−	−	−	−	++	−	+
Plicatulum paspalum	+	+	+	++	−	+++	−	+
Setaria sphacelata	+++	++	+++	+++	−	+++	−	+
Tripsacum laxum	++	−	−	+	−	+++	−	+

[a] Toxins are represented by abbreviations as follows: FA = ferulic acid; SA = syringic acid; *p*CA = *p*-coumaric acid. VA = vanillic acid; *p*HBA = *p*-hydroxybenzoic acid; *o*HPAA = *o*-hydroxyphenylacetic acid. Quantitative comparison of toxins (e.g., ++++ > +++ > ++ > + > −) based on 3–4 analyses of each source.

TABLE 4. THE DYNAMICS AND DISTRIBUTION OF PHYTOTOXINS IN VARIOUS GRASS SOILS[a]

Species	Compound							
	FA	*tp*CA[b]	*cp*CA[b]	SA	VA	*p*HBA	*o*CA	*o*HPAA
Acroceras macrum	++	++	+	+	++	−	−	+++
Andropogon nodosum	++	+++	++	+	++	+	−	+++
Brachiaria mutica	++	++	++	+	++	++	+++	++
Chloris gayana	++	+++	++	+	+++	++	−	++
Cortaderia selloana	+	++	+	+	+++	++	−	+++
Cynodon dactylon	++	++++	++	+	++++	++	−	+++
Digitaria decumbens	+	+++	+	−	+++	++	−	+++
Eragrostis curvula	++	+++	+	−	+++	++	−	++
Panicum maximum	+	+++	+	−	++	++	−	++
Plicatulum paspalum	++	++++	+	−	+++	++	−	+++
Setaria sphacelata	+	++	+	+	+++	++	++	++
Tripsacum laxum	++	++	+	−	+++	++	−	++
Control (without grass)	+	+	+	−	+	+	−	−

[a] Toxins are represented by abbreviations as follows: FA = ferulic acid; *tp*CA = *trans-p*-coumaric acid; *cp*CA = *cis-p*-coumaric acid; SA = syringic acid; VA = vanillic acid; *p*HBA = *p*-hydroxybenzoic acid; *o*CA = *o*-coumaric acid; *o*HPAA = *o*-hydroxyphenylacetic acid. Quantitative comparison of toxins (e.g., ++++>+++>++>+>−) based on 3–4 analyses of each source.

[b] The *cis-* and *trans-p*-coumaric acid may exist in an equilibrium of some sort in an aqueous solvent such as 2% acetic acid; however, they do separate clearly on the chromatogram of this study.

ment, resulting in the accumulation of toxins in soils. An attempt was made to extract and identify the toxic substances from the grass soils. The method of extracting soil phytotoxins was based on alkaline hydrolysis, and the technique used was a modification of that of Wang et al. (1967*a*). Soil phenolics were identified by means of paper chromatography, as described above. Phenolic toxic compounds identified from the grass soils included ferulic, *cis-* and *trans-p*-coumaric, syringic, vanillic, *o*-coumaric, *p*-hydroxybenzoic, and *o*-hydroxyphenylacetic acids (Table 4). Some of them were found in the control soil (an open area without herb growth), but the amount of toxins was far less than in the grass soils. The extractable phenolics found in the soils were almost identical with those found in the grasses. This indicates that the toxins present in soils are of plant origin. However, the toxins in the plants and soils did not fully agree with each other in their concentrations.

DISCUSSION

The compounds identified from the above grasses have been recognized as phytotoxins by many scientists (Börner, 1960; Rice, 1967; Patrick, 1971, Chou and Muller, 1972; Wang et al., 1967*b*; McPherson et al., 1971), and appear in a variety of plants and soils (Whittaker, 1971). Using the same bioassay techniques, McPherson et al. (1971) reported that compounds, such as ferulic, vanillic, and *p*-hydroxybenzoic acids can suppress the growth of lettuce at a concentration of 200 ppm, while *o*-hydroxyphenylacetic acid suppresses it at 100 ppm (Chou, 1973). Two other toxic spots have not been identified, but they appear to be amino acids or other nitrogen containing compounds because of their positive ninhydrin reaction.

It is also clear that most of the compounds identified in the grasses are found in their associated soils. A positive correlation between the toxic concentration in the grasses and soils was found. Furthermore, the amounts of compounds present in the control soil were significantly less than that in the grass soils. Without doubt, the phenolics that had accumulated in grass soils were of plant origin. These compounds in soils are possibly caused by the leaching of plant metabolites, or to root exudation or to the decomposition of grass residues in the soil. Tukey (1971) reported that the metabolites leached from the plants consisted of a variety of substances, such as mineral nutrients, carbohydrates, amino acids, and other organic compounds. These substances sometimes inhibit plant growth and sometimes stimulate plant growth, depending on the concentration of substances leached out. The leachability of substances varies with the age of the plants and with the seasons. The concentration of toxic metabolites is usually higher in dry seasons than in wet seasons (Muller, 1970; Whittaker, 1971). Tukey (1971)

indicated that the amount of substances lost from a plant increased with the maturity of its tissue, resulting in a peak at senescence. In this study we have no evidence to support this, although our plant materials were collected in the summer season.

The biochemical interaction among grasses is not yet clear, and some field studies need to be performed. At present, we lack information concerning the interaction of the physical factors, such as competition for nutrients, soil moisture, and light. However, the evolution and differentiation of phytotoxins in grasses may partly explain one of the mechanisms determining the process of dominance.

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SEX PHEROMONE OF THE FACE FLY, *Musca autumnalis* De Geer (DIPTERA: MUSCIDAE)

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Abstract—Components of a sex pheromone that cause male face flies to strike at females were found to be the straight-chain monoalkenes (*Z*)-14-nonacosene, (*Z*)-13-nonacosene, and (*Z*)-13-heptacosene. Although these compounds were found in the extracts of both sexes, extracts from sexually mature males contained a much higher proportion of nonacosane and heptacosane, which attenuated the activity of the active monoalkenes. The monoalkenes were readily synthesized by a Wittig reaction modified by the use of hexamethylphosphoric triamide as a cosolvent with tetrahydrofuran to produce a product containing 94–96% (*Z*) isomer.

Key Words—face fly, *Musca autumnalis* De Geer, pheromone, sex pheromone, behavioral bioassay, (*Z*)-14-nonacosene, (*Z*)-13-nonacosene, (*Z*)-13-heptacosene, mating strike.

INTRODUCTION

The face fly, *Musca autumnalis* De Geer, long recognized as a serious cattle pest has a predilection for mucoid substances about the eyes and nose of cattle and horses (Teskey, 1960). Although inferences have been drawn from the concurrence of cattle pinkeye with heavy face fly infestations, there has yet been no demonstration of any causal relationship. Nevertheless, the female face fly lays eggs in cow manure, and the flies frequent the mucous membranes of the cattle and their body wounds, so the insect must be viewed as a potential vector of disease.

In 1970, Lodha et al. reported that vision and an olfactory stimulus

might be involved in the sex recognition and mating behavior of the face fly. Subsequently, Chaudhury et al. (1972) reported a volatile sex pheromone in the extract of mature (5–6-day-old) virgin female face flies. Preliminary chemical tests suggested that the pheromone was an unsaturated hydrocarbon. The pheromone appeared to attract the male to the pheromone source and sexually stimulated him to initiate copulation. This behavior was consistent with an unpublished observation from our station in which we observed that caged male face flies did not make "mating strikes" toward males that had been isolated from females. However, males that were caged with mature females frequently became contaminated with the female pheromone and then became the objects of "mating strikes" by other males. We report here the identification and synthesis of components of this pheromone that initiate sexual activity.

METHODS AND MATERIALS

The most successful of the Chaudhury et al. (1972) devices for measuring extract activity was a glass cylinder olfactometer. However, we obtained more satisfactory results by using the following procedure.

Male and female face flies within 24 hr after eclosion were immobilized by cooling (Wolf et al., 1967) and separated by sex. Ten male flies were put into a quart mason jar that was placed on its side, and the jar was closed with a screened lid that had a small hole plugged with cotton. The flies were provided a dry milk-sugar mixture and water in small containers. Tests were conducted over a 2-hr period beginning at 0800 with male flies between 3–9 days of age. Male flies, the same age as the flies in the jars, were impaled on pins taped to soda straws. The test materials in hexane were placed on the dorsal part of the thorax and abdomen of the impaled flies, which were then introduced into the jars through the holes in the lids. The number of mating strikes at the test fly during a 5-min period was recorded. Five replicates of each dose level of each material were made in a single day, and each material was tested on at least four different days. Strike data were obtained with impaled females and served as a basis for comparison.

On any day of testing an individual jar of flies was used only one time. Values obtained were expressed as the ratio of mating strikes at the males bearing the test materials to the number of strikes at the females; this ratio is called the activity quotient. The flies used in the bioassay and for preparation of extracts were from a laboratory colony at Beltsville, Maryland, that had been in colonization for 14 years. Initial extracts were from dead face flies collected from the colony cages and sexed. Later, extracts were from 6-day-old (or other ages in some cases) unmated males and virgin females.

$$RCHO + Ph_3P = CHR' \xrightarrow[HMPT]{THF} RCH = CHR' \quad (94\text{–}96\%\ Z)$$

$$C_{13}H_{27}CHO + Ph_3P = CHC_{14}H_{29} \xrightarrow{\text{toluene}} C_{13}H_{27}CH = CHC_{14}H_{29} \quad (\sim 60\%\ E)$$

FIG. 1. Synthesis of alkenes.

Cuticular lipids were obtained by surface-rinsing males or females with petroleum ether. Homogenization of the flies with anhydrous sodium sulfate and petroleum ether served to provide the tissue extracts. The lipids were separated into their respective classes by chromatography on Florisil columns (containing about 5% water) with hexane followed by increasing concentrations of diethyl ether in hexane.

The hydrocarbon fraction (hexane eluate) was separated into saturated and unsaturated components by column chromatography on 25% silver nitrate-impregnated Florisil, and the unsaturated hydrocarbons were checked for purity and configuration by thin layer chromatography on 20% silver nitrate impregnated silica gel. Gas liquid chromatography (GLC) of the unsaturates (5% SE-30 on Gas Chrom Q programmed from 185° to 265°C at 1°/min) showed the same peaks were present in all extracts. Substances from the three major peaks were trapped.

Mass spectrometric investigations of the three trapped materials were performed with a Finigan Corp 1015 quadrupole mass spectrometer interfaced with a glass Gohlke separator to a gas chromatograph equipped with a glass column containing 3% OV-1 on Varaport.

Double bonds were located by ozonolysis and gas chromatographic analysis (5% SE-30 on 70/80 Anakrom ABS programmed from 80° to 240°C) of the resulting aldehydes by the method of Beroza and Bierl (1967).

The C_{27} and C_{29} alkenes, as well as a few of the C_{25} alkenes were all synthesized by a directed Wittig reaction (Figure 1). The proportion of *Z* olefin present in the product of a Wittig reaction is due in large measure to the nature of the solvent for a given pair of reactants. We noted that addition of a small amount of hexamethylphosphoric triamide to the solvent system we had been employing (tetrahydrofuran) yielded *Z* olefins of 94–96% geometrical purity. This purity was estimated from the intensity of the 970 cm^{-1} infrared band due to *E* olefin, with *E*-9-octadecene as the standard.

RESULTS AND DISCUSSION

Typically, the crude surface rinses and tissue extracts from the females and the hydrocarbon fractions obtained therefrom by Florisil column

chromatography were only weakly active. However, once the saturated compounds were separated from the hydrocarbon fraction, the residual unsaturates from both males and females were active. Argentation thin-layer chromatography of the unsaturates indicated that only monoolefins were present and that their configuration was *Z*, i.e., no *E* isomer was detected. A sample of muscalure, (*Z*)-9-tricosene, containing about 3–4% *E* isomer was applied on the same thin layer plate in an amount identical to that of the unknown monoolefins, and the *E* isomer was readily observed.

Activity resided in the trappings of two of the three major peaks. Trappings of the third peak, although inactive, were also examined since they might not be active unless combined with the active fractions. However, recombinations of the inactive fraction with the active materials did not improve their activity.

Mass spectrometry of the three materials revealed that the parent masses were 350, 378, and 406, corresponding to $C_{25}H_{50}$, $C_{27}H_{54}$, and $C_{29}H_{58}$, respectively; the last two fractions were active. None of the spectra indicated the presence of branching. Kovat's retention indices were also consistent with an absence of branching. (*Z*)-9-tricosene, employed as the standard alkene, gave a retention index of 2270.

Reductive ozonolysis and examination of the resulting aldehydes showed that the proportions of the various C_{27} and C_{29} monoolefins in the male and female extracts were roughly similar, and those isomers in which the olefinic linkage was centrally located predominated (Table 1). Only in the C_{25} monoolefin fraction from females were isomers found in which the double bonds were far removed from the center of the chain. This double-

TABLE 1. COMPOSITION OF C_{25}, C_{27}, AND C_{29} MONOALKENES IN TISSUE EXTRACTS OF 6-DAY-OLD MALE AND FEMALE FACE FLIES

Position of double bond	Percent of total				
	C_{25} alkenes in	C_{27} alkenes in		C_{29} alkenes in	
	♀	♀	♂	♀	♂
6	19	—	—	—	—
8	8	—	—	—	—
10	32	14	11	16	18
11	19	9	10	5	7
12	22	20	36	10	21
13	—	57	43	35	28
14	—	—	—	33	29

TABLE 2. ACTIVITY QUOTIENTS OF SYNTHETIC COMPOUNDS[a]

Compound	100 μg	200 μg	300 μg
(*Z*)-10-heptacosene	0.09	0.16	0.04
(*Z*)-11-heptacosene	0.03	0.06	0.11
(*Z*)-12-heptacosene	0.26	0.25	0.22
(*Z*)-13-heptacosene	0.25	0.56	0.45
(*Z*)-10-nonacosene	0.00	0.01	0.00
(*Z*)-11-nonacosene	0.01	0.05	0.21
(*Z*)-12-nonacosene	0.10	0.14	0.16
(*Z*)-13-nonacosene	0.46	0.46	0.74
(*Z*)-14-nonacosene	0.75	0.96	0.94

[a] Activity quotient is defined in the text. The synthetics contain 4–6% *E* isomer.

bond distribution was manifest in the asymmetry of the C_{25} peak in the original gas chromatogram of the unsaturates. The C_{25} peak and the C_{25} synthetics that we prepared and tested showed no activity either alone or in combination with the trapped C_{27}, C_{29}, and various active C_{27} and C_{29} synthetics. The C_{25} peak from the male extracts was not investigated; it was symmetrical and presumably contained a roughly centrosymmetric spectrum of alkenes similar to those from the C_{27} and C_{29} peaks of both sexes.

The activity quotients of these synthetics alone and in mixtures that reproduced the chemical composition found in the male and female face flies are given in Table 2. Activity increased markedly as the double bond moved toward the center of the chain. Admixtures of the male and female olefinic fractions with the synthetics did not produce materials of significantly enhanced activity. The most active compounds (in order of activity) were (*Z*)-14-nonacosene, (*Z*)-13-nonacosene, and (*Z*)-13-heptacosene. Since the synthetics had ca. 5% *E* isomer and the tlc investigation described earlier had denied the presence of that isomer in the extracts, a sample of (*Z*)-14-nonacosene was freed of its *E* isomer by column chromatography on $AgNO_3$ impregnated Florisil; but the activity of the pure *Z* isomer was not materially greater than that of the original synthetic. A sample of pure (*E*)-13-heptacosene was prepared by repeated recrystallization of a sample of heptacosene synthesized by the same Wittig reaction, but in a solvent (toluene) in which a higher proportion (ca. 60%) of *E* isomer is obtained. This compound, in contrast to the *Z* isomer, was devoid of activity.

Thus, at this time we were confronted with two enigmatic results: the activities of our fractions and our synthetics were materially less than that

TABLE 3. PERCENTAGES OF C_{25}, C_{27}, AND C_{29} MONOOLEFINS AND SATURATES IN CUTICULAR RINSES OF MALE AND FEMALE FACE FLIES OF DIFFERENT AGES

Age (days)	C_{25}		C_{27}		C_{29}	
	Olefin	Saturate	Olefin	Saturate	Olefin	Saturate
0 (♂)	3	19	12	22	34	9
1	9	20	25	13	28	4
3	23	23	25	17	9	4
5	19	27	17	25	6	6
0 (♀)	3	20	15	20	34	8
1	12	24	27	12	22	3
2	18	29	29	10	13	1
3	16	32	29	11	16	2
5	9	30	24	14	20	2

of a live female face fly, and the male, although unattractive, nevertheless secreted the same active olefins in proportions very similar to that of the female. We therefore examined the total hydrocarbon content of males and females as a function of age and found several key differences. The extracts of newly emerged adults were low in hydrocarbon content relative to those from older insects. Males and females less than 24-hr-old had an average of 10.6 and 11.7 μg of hydrocarbon, respectively. The saturates made up 50% of the male and 48% of the newly-emerged female hydrocarbons. Mature 5-day-old males had an average of 20.4 μg of hydrocarbons with 58% satura-

TABLE 4. EFFECTS OF ADDED SATURATED HYDROCARBON ON ACTIVITY OF ACTIVE SYNTHETIC ALKENES

Compound(s)	Amount (μg)	Activity quotient[a]
(*Z*)-13-heptacosene	200	.41
	300	.55
(*Z*)-13-heptacosene + heptacosane (1 :1)	400	.22
	600	.34
(*Z*)-14-nonacosene	200	1.55
	300	1.62
(*Z*)-14-nonacosene + nonacosane (1 :1)	400	.66
	600	.89

[a] Obtained with 6–9-day-old flies. Total of twenty 5-min observations for each material.

TABLE 5. ACTIVITY OF (*Z*)-14-NONACOSENE APPLIED TO NEWLY EMERGED AND 5-DAY-OLD FLIES

Sex of fly	Age	Amount applied (μg)	Activity quotient[a]
♀	0	300	0.41
♂	0	300	0.70
♀	5	300	2.19
♂	5	300	1.96

[a] Five 5-min observations for each treatment.

ted, while the females had 28.1 μg of hydrocarbons with 46% saturated. The ratios of the unsaturated to saturated C_{27} and C_{29} hydrocarbons increase for the female during maturation (Table 3). However, the proportion of saturated to unsaturated C_{27} and C_{29} in males remains high. This finding suggested that the higher proportion of saturates may aid the males in differentiating the sexes. Indeed, addition of heptacosane or nonacosane to the active alkenes materially reduced their activity (Table 4). Furthermore, when the total C_{27} and C_{29} hydrocarbon fraction of each sex was reconstituted with our synthetics, the female mixture was active; the male mixture was not.

The activity of (*Z*)-14-nonacosene placed on male and female face flies of different ages is reported in Table 5. If the male fly carried inhibitors and a visual factor was operative, then perhaps a newly emerged fly might better serve as a support for test materials. Curiously, newly emerged flies were poor supports for the active alkenes, and even dose levels of 300 μg produced activity quotients less than unity.

CONCLUSION

Unlike the sex pheromones of Lepidoptera, which are highly active and attract males to females from a distance, the pheromone of the female face fly is only weakly active, and it stimulates only proximate males to exhibit mating behavior. The most active components identified in the active extract of the insect are (in order of their activity) (*Z*)-14-nonacosene, (*Z*)-13-nonacosene, and (*Z*)-13-heptacosene. The activity of these materials is attenuated by the presence of the saturated analogs, heptacosane and nonacosane. Both males and females have these same saturates and unsaturates, but the higher proportion of saturates and lower proportion of active unsaturates in the male may account for the ability of male face flies to distinguish females from males.

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ISOLATION, IDENTIFICATION, AND SYNTHESIS OF THE SEX PHEROMONE OF THE TOBACCO BUDWORM[1,2]

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Abstract—The sex attractant of adult tobacco budworms, *Heliothis virescens* (F.) was isolated from ether washes of "calling" females and verified as active by field cage bioassays. The components of the pheromone were identified as (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal by spectroscopic and microdegradative methods. The two components are inactive when tested separately, but when they are mixed in the ratio in which they occur in female washes (16:1, respectively), the synthesized mixture is equivalent to the natural one in attracting males in large cages. In field tests, 53 μg of synthesized mixture was competitive with four live females.

Key Words—sex pheromone, *Heliothis virescens* (F.), (*Z*)-11-hexadecenal, (*Z*)-9-tetradecenal.

INTRODUCTION

The tobacco budworm, *Heliothis virescens* (F.), is one of the most important pest insects in the United States. It attacks a wide variety of crops throughout

[1] Mention of a pesticide or a commercial or proprietary product in this paper does not constitute a recommendation or an endorsement of that product by the U.S. Department of Agriculture.

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the country and is highly resistant to most available insecticides. Efforts to identify the sex pheromone of this species were begun as early as 1963 when Gaston and Shorey (1964) demonstrated that *H. virescens* males responded to a crude ether extract of female abdominal tips in the laboratory. At about the same time, Gentry et al. (1964) reported that males were caught in a greenhouse in traps baited with ether or hexane extracts of females. However, subsequently, Berger et al. (1965) were not able to obtain activity from crude extracts of female abdominal tips, but males did respond to a component of the crude extract eluting from a gas chromatograph. Later, Shorey and Gaston (1967) reported equal responsiveness in laboratory assays of *H. virescens* males to a crude methylene chloride extract of female abdominal tips and to the GC-purified pheromone obtained from the same crude extract.

Jacobson et al. (1972) reported that a mixture of (*Z*)-9-tetradecen-1-ol formate and (*E*)-9-tetradecen-1-ol was a sex attractant for *H. virescens* males; however, in subsequent tests in large field cages, these compounds were not significantly attractive to males compared with crude ether washes of attractive females (Hendricks, unpublished data).

In our initial studies (Mitchell et al., 1974), we obtained a highly active crude material by washing "calling" *H. virescens* females with ether. This female wash was competitive with live females in attracting native *H. virescens* males in field tests and also attracted large numbers of laboratory-reared males in field-cage bioassays (Hendricks and Tumlinson, 1974). Recently, from laboratory olfactometer and electroantennogram assays, Roelofs et al. (1974) reported that the sex pheromone of *H. virescens* is a mixture of (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal. However, their field tests were inconclusive. We here confirm and extend their findings as a result of our concurrent isolation and identification of this pheromone and our field-cage bioassays and field tests of the synthesized mixture vs. live *H. virescens* females.

METHODS AND MATERIALS

Extraction and Bioassay

All tobacco budworm moths used in the investigation were reared at the Southwestern Cotton Insects Research Laboratory, Brownsville, Texas (Raulston and Lingren, 1972). Virgin female adults that were exhibiting calling behavior were washed with ether, and the ether wash was concentrated by distillation through a dufton column as described by Mitchell et al. (1974). Each step in the subsequent purification and identification was monitored with the large cage bioassay described by Hendricks and Tumlin-

son (1974). The concentrated crude female wash was used as a standard for comparison in each test since it had previously been shown to be competitive with live females in attracting native males in the field (Mitchell et al., 1974). A sample of each fraction (about 50 female equivalents [FE] in 0.5 ml solvent) was poured onto a piece of filter paper that was suspended in the middle of a cylindrical electric trap (Mitchell et al., 1972). Seven of these traps were placed in a large screen wire outdoor cage ($12 \times 17 \times 3$ m) containing from 800 to several thousand laboratory reared males. The traps were baited at night to coincide with the activity period of the insects (Mitchell, et al., 1974).

For the field tests, 18 saucer traps (Hendricks, et al., 1973) were placed about 50 ft apart in a straight line across a cotton field near Brownsville, Texas the nights of May 13 and 14, 1974. The direction and location of the line of traps was changed each night. Three replicates, internally randomized, were used both nights. The traps were baited at 2400 hr CDT with (1) 4 virgin females, (2) 20 FE of crude whole-body female wash, (3) a mixture of 50 μg of (*Z*)-11-hexadecenal and 3.13 μg of (*Z*)-9-tetradecenal, (4) 0.5 μg of (*Z*)-11-hexadecenal and 0.03 μg of (*Z*)-9-tetradecenal, (5) a mixture of 50 μg of (*Z*)-11-hexadecenal, 3.13 μg of (*Z*)-9-tetradecenal, and 0.13 μg of (*E*)-9-tetradecenal, and (6) 0.5 μl hexane. The chemical baits were placed on filter paper wicks. Trap catches were tabulated daily.

Isolation

The concentrated crude female wash was first injected onto a 1.27-cm (ID) glass liquid chromatographic column packed to a height of about 93 cm with Poragel® 60 A, 37–75 μm (Waters Associates). The column was eluted with hexane at a flow rate of 300 ml/hr and a column inlet pressure of about 40 lb/in^2. About 2500 FE of the crude wash was injected at one time on the Poragel column, and thirty 10-ml fractions were collected. Field-cage bioassays (3 replicates) were made with about 50 FE (0.2 ml) of each fraction.

All the micropreparative and analytical gas-liquid chromatography (GLC) was performed in a Packard Model 804 and a Varian Model 1400 gas chromatograph, both equipped with flame ionization detectors. Stainless steel columns were used, and the effluent from the packed columns was split so about 97% was collected in cooled (dry ice) glass capillaries and 3% was routed to the detector.

The active fractions from the Poragel column were concentrated by distillation at atmospheric pressure through an 8-cm vigreux column and further purified by GLC on a 2-m $\times$ 2.3-mm (ID) column packed with 5% OV-101 on 80/100 mesh Chromosorb G-HP; the column temperature was held at 80°C for 5 min and then programmed to 210°C at 6°/min; the He flow was 20 cc/min. None of the individual fractions collected from this

column were active, but activity was regained when fraction 2, collected 9.2 to 11 min after injection and fraction 4, collected 14–15.5 min after injection, were combined.

Fraction 2 was further purified by GLC on a 7.3-m × 2.3-mm (ID) stainless steel column packed with 5% Hi Eff 1BP (DEGS) on 80/100 mesh Chromosorb G-HP and operated isothermally at 190°C; the He flow was 20 cc/min. A component (fraction 2d) eluting from this column between 24 and 25.6 min was active when recombined with fraction 4 from the OV-101 column. Fraction 2d produced only one peak that was at least 99% pure when rechromatographed on the OV-101 and DEGS columns and when analyzed on a Dexsil capillary column (Dexsil 300 GC, 60 m × 0.762 mm [0.03 in] [ID], column temp 175°C, He carrier gas velocity 22.5 cm/sec). Fraction 2d contained a 3% impurity appearing as a shoulder on the front of the peak when analyzed on a Carbowax capillary column (Carbowax 20M, 60 m × 0.762 mm [0.03 in] [ID], column temp 190°C, He carrier gas velocity 25 cm/sec).

Fraction 4 from the OV-101 column was further purified by GLC on a 2-m × 2.3-mm (ID) stainless steel column packed with 5% Carbowax 20 M on 80/100 mesh Chromosorb G-HP and operated isothermally at 180°C. Fraction 4b, which eluted between 10.6 and 12 min after injection, was active when recombined with fraction 2d. Fraction 4b, analyzed by GLC on all 5 columns, was determined to be at least 99% pure.

Identification

The 2 components of the pheromone were identified by infrared and chemical ionization mass spectrometry and by reduction and microozonolysis of the olefinic bonds. All mass spectra were obtained with a Finnigan Model 1015C chemical ionization mass spectrometer equipped with a gas chromatographic inlet. The total effluent of a 2-m × 2.3-mm column packed with 3% SE-30 on 100/120 mesh Varaport 30 was introduced directly into the ionization source. Methane was the GLC carrier gas and the reagent gas. Data acquisition and reduction were accomplished with a Systems Industries Disc System 150 computer interfaced to the mass spectrometer.

The infrared spectra were obtained with a Perkin-Elmer Model 467 infrared spectrometer equipped with a Barnes-Engineering beam condenser and ultramicro cavity cells (2 μl volume). Samples were dissolved in CCl_4. About 2 μg of compound 2d was reduced with hydrogen and neutral palladium catalyst in the inlet of the gas chromatograph equipped with the 2-m × 2.3-mm Carbowax 20M column in the manner of Beroza and Sarmiento (1966). Microozonolysis of the two components of the pheromone was carried out in CS_2 at −78°C, and the ozonide was reduced with triphenyl-

phosphine in the manner of Beroza and Bierl (1966, 1967). The ozonolysis products were chromatographed on the Carbowax 20M GLC column, and retention times were compared to standard aldehydes and to 1,10-decanedial obtained by ozonolysis of synthetic (*E,Z*)-3,13-octadecadien-1-ol acetate, the pheromone of the lesser peachtree borer, *Synanthedon pictipes* (Grote and Robinson), (Tumlinson et al., 1974). Additionally, the ozonolysis products were collected and further analyzed by chemical ionization mass spectrometry.

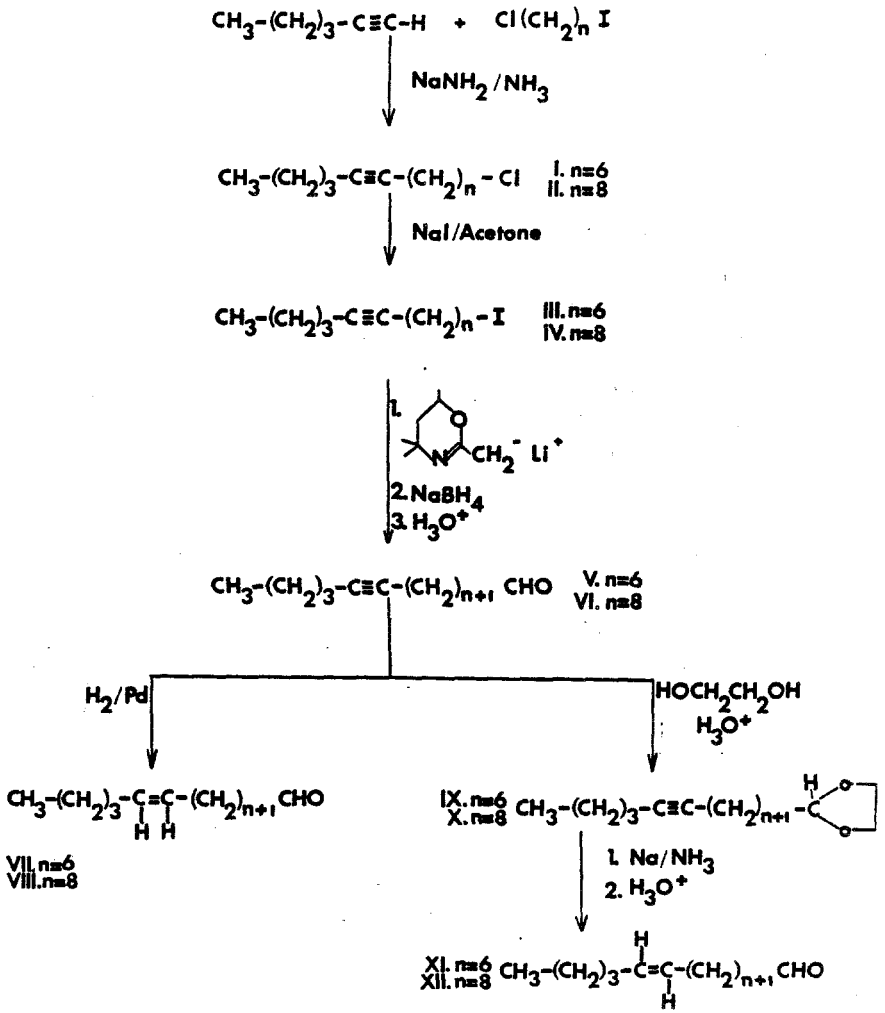

FIG. 1. Synthesis routes for (*Z*)- and (*E*)-11-hexadecenal and (*Z*)- and (*E*)-9-tetradecenal.

Synthesis

The *Z*- and *E*-isomers of 9-tetradecenal and 11-hexadecenal were synthesized by the same general procedure (Figure 1). Hexamethylene chloroiodide (1-chloro-6-iodiohexane) (Columbia Organic Chemicals) and octamethylene chloroiodide (1-chloro-8-iodooctane) were reacted with the sodium salt of 1-hexyne in liquid ammonia by the procedure of Ahmad et al. (1948) to produce 1-chloro-7-dodecyne, I, and 1-chloro-9-tetradecyne, II, respectively, in 80–90% yields. The chlorides, I and II, were converted to the iodides, III and IV, respectively, by refluxing with an excess of sodium iodide in acetone for 24 hr (90–95% yields). The iodides, III and IV, were then reacted with the lithium salt of 5,6-dihydro-2,4,4,6-tetramethyl-1,3(4H)-oxazine (Columbia Organic Chemicals); the crude intermediate alkylated oxazines were reduced with sodium borohydride; and the tetrahydrooxazines were cleaved to the aldehydes, V and VI, with aqueous acid (Meyers et al., 1973). The overall yield for these three steps was only about 35%. The acetylenic aldehydes, V and VI, were hydrogenated over palladium on barium sulfate or palladium on calcium carbonate (Lindlar) catalyst in cyclohexane with quinoline (224 μl/g of catalyst) added. (*Z*)-9-tetradecenal, VII, and (*Z*)-11-hexadecenal, VIII, were produced in 80% yield and greater than 90% isomeric purity with both catalysts.

(*E*)-9-tetradecenal, XI, and (*E*)-11-hexadecenal, XII, were prepared by converting the acetylenic aldehydes, V and VI, into the acetals, IX and X (83% yield), with ethylene glycol and a catalytic amount of *p*-toluenesulfonic acid in benzene (Heywood and Phillips, 1960). The water produced in the reaction was removed by azeotropic distillation. Sodium and liquid ammonia reduction (Schwarz and Waters, 1972) and subsequent hydrolysis with aqueous acidic tetrahydrofuran produced the *trans* aldehydes, XI and XII, in 50% yield.

The synthesized compounds were distilled and further purified by high pressure liquid chromatography on a 1.27-cm (OD) × 50-cm stainless steel column packed with $AgNO_3$ treated silica gel (Adsorbosil-CABN, Applied Science Laboratories). The column was eluted with benzene at a flow rate of 4.0 ml/min and an inlet pressure of 2400 lb/in^2. A center cut was collected from the major peak in each case, rechromatographed on the same column, and then further purified by preparative GLC on an SE-30 column.

RESULTS AND DISCUSSION

The crude ether wash of *H. virescens* females used as a standard for the bioassays throughout this investigation was consistently active in the cage tests at the 10–20 FE level. The number of males captured in the field cages

in one electric trap in one night with 20 FE of the crude female wash ranged from 50 to 3,000, depending on the male population in the cage. Usually, the population was maintained at such a level that the standard baited traps captured between 100 and 500 males, and empty traps or solvent blanks captured 0 to 30 males.

The crude female wash was chromatographed on Poragel to remove the high molecular weight compounds. The active fraction was eluted from this column in a narrow band with a retention volume of 100–120 ml. Fifty FE of this 20-ml fraction was as attractive as 20 FE of the crude wash. Activity could not be increased by recombination of all the fractions from this column. Since Poragel separates by molecular size and not by adsorption, it is unlikely that a component of the pheromone was lost on the column.

GLC of the active Poragel fraction on the OV-101 column yielded two components, fractions 2 and 4, that retained all the activity of the Poragel fraction when they were combined. However, individually they were completely inactive.

Further GLC purification of fractions 2 and 4 on DEGS and Carbowax 20M columns, respectively, yielded products that gave single peaks when analyzed on the three packed columns and the two capillary columns. However, on the Carbowax 20M capillary, component 2d appeared to have a slight shoulder on the front, comprising about 3% of the total peak area. The location of this shoulder is consistent with the retention characteristics of the *trans* isomer of this component as we later determined by synthesis. About 15 μg of compound 2d and 240 μg of 4b were obtained from 160,000 female tobacco budworm moths.

GLC-methane ionization mass spectrometry of the two pure components gave further evidence that each was a single compound. Mass spectra obtained at several different points on the front and back of each peak were identical. A molecular weight of 210 for compound 2d was established by peaks at *m/e* 209 (P−1), 211 (P+1), 239 (P+29), and 251 (P+41) in its mass spectrum (Figure 2). The peak at *m/e* 193 (P+1–18), which indicated a loss of H_2O, and the remaining paraffinic character of the spectrum suggested a straight chain aldehyde or alcohol since both lose H_2O in methane ionization mass spectrometry. The mass spectrum of compound 4b was almost identical to that of 2d, except that the P+1, P+29, P+41, and P+1−H_2O peaks were 28 mass units higher, evidence of a molecular weight of 238. Thus, compounds 2d and 4b were 14 and 16 carbon alcohols with two double bonds, respectively, or the corresponding aldehydes with one double bond.

The infrared spectrum of 4b showed a strong carbonyl absorption at 1735 cm^{-1} and no absorption in the 950–1000 cm^{-1} region, confirmation that this compound was a 16 carbon aldehyde and that the single olefinic bond was in the *cis* configuration. Since only about 15 μg of 2d was available

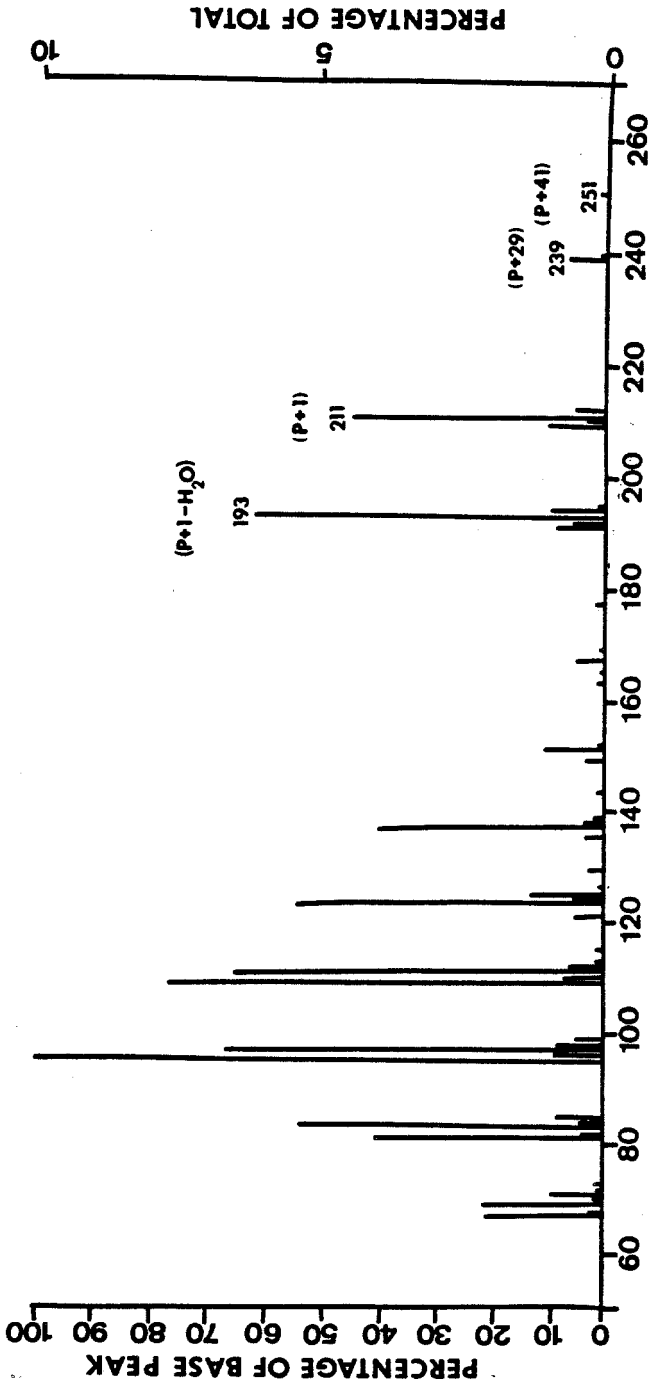

FIG. 2. Methane ionization mass spectrum of compound 2d [(Z)-9-tetradecenal].

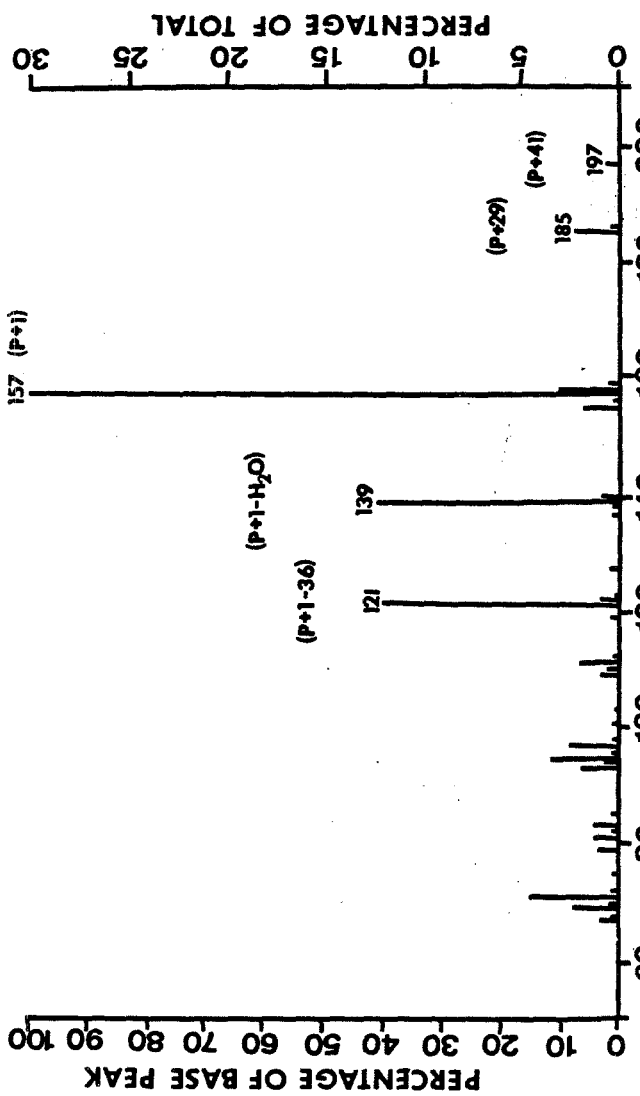

FIG. 3. Methane ionization mass spectrum of second peak (1,9-nonanedial) from ozonolysis of compound 2d.

for an infrared spectrum, the resulting spectrum was too weak to yield much information. However, a carbonyl band was present at 1735 cm^{-1}. The aldehydic structure of 2d was confirmed by hydrogenation over neutral palladium catalyst in the GLC injector port. The product had the same retention time on a Carbowax 20M column as tetradecanal.

Microozonolysis of 2d and 4b yielded two products in each case. The GLC retention on the 2 m Carbowax 20M column and the mass spectrum of the first peak from both ozonolyses was identical to that of valeraldehyde. The second product from 2d was consistent with 1,9-nonanedial in retention time on Carbowax 20M and mass spectrum (Figure 3). Similarly, the second product from 4b was demonstrated to be 1,11-undecanedial. Thus, 2d was either (*Z*)- or (*E*)-9-tetradecenal and 4b was (*Z*)-11-hexadecenal.

Both isomers of the 2 pheromone components were synthesized by the route shown in Figure 3. The synthetic compounds were purified by distillation, liquid chromatography on $AgNO_3$ impregnated silica gel, and GLC on a preparative SE-30 column. All four isomers were obtained in at least 99% purity in this way.

Synthesized (*Z*)-11-hexadecenal was identical in GLC retention times and infrared and mass spectra to the naturally derived compound 4b. Similarly (*Z*)-9-tetradecenal was identical in mass spectrum and GLC retention times to compound 2d. Compound 2d and (*Z*)-9-tetradecenal had identical retention times on the Carbowax 20M capillary, which separated the *Z*- and *E*-isomers of both aldehydes. The retention time of the 3% impurity

TABLE 1. MEAN CAPTURES OF NATIVE MALE TOBACCO BUDWORM MOTHS IN FIELD TRAPS BAITED WITH SYNTHETIC PHEROMONE, CRUDE FEMALE WASH, OR VIRGIN FEMALES
(3 Replicates/test. Brownsville, Texas, May, 1974)

Bait	Concentration	$\bar{X}$ number males/trap[a] Test 1	Test 2
Virgin females (4)	—	34.3 a	13.7 a
Crude whole-body female wash	20 FE	0.7 b	1 c
Z-11-hdal[b]+Z-9-tdal[b]	50 μg+3.12 μg	24.3 a	6.3 b
Z-11-hdal+Z-9-tdal	0.5 μg+0.03 μg	0 b	0 c
Z-11-hdal+Z-9-tdal+E-9-tdal[b]	50 μg+3.12 μg+0.12 μg	24.3 a	5.3 b
Hexane control	0.5 ml	0 b	0 c

[a] Means in the same test followed by the same letter are homogeneous ($P = 0.05$, Duncan's multiple range test).
[b] Z-11-hdal = (*Z*)-11-hexadecenal; Z-9-tdal = (*Z*)-9-tetradecenal; E-9-tdal = (*E*)-9-tetradecenal.

in 2d, which only separated on the Carbowax 20M capillary, was consistent with (*E*)-9-tetradecenal.

For the cage bioassays, mixtures of synthesized (*Z*)-11-hexadecenal/(*Z*)-9-tetradecenal were tested in the following ratios: 100/1, 50/1, 20/1, 16/1, 10/1, and 5/1. The amount of (*Z*)-11-hexadecenal in each of these mixtures was 100 ng. Mixtures at all of the above ratios were also tested with (*Z*)-11-hexadecenal amounts of 200 ng and 500 ng. Additionally, these 2 compounds were tested separately and in mixtures with the (*E*)-isomers. The individual compounds were inactive alone, and mixtures containing (*Z*)-11-hexadecenal and (*E*)-9-tetradecenal, or (*E*)-hexadecenal and (*Z*)-9-tetradecenal were not attractive to males in cage tests. At any of the above concentrations, the mixtures of (*Z*)-11-hexadecenal/(*Z*)-9-tetradecenal were equally attractive in ratios ranging from 5/1 to 20/1. These synthetic mixtures were equivalent in attraction to the pure natural pheromones obtained from female washes when they were tested at about equal concentrations. The 100/1 and 50/1 mixtures were less active at all concentrations.

In the cage bioassay, the crude female wash (20 FE) and 500 ng of the 16/1 synthetic mixture were equivalent in attractiveness to males for 3 hr.

A mixture of synthesized (*Z*)-11-hexadecenal (50 μg) and (*Z*)-9-tetradecenal (3.13 μg) (the same ratio that these compounds occur in female washes, 16/1) was as attractive to native males in the field as four live virgin females (Table 1). Furthermore, addition of about 3% (*E*)-9-tetradecenal to the (*Z*) isomer did not change the attractiveness of this mixture. Thus, the appearance of what seemed to be 3% *trans* isomer in naturally derived compound 2d cannot be explained. The individual (*Z*) and (*E*) isomers of both compounds were completely inactive in field and cage tests at concentrations of 50 ng to 50 μg.

We conclude that the sex pheromone produced by *H. virescens* females consists of two synergistic components, (*Z*)-9-tetradecenal and (*Z*)-11-hexadecenal. However, 20 FE of the crude ether wash of females attracts more males in cage tests than either 20 FE of the pure natural pheromone or 100 ng of the synthesized mixture, so other components as yet unidentified, may also be involved. Alternatively, the lesser activity of the pure materials may be the result of losses during purification or lack of formulation. However, 53 μg of the synthetic mixture were competitive with live females in trapping native males in the field. Thus, these two compounds together are true sex attractants. When they are properly formulated, we should find them useful for survey and for control of this important pest.

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SEX PHEROMONE OF THE ORANGE TORTRIX MOTH, *Argyrotaenia citrana* (LEPIDOPTERA: TORTRICIDAE)

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Abstract—Female orange tortrix, *Argyrotaenia citrana* (Fernald), abdominal tip extracts, and effluvia were analyzed, and two pheromone components were identified as *cis*-11-tetradecenal (c11-14:ALD) and *cis*-11-tetradecenyl acetate (c11-14:Ac). Female extracts contained such very low quantities of c11-14:ALD relative to c11-14:Ac that the aldehyde was frequently undetectable, whereas female effluvia contained these compounds in a ratio of ca. 15:1. Field attractancy tests showed that traps baited with c11-14:ALD were attractive, whereas c11-14:Ac possessed no intrinsic attractiveness. Additionally, traps containing c11-14:ALD in a range of ratios to the component c11-14:Ac (5:1 to 1:10) are more competitive for *A. citrana* males than are live female traps. The females produce pure *cis* isomers (<0.5% *trans*), and addition of >5% t11-14:ALD (the geometric isomer of the primary component) reduces attractancy, whereas addition of t11-14:Ac (the geometric isomer of the secondary component) apparently does not affect attractancy significantly unless c11-14:Ac is absent.

Key Words—sex pheromone, sex attractant, *Argyrotaenia citrana*, orange tortrix moth.

INTRODUCTION

The orange tortrix moth, *Argyrotaenia citrana* (Fernald), is a major pest of grapes and other deciduous fruit in the western United States. Infestations could be monitored and possibly controlled by sex pheromone if the phero-

mone components were available. This paper reports the identification of two pheromone components and the results of field attractancy tests.

METHODS AND MATERIALS

Moths were from a laboratory culture or were field collected as larvae in California. After segregating by sex and mailing to Geneva, New York, pupae were placed on a 16:8 light:dark regime at 24°C. Abdominal tips of emerged females were excised after 4–5 hours of scotophase and were extracted with methylene chloride.

Airborne pheromones were collected by a procedure similar to the method described by Byrne et al. (1975). Air was drawn over 20–50 females (1–4 days old) held in a glass tube (9 cm OD × 1.2 m), stoppered at both ends with glass wool, and into a glass column (2.2 cm OD × 45 cm) packed with either Polypak or Porapak Q to a length of ca. 20 cm. These packing materials had been preconditioned by continuous extraction with Skelly B for at least 24 hrs, followed by drying in an oven at 100°C for at least 24 hours. Air flow rates were approximately 2 liter/min. Moths were maintained on a 16:8 light:dark regime during collections. At a temperature of 16°C, females were observed calling (protruding the pheromone-producing gland), even several hours prior to scotophase, whereas at 24–26°C only a few females were observed in the calling position. In addition, larger amounts of pheromones were collected at the lower temperature.

Chemical analyses of abdominal tip extracts were carried out essentially as described elsewhere (Roelofs et al., 1971*b*; Hill et al., 1974). All solvents were redistilled prior to use. Glass GLC columns were packed with 3% OV-1 (methyl silicone, 2 m × 4 mm) on 100–120 mesh Gas-Chrom Q, 3% PDEAS (phenyldiethanolamine succinate, 4 m × 2 mm) on Chromosorb W-AW-DMCS or 3% CHDMS (cyclohexanedimethanol succinate, 2 m × 4 mm) on 100–120 mesh Gas-Chrom Q; hydrogen flame ionization detection was used.

By preparative TLC using benzene elution on $AgNO_3$-impregnated silica gel-G plates, *cis*-11-tetradecenyl acetate (c11-14:Ac) (Farchan Chemical Company) was freed of *trans*-11-tetradecenyl acetate (t11-14:Ac). By saponification with NaOH-ethanol followed by oxidation with chromium trioxide-pyridine in methylene chloride (Ratcliffe and Rodehorst, 1970), *cis*-11-tetradecenal (c11-14:ALD) was prepared from the acetate. If there was *cis*-11-tetradecen-1-ol (c11-14:OH) remaining in the product, it was removed by column chromatography on Florisil using benzene.

Chemicals were placed either on rubber septa (5 × 9 mm rubber stoppers, sleeve-type, Arthur H. Thomas Company) or in polyethylene caps (OS-6 natural polyethylene closures, Scientific Products).

Electroantennograms (EAG) were carried out as previously described (Roelofs and Comeau, 1971*a*) and were used for assaying GLC collections of female tip extracts (Roelofs et al., 1971*a*) and for determining normalized response profiles for several series of long-chain acetates, alcohols, and aldehydes (Hill et al., 1974).

Field tests were conducted at the Paul Masson Vineyards, Soledad, California, on Emerald Riesling grapes using Pherocon® IC traps (Zoecon Corporation, Palo Alto, California) placed at a height of 1 m and 18 m apart in a randomized complete block design. Treatments were replicated 4 or 5 times. Data were transformed to $\sqrt{X+0.5}$ and submitted to an analysis of variance. Throughout, means of males/trap followed by the same letter are not significantly different at the 5% level as determined by Duncan's new multiple range test (Steel and Torrie, 1960).

RESULTS

EAG Responses of Standards

Normalized EAG responses of male *A. citrana* antennae to a series of 14-carbon chain acetates, alcohols, and aldehydes (Figure 1) show that the largest response is to c11-14:ALD, and that the next largest responses are to the c11-14:Ac in the 14-carbon acetate series and to c11-14:OH in the 14-carbon alcohol series. The high activity of aldehyde was not found with some related tortricids utilizing c11-14:Ac as the primary pheromone component (Roelofs and Cardé, 1974); this indicated the possible role of an aldehyde, such as c11-14:ALD, in the pheromone system of *A. citrana*. Good antennal activity in the other *cis*-11 compounds does not necessarily imply that they are possible pheromone components, since this activity could result from structural similarities to the components.

Pheromone Preparation

Female tip extract (ca. 25 FE) was injected onto the OV-1 column (175°C), the effluent was collected in 1-min fractions, and the fractions were assayed by EAG. EAG activity was located at 5–6 min (component A) and at 11–12 min (component B), corresponding approximately to the retention times of tetracecanal (14:ALD), 5.2 min, and tetradecyl acetate (14:Ac), 11.3 min. A GLC trace from OV-1 of the crude extract showed two peaks attributable to components A and B in the approximate ratio of 1:100.

Examination of materials recovered from airborne collections of calling female effluvia showed the same two EAG active areas on OV-1 (173°C). Component A eluted at 3–4 min (14:ALD at 3.7 min) and B at 7.5–8.5 min

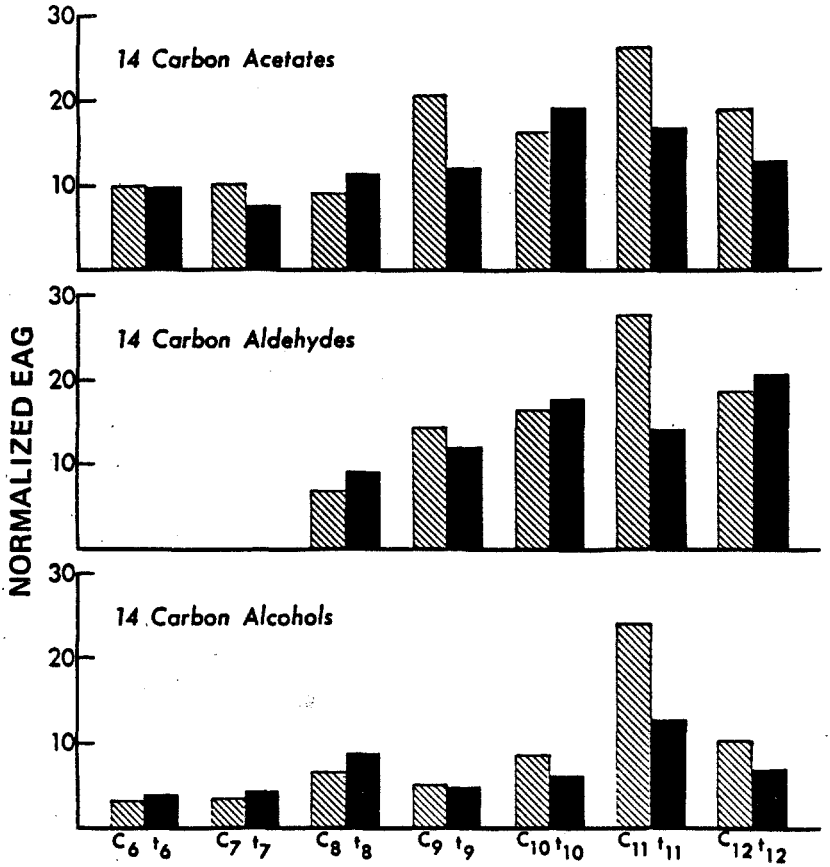

FIG. 1. Normalized EAG responses of male *A. citrana* to 14-carbon acetates, aldehydes, and alcohols using *cis*-7-tetradecenyl acetate as the standard. Normalization consisted of multiplying the average value of the EAG determinations (amplitude in mV) for each compound by 10 and dividing by the value of the standard (determined just prior to the test compound). The abscissa gives the geometric configuration and position of unsaturation for each monounsaturated standard used.

(14:Ac at 8.0 min). The airborne collection possessed substantially more of component A relative to B (15:1) than did female tip extracts.

Characterization of Component A

Component A was considerably more abundant in the airborne collected materials, and so these were used as a source for characterization of A. Component A was collected from OV-1 (170°C) at 3–4 min, and recollected

from CHDMS (160°C) at 4–5 min; the presence of EAG activity in these fractions was confirmed after each of these collections. On OV-1 (160°C) and CHDMS (160°C), the collected material showed one peak with retention times identical to those of c11-14:ALD (10.2 and 6.85 min, respectively) on these two columns. A sample collected from OV-1 was injected onto the PDEAS column (173° C) and showed EAG activity at 7.5–8.5 min and a peak at 7.8 min; c11-14:ALD and t11-14:ALD had retention times of 7.85 and 7.4 min, respectively. No peak was seen at 7.4 min, indicating the absence (<0.5%) of t11-14:ALD in component A. The EAG active sample of A collected from PDEAS was ozonized to produce a material with retention times on OV-1 (150°C) and CHDMS (160°C) of 8.3 and 14.55 min, respectively; those for undecanedial produced by ozonolysis of c11-14:ALD were 8.3 and 14.5 min, respectively. This reaction confirmed the assignment of the double bond in the 11-position and further supported an aldehyde functionality of component A.

These data in conjunction with the EAG responses to standards support the characterization of component A as c11–14:ALD.

Characterization of Component B

Component B isolated from female tip extracts was used for identification of this material. B was recovered after collection from OV-1 and recollected from CHDMS (175°C). EAG activity was found at 4–5 min, corresponding to the retention of 14:Ac (4.2 min). Ozonolysis of this OV-1 and CHDMS collected material yielded a product with retentions on OV-1 (175°C) and CHDMS (175°C) of 9.25 and 7.8 min, respectively, similar to those of 11-acetoxyundecanal (9.2 and 7.85 min, respectively). This reaction showed that the double bond was in the 11 position.

Component B was collected from OV-1 (174°C) at 7–8.25 min, found to be EAG active, recovered, and saponified using NaOH–ethanol. Saponified B was collected from OV-1 (174°C) and EAG activity was found at 4–5 min, but was absent at 7–8 min; c11–14:OH had a retention time of 4.6 min. Saponified B showed a peak on PDEAS (174°C) at 9.75 min; c11-14:OH and t11-14:OH were at 9.8 and 9.2 min, respectively, supporting a *cis* assignment for this component. Saponified B was recovered, acetylated with acetyl chloride, and the product collected from OV-1 (174°C). EAG activity was restored at 7–8.5 min and was absent at 4–5 min. These series of reactions show that the compound is an acetate. Reacetylated B had a retention on PDEAS (174°C) of 9.45 min; those of c11-14:Ac and t11-14:Ac were 9.45 and 8.9 min, respectively, again supporting a *cis* structure for component B. This assignment was further confirmed by collection of untreated component B from OV-1 which was injected onto PDEAS (155°C). It had the same re-

tention time as c11-14:Ac (10.45 min), whereas no peak was visible at the retention time of t11-14:Ac (9.9 min), indicating the absence (<0.5%) of the *trans* isomer. The same observation was made for B recovered from airborne collection of female effluria. The mass spectra of component B and c11-14:Ac are identical.

These data in conjunction with the EAG responses to standards support the characterization of B as c11-14:Ac.

Field Attractiveness

Initial evaluation experiments conducted April 12–18, 1973 utilizing rubber septa dispensers indicated that 100 μg c11-14:ALD is attractive to *A. citrana* males (mean males/trap, $\bar{X}$ = 102.8a) and that the addition of c11-14:Ac to the trap can increase male catches (30 μg c11-14:ALD + 100 μg c11-14:Ac, $\bar{X}$ = 153.4b). By itself c11-14:Ac at 100 μg was essentially unattractive ($\bar{X}$ = 1.6c). More extensive trials conducted from May 16–17 with c11-14:ALD and c11-14:Ac combinations revealed that the ratio of these components was not especially critical to the level of attractancy. When compared to 100 μg c11-14:ALD on rubber septa dispensers ($\bar{X}$ = 27.4b), added amounts of 20–1000 μg c11-14:Ac (ratios of 5:1 to 1:10) produced means that ranged from 79.8a to 108.0 a. Two, 2-day-old females, attracted $\bar{X}$ = 36.8b males, and unbaited traps caught $\bar{X}$ = 0.2c males.

A similar experiment conducted June 4–6, 1973 utilizing polyethylene cap dispensers compared 2 mg of c11-14:ALD alone to 2 mg of c11-14:ALD plus 1–8 mg of c11-14:Ac. The dispersers with both components attracted means of 15.0ab and 30.0a males, whereas 2 mg c11-14:ALD alone attracted $\bar{X}$ = 2.0c males. Two females/trap caught $\bar{X}$ = 1.2c males and unbaited traps lured 0.0c males. In this same test rubber septa baited with 100 μg c11-14:ALD and 40 μg c11-14:Ac caught $\bar{X}$ = 6.0bc males suggesting that high dispenser loads are more effective.

Since both the naturally occurring c11-14:ALD and c11-14:Ac appeared to be isomerically pure (<0.5% *trans*), the possible effects on attraction of the *trans* isomers were investigated (Tables 1 and 2). The primary component alone (c11-14:ALD) was most effective with <0.5 to 2% *trans* isomer. Five percent or more t11-14:ALD appreciably diminished attractancy, whereas 20 and 50% t11-14:ALD essentially eliminated attractiveness. A preliminary test (Table 1) showed that addition of 10% t11-14:Ac to the c11-14:ALD and c11-14:Ac blend did not affect attractancy. More extensive evaluation (Table 2) utilizing <0.5 *trans* to 100% t11-14:Ac indicated that the synergistic effect of the acetates was not significantly affected by the presence of as much as 50% *trans* isomer, although 100% *trans* isomer significantly reduced the attractancy of the aldehyde.

TABLE 1. FIELD ATTRACTANCY OF *A. citrana* MALES TO THE PHEROMONE COMPONENTS CONTAINING VARIOUS AMOUNTS OF THE GEOMETRICAL ISOMERS
(Conducted November 19–21, 1973)
19–21, 1973)

Treatment on rubber septum	Mean/trap[a]
100 μg c11-14 :ALD	
(0.5% *trans*)	84.2d
(2% *trans*)	89.0d
(5% *trans*)	22.1f
(10% *trans*)	20.0f
(20% *trans*)	5.5g
(50% *trans*)	4.8g
(0.5% *trans*)+1 μg c11-14 :Ac (0.5% *trans*)	142.3c
(0.5% *trans*)+3 μg	214.7ab
(0.5% *trans*)+10 μg	200.3b
(0.5% *trans*)+30 μg	257.1a
(0.5% *trans*)+100 μg	260.2a
(0.5% *trans*)+30 μg c11–14 :Ac (10% *trans*)	270.7a
100 μg t11-14 :ALD	0.8g
2 (3-day-old) virgin females	58.8e
Unbaited	2.8g

[a] Means in the same column followed by the same letter are not significantly different at the 0.05 level.

TABLE 2. FIELD ATTRACTANCY OF *A. citrana* MALES TO THE PHEROMONE COMPONENTS WITH VARIOUS AMOUNTS OF t11-14 :Ac
(Conducted January 29–30, 1974)

Treatment on rubber septum	Mean/trap[a]
100 μg c11-14 :ALD (<0.5% *trans*)	5.0b
+30 μg c11-14 :Ac (0.5% *trans*)	23.8a
+30 μg c11-14 :Ac (2% *trans*)	23.8a
+30 μg c11-14 :Ac (5% *trans*)	26.8a
+30 μg c11-14 :Ac (10% *trans*)	26.2a
+30 μg c11-14 :Ac (30% *trans*)	27.0a
+30 μg c11-14 :Ac (50% *trans*)	15.6a
+30 μg c11-14 :Ac (100% *trans*)	0.8c
2 (2-day-old) virgin females	6.2b
Unbaited	0.4c

[a] Means in the same column followed by the same letter are not significantly different at the 0.05 level.

TABLE 3. FIELD ATTRACTANCY OF *A. citrana* MALES TO VARIOUS PHEROMONE BLENDS IN SEVERAL TYPES OF CARRIERS
(Conducted February 20–21, 1974, using compounds containing 2% *trans* isomer)

Treatment	Mean/trap[a]
Rubber septum	
0.1 mg c11-14:ALD+0.3 mg c11-14:Ac	50.8b
1 mg c11-14:ALD+0.3 mg c11-14:Ac	61.0b
1 mg c11-14:ALD+1 mg c11-14:Ac	105.8ab
3 mg C11-14:ALD+1 mg c11-14:Ac	110.8a
3 mg c11-14:ALD+3 mg c11-14:Ac	87.2ab
Polyethylene cap	
2 mg c11-14:ALD+0.5 mg c11-14:Ac	86.0ab
2 mg c11-14:ALD+1 mg c11-14:Ac	85.2ab
2 mg c11-14:ALD+2 mg c11-14:Ac	74.8ab
2 (2-day-old) virgin females	10.8c
Unbaited	2.2c

[a] Means in the same column followed by the same letter are not significantly different at the 0.05 level.

A final test (Table 3) of pheromone blends in rubber septa and in polyethylene dispersers showed that the rubber septum carrier containing ca. 1–3 mg c11-14:ALD and 1 mg c11-14:Ac was a highly effective attractant when contrasted with virgin female moths.

DISCUSSION

Most of the sex pheromone components characterized to date in the tortricine subfamily are 14-carbon chain compounds with a double bond in the 11 position (Roelofs and Cardé, 1974). The present identification of c11-14:ALD as the primary component for *A. citrana* provides the first report that this compound is a tortricid pheromone, although the geometric isomer, t11-14:ALD has been found in *Choristoneura fumiferana* (Clemens) (Weatherston et al., 1971). The secondary component, c11-14:Ac, found in *A. citrana* is commonly used as a pheromone component of tortricine species. In the genus *Argyrotaenia*, it has been found to be the primary component of *A. velutinana* (Walker) (Roelofs and Arn, 1968), along with t11-14:Ac (ca. 8%, Klun et al., 1973; Roelofs et al., 1975) and 12:Ac (ratios of 1:1 or higher) (Roelofs and Comeau, 1971*b*; Roelofs et al., 1975).

The secondary pheromone component role of c11-14:Ac with *A. citrana* appears to be quite similar to the role of t11-14:Ac with *Platynota*

ideausalis (Walker) which uses t11-14:OH as the primary pheromone component (Hill et al., 1974). In both cases, the ratios of the two components are not critical for their effect, and the inhibitory effect of the geometric isomer of the secondary component is considerably less than that of the geometric isomer of the primary component. With *P. ideausalis*, t11-14:Ac was about equally effective in ratios to t11-14:OH of approximately 3:5 to 8:5. The activity of the secondary component was only slightly reduced by addition of the geometrical isomer c11-14:Ac, whereas additions of c11-14:OH, the geometric isomer of the primary component, greatly reduced male attractancy.

Similarly, in the present case, c11-14:Ac is effective throughout a range of ratios (1:2 to 4:1) to the primary component for male *A. citrana* attractancy, and addition of t11-14:Ac, the geometric isomer of the secondary component is not nearly as inhibitory to male attractancy as addition of t11-14:ALD, the geometric isomer of the primary component.

Among the tortricine it appears that some species use mixtures of geometric isomers to create unique pheromonal blends (Hill and Roelofs, 1975; Klun et al., 1973; Persoons et al., 1974; Roelofs et al., 1974; Roelofs et al., 1975), but other species use positional isomers or functional group analogs as pheromone components (Roelofs and Cardé, 1974; Tamaki et al., 1971*a*, 1971*b*; Meijer et al., 1972; Minks et al., 1973; Hill et al., 1974). In *A. citrana*, as in *P. idaeusalis*, the functional group analog components are apparently isomerically pure.

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SEX PHEROMONE OF THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (LEPIDOPTERA: PYRALIDAE), IN NEW YORK

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Abstract—*trans*-11-Tetradecenyl acetate (96%) and *cis*-11-tetradecenyl acetate (4%) are pheromone components of the European corn borer, *Ostrinia nubilalis* (Hübner), from New York. This isomeric blend extracted from the female abdominal tips is very similar to the most attractive synthetic mixture in the field in New York. An *O. nubilalis* strain from London, Ontario, was found to produce 97% *cis*-11-tetradecenyl acetate and 3% *trans*-11-tetradecenyl acetate.

Key Words—Pyralidae, European corn borer, sex pheromone, sex attractant, pheromone specificity, *Ostrinia nubilalis*.

INTRODUCTION

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), was introduced into North America on at least two and probably three separate occasions during the 20th century (Caffrey and Worthley, 1927). It is now a widespread pest of corn and some other crops in eastern and midwestern North America. Because of the economic importance of this pest, there has been considerable effort in the elucidation of its sex pheromone. The preliminary pheromone isolation was reported by Klun (1968) and the primary component was proposed to be *cis*-11-tetradecenyl acetate (c11-14:Ac) on the basis of gas chromatographic (GLC) retention times (Klun and Brindley, 1970). This material was found (Klun and Robinson, 1971) to be attractive to male ECB in the field in Iowa. Subsequently, an isomer mixture (96% *cis*, 4% *trans*) was shown to be optimum for trapping male ECB in Iowa (Klun et al., 1973).

In early field screening experiments in New York (Roelofs and Comeau, 1971; Roelofs et al., 1972), male ECB were attracted to *trans*-11-tetradecenyl acetate (t11-14:Ac) (no GLC detectable c11-14:Ac; <1%). This paper reports the chemical identification of pheromone components from abdominal tip extracts of two different populations of ECB.

METHODS AND MATERIALS

Insect Rearing and Collection

A multivoltine ECB strain from Aurora, New York was used for the chemical work unless otherwise noted. The culture was established from 2 dozen diapausing larvae collected from corn in the spring of 1972. ECB from similar cultures originating in the Hudson Valley of New York and Geneva, New York were obtained from Dr. A.C. Davis of this department. A culture of borers from London, Ontario, was established with insects obtained from Dr. G. McLeod of the University Research Institute in London, Ontario.

ECB larvae were reared individually on modified (Miles, 1970) wheat germ medium (Chippendale and Beck, 1964) in 45-ml plastic jelly cups (Premium Plastics Company, Chicago) at 25°C under a 16-hr photoperiod. Abdominal tips from 1-day-old frozen virgin females were excised and extracted with methylene chloride. Extracts were stored at −20°C.

Chemical Determinations

GLC employed flame ionization detectors and glass columns 2 m × 4 mm packed with 3% methyl silicone (OV-1) on Gas-Chrom Q or with 3% cyclohexanedimethanol succinate (CHDMS) on 100/120-mesh Chromosorb W. *cis-trans*. Ratios were determined on a 3.7-m × 2-mm column of 3% phenyldiethanolamine succinate (PDEAS) on 100/120 Chromosorb W-AW-DMCS.

Preliminary cleanup of some samples was carried out by high-pressure liquid chromatography (HPLC) using a 1.5-m × 12-mm stainless steel column packed with a steric exclusion resin (Bio-Beads SX-2, Bio-Rad Laboratories). Benzene was used as eluent at 1.0 ml/min. High molecular weight materials started eluting with about 30 ml of solvent. The EAG-active material was collected with 78–90 ml of eluent.

All solvents were distilled in glass before use to remove possible high-boiling impurities. Electroantennograms (EAG) were conducted as previously described (Roelofs, et al., 1971).

Field Studies

Various *cis-trans* mixtures were made up in quantities sufficient for all tests. They were prepared volumetrically from analyzed samples of c11-14 :Ac and t11-14 :Ac (Farchan Chemical Company [*cis*] and by acetylation of the corresponding alcohol [Farchan, *trans*]). GLC analysis indicated that the mixtures contained, respectively, 0, 1.2, 3.1, 5.1, 8.0, 20, 50, 70, 94.2, 96.5, 98.2, and 100% *trans*. The pure *cis* isomer was prepared by preparative scale thin layer chromatography (TLC) on 30% $AgNO_3$/Silica Gel G and contained no *trans* isomer detectable by GLC (<0.5%).

Pheromone dispensers of silicone rubber septa (rubber stoppers, sleeve type, 5×9 mm, Arthur H. Thomas Company) were charged with 100 μl of a 1% (v/v) solution of the appropriate mixture in Skellysolve B. This dose corresponds to ca. 750 μg of mixed pheromone.

Dispensers were placed in the bottom center of XC-26 Sectar® traps (3M Company) supported 1 m off the ground on stakes placed in weeds around the edges of cornfields near Geneva and Pittsford, New York. Traps were set out in a randomized complete block design, sampled and rerandomized at least weekly, and supplied with new dispensers once during each flight. Treatments were replicated five times in each locality and data were submitted to an analysis of variance.

RESULTS

Pheromone Identification

Crude abdominal tip extract from Aurora ECB females was injected onto the OV-1 and CHDMS GLC columns and the effluent collected in 1-min fractions for assay by EAG. The only EAG-active area corresponded to a visible peak representing 600–900 pg/female and having the same retention time as t11-14 :Ac on OV-1 and CHDMS (16.2 and 15.9 min, respectively, at 150°C). This activity was found in the 4–5 min fraction from OV-1 at 200°C. Activity at this time was retained on treatment of the collected material with acetyl chloride and recollection. Activity at this retention time was destroyed by bromination (Br_2/CCl_4), saponification (KOH/CH_3OH), and reduction (Red-Al®). Collection of the 14-carbon alcohol region (2–4 minutes at 200°C) from the saponification and from the reduction reactions and subsequent acetylation of the collected material restored activity at 4–5 min on recollection in each case. These data indicate that the primary pheromone component is a 14-carbon acetate.

Two hundred female equivalents of extract from Geneva ECB were collected from OV-1 (4–5 min at 200°C) and further chromatographed by

TLC on silver nitrate-impregnated silica gel using benzene as eluent. Good EAG activity (2.7 mv above background) was found in ether extracts of the *trans* area of the plate ($R_f \sim 0.5$) but only slight activity (0.5 mv above background) occurred in similar extracts of the *cis* area ($R_f \sim 0.35$). Injection of these extracts on a CHDMS GLC column at 170°C gave a visible peak at the same retention time as t11-14 :Ac (8.3 min) from the *trans* area, but no such peak was observed from the *cis* area. These data indicate that the predominant pheromone component is *trans*.

Hydrogenation of Geneva ECB extract (collected from OV-1) over PtO_2 in methanol-ether overnight at 25°C yielded material with the same retention time as 14 :Ac on OV-1 and CHDMS (9.1 and 7.4 min, respectively, at 170°C). The retention time of 12-methyltridecyl acetate was significantly shorter than that of the straight-chain compound (8.0 and 6.2 min, respectively, on OV-1 and CHDMS at 170°C). The branched-chain compound represents the hydrogenation product of an isomer, 12-methyl-11-tridecenyl acetate, that would give the same major fragment on ozonolysis as t11-14 :Ac. These data indicate that the major component of the pheromone is a straight-chain 14-carbon acetate.

Microozonolysis in CS_2 (Beroza and Bierl, 1967) of Aurora ECB extract collected from HPLC, OV-1 (10–12 min at 160°C), and CHDMS (10–12 min at 155°C) gave a product with a retention time similar to synthetic 11-acetoxyundecanal on both OV-1 and CHDMS (10.6 and 37.0 min, respectively, compared to the standard at 10.6 and 36.7 min at 150°C). The difference between natural and synthetic on the CHDMS column was within experimental error at the long retention time. These data support the assignment of the double bond to the 11-position, which confirms the identification of the primary pheromone component as t11-14 :Ac.

Collection of active fractions representing approximately 300 abdominal tips from HPLC and then from OV-1 (8.8–9.5 min at 175°C) gave a 14-carbon acetate fraction that was used to determine *cis-trans* ratios. Injection on a PDEAS column at 155°C gave a chromatogram consisting of three peaks. The first had a retention time equal to that of tetradecyl acetate (14 :Ac, 12.7 min). A similar peak appeared also on CHDMS (14.3 min at 150°C) and OV-1 (16.2 min at 150°C) and the compound was unchanged on attempted microozonolysis. The other two peaks on PDEAS had retention times identical to the identified pheromone t11-14 :Ac (15 min) and its geometrical isomer c11-14 :Ac (16.0 min). Extracts of ECB from London, Ontario, and the Hudson Valley of New York were worked up similarly, and the ratios of the three GC peaks of the ECB populations calculated (Table 1).

The analyses show that both Aurora and Hudson Valley ECB females possess about 96% *trans* isomer, whereas the London females secrete 97% *cis*, similar to attractancy for Iowa ECB (Klun et al., 1973). The major

TABLE 1. RATIOS OF 14-CARBON ACETATE COMPOUNDS FROM PURIFIED ABDOMINAL TIP EXTRACTS OF VARIOUS EUROPEAN CORN BORER POPULATIONS (Determinations made on a PDEAS GLC column at 155°C)

Origin	14 :Ac	c11-14 :Ac	t11-14 :Ac	*c/t*
London, Ontario	9.5%	87.6%	2.9%	96.8/3.2
Aurora, New York	2.2	3.6	94.2	3.7/96.3
Hudson Valley, New York	19.5	3.5	77.0	4.3/95.7

component of the London ECB female extract was collected as described above and shown to be c11-14 :Ac as follows.

1. The purified compound had retention times identical to those of c11-14 :Ac on OV-1 (9.1 min at 175°C), CHDMS (16.7 min at 150°C), and PDEAS (16.0 min at 155°C).

2. Microozonolysis of the purified compound gave a product with retention times identical to those of 11-acetoxyundecanal on OV-1 and CHDMS (10.6 and 37.0 min, respectively, at 150°C).

Field Attractancy in New York

The field test (Table 2) of male ECB attractancy to a number of *cis-trans* tetradecenyl acetate blends clearly indicates that ca 98% *trans* isomer was the most effective mixture. The pure *trans* isomer and the blend containing 96.5% *trans* were less attractive. Tetradecyl acetate, also indicated to be

TABLE 2. FIELD TRAPPING OF MALE ECB IN GENEVA AND PITTSFORD, NEW YORK, WITH VARIOUS *cis-trans* MIXTURES OF 11-TETRADECENYL ACETATES, 1973

Treatment	Mean no./trap[a]	
Percent *trans*	**June**	**August**
96.5	1.5b	1.2bc
98.2	2.7a	2.9a
100.0	0.7bc	1.6b
Others[b]	0.0–0.7c	0.0–0.7c

[a] Means in the same column followed by the same letter are not significantly different at the 5% level, as determined by Duncan's new multiple range test.

[b] All other treatments (see Methods and Materials) and unbaited traps.

present in extracts of female abdominal tips, was evaluated for its effect on male attraction to the 98.2% *trans* mixture. When added to the pheromone dispenser at rates of 1–20% of the pheromone, 14:Ac had no effect on attractancy.

DISCUSSION

It has been shown that female ECB in New York produce t11-14:Ac as the primary pheromone component, accompanied by ca 4% c11-14:Ac, and that New York male ECB in the field are maximally attracted to a mixture containing ca 2% c11-14:Ac. This difference may be due to the emission during calling of a mixture slightly different from that extracted from excised tips. Female ECB from London, Ontario produce the pheromones in an opposite ratio with c11-14:Ac as the primary pheromone component and only 3% of t11-14:Ac. Male ECB in London have been attracted to traps emitting mainly c11-14:Ac (McLeod, personal communication), similar to the attractant blend found for ECB males in Iowa (Klun et al., 1973). In both Iowa (Klun et al., 1973) and New York (this paper), an increase in the amount of the respective minor component eliminates male attraction. This would presumably cause reproductive isolation in the field and would support a separate species status for the two populations. These differences in pheromone systems among ECB populations in North America are probably the result of several introductions of ECB into the United States (Caffrey and Worthley, 1927) from different regions of the old world. Disparate biological characteristics (food plant specificity, number of yearly generations, and diel rhythm of mating in the laboratory) have been observed for at least two of them (Arbuthnot, 1944; Caffrey and Worthley, 1927). The *cis* population appears to be predominant, since female ECB from Georgia, Iowa, Minnesota, and Quebec attract ECB males in the field in Iowa. New York ECB females are not attractive to Iowa ECB males (Showers et al., 1974). The *trans* population has only been found in New York and Pennsylvania. In the latter state, the two populations apparently occur sympatrically (Cardé et al., 1975), since males were attracted to mixtures similar to the best for New York and Iowa, but not to intermediate mixtures as might be expected for hybrids.

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BARK BEETLE OLFACTION. III. ANTENNAL OLFACTORY RESPONSIVENESS OF *Dendroctonus frontalis* Zimmerman AND *D. brevicomis* Le Conte (COLEOPTERA: SCOLYTIDAE) TO AGGREGATION PHEROMONES AND HOST TREE TERPENE HYDROCARBONS[1]

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Abstract—Electroantennograms from males and females of *Dendroctonus frontalis* and *D. brevicomis* in response to serial dilutions of the pheromones *exo* brevicomin and frontalin and the host terpene hydrocarbons 3-carene and α-pinene show no significant differences in the threshold concentration for response to the compounds for either sex or species. The intensity of response was greater to higher concentrations of the pheromones (10 to $>10^4$ μg) than the terpene hydrocarbons, suggesting the presence of more receptors on the antennae for pheromones than terpene hydrocarbons. Antennal olfactory responses to the compounds did not correlate to published data on behavioral responses by the beetles to the compounds in both field and laboratory studies. Adaptation experiments indicated that for *D. frontalis*, both frontalin and *exo* brevicomin share the same receptors on the antennae. The results indicated that the terpene hydrocarbons share some, but not all, of the same receptors.

Key Words—electroantennogram, pheromones, bark beetles, *Dendroctonus*, olfactory, receptors, electrophysiology.

INTRODUCTION

The southern pine beetle (*Dendroctonus frontalis* Zimmerman) and the western pine beetle (*D. brevicomis* Le Conte) are closely related, allopatric species.

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Both respond to some of the same compounds that play important roles in the aggregation behavior of the beetles in nature (Kinzer et al., 1969; Pitman et al., 1969; Vité and Pitman, 1970).

Considerable research has been conducted on the behavioral aspects of aggregation pheromones and host tree terpene hydrocarbons in *D. frontalis* and *D. brevicomis* (Bedard et al., 1969; Kinzer et al., 1969; Pitman and Vité, 1971; Vité and Pitman 1969, 1970; Vité and Renwick, 1971). However, none of these studies involved investigation of response at the antennal receptor level.

Antennal olfactory responsiveness of bark beetles to aggregation pheromones and host tree terpene hydrocarbons has been measured successfully with electrophysiological techniques. Payne (1971) recorded response to frontalin from the antennae of male and female *D. frontalis*. In a preliminary study, both males and females of *D. frontalis* and *D. brevicomis* were responsive at the antennal receptor level to aggregation pheromones and host tree terpene hydrocarbons presented in undiluted form (Payne, 1970). The study demonstrated antennal olfactory responsiveness of the beetles to those compounds. However, since the compounds were not presented as serial dilutions, the relative sensitivities of the beetles to each compound were not demonstrated.

Electrophysiological techniques were employed in the following study to provide:

(1) a measure of the relative sensitivities of male and female *D. frontalis* and *D. brevicomis* at the antennal receptor level to 3-carene, α-pinene, *exo* brevicomin, and frontalin;

(2) information on antennal olfactory responsiveness that might correlate to whole organism (behavioral) responsiveness to the compounds; and

(3) an insight into the specificity of the olfactory receptors for the compounds.

METHODS AND MATERIALS

The study was carried out using the electronantennogram (EAG) as a measure of antennal olfactory responsiveness. The methods used here for obtaining EAGs are described elsewhere (Payne, 1970; Payne et al., 1970) and are modifications of a technique described earlier (Schneider, 1957; Kaissling, 1971).

Responses were recorded with Ag–AgCl capillary electrodes filled with 3M KCl. To obtain information on (1) and (2), the responses to serial dilutions of 3-carene, α-pinene, *exo*-brevicomin (exo-7-ethyl-5-methyl-6,8-dioxabicyclo [3.2.1] octane [Silverstein et al., 1968]) and frontalin (1,5-dimethyl-6,8-dioxabicyclo [3.2.1] octane [Kinzer et al., 1969]) were

recorded from the intact antennae of male and female adults of both *D. frontalis* and *D. brevicomis.*[2]

A dilution series of 10^{-3} to 10^{4} μg (undiluted) of compound per 10 μl of 70% ethanol was prepared for each compound. Each antennal preparation was exposed to the 4 compounds at random. For every compound, each antennal preparation received the following stimulation: 10 μl of 70% ethanol (control); 10 μl of each dilution presented in increasing concentration; 10 μl of undiluted compound; 10 μl of frontalin at about 10^{4} μg/10 μl (standard); and 5 mV calibration stimulus.

Since a combination of frontalin and α-pinene is known to elicit aggregation behavior in *D. frontalis* (Renwick and Vité, 1969), antennal preparations of the beetle were exposed to a mixture of the compounds, ca. 10^{4} μg/10 μl, to determine the influence of the mixture at the antennal receptor level. A similar synergistic effect occurs in *D. brevicomis* to a mixture of *exo* brevicomin, frontalin, and myrcene (Vité and Pitman, 1969; Bedard et al., 1970; Wood, 1972); however, data were not obtained on response by *D. brevicomis* at the antennal receptor level to a mixture of those compounds. A 3-min interval was allowed between each stimulation for all stimuli for each compound and a 5-min interval was allowed between each compound to control for sensory adaptation and possible air-flow contamination. Both intervals were found to be more than sufficient to allow for complete recovery of olfactory receptors to the initial response level (Payne, unpublished). In a lepidopterous species, *Trichoplusia ni*, complete recovery occurred in less than 1 min (Payne et al., 1970).

To obtain information on (3), receptor specificity, antennal preparations of male and female *D. frontalis* were each exposed to 10^{4} μg of each pheromone or terpene hydrocarbon alone until the antennal receptors became adapted. Each preparation was then immediately (within msec) exposed to 10^{4} μg of the same or second pheromone or terpene hydrocarbon. If two compounds were to stimulate identical receptors, then it was assumed that adaptation to one of them should eliminate peripheral response to immediate stimulation by the other. In addition, if two compounds were to share some, but not all, of the same receptors, then it was assumed that response to one compound would be reduced, but not eliminated, when stimulation took place during adaptation to the other compound.

In all cases the ethanol and compound samples were placed on filter paper and delivered over each antennal preparation via a 100 ml/min air flow through a Varian-Aerograph gas sample valve (Payne et al., 1970) or a 3-way solenoid-operated gas valve. Although it is the amount of compound that evaporates *from* the filter paper that is involved in stimulating the antenna, for convenience, later discussion refers to stimulation in terms

[2] Compound purity > 99.5% with GLC.

of the amount placed *on* the paper. The amplitude of the rapid initial negative change in potential upon stimulation was used as the quantitative measure of the intensity of the EAG. Responses to the compounds were adjusted by subtraction of any response to the control and by conversion to percent of the response, to response to the standard. Standard errors of the mean responses were measured. Responses were recorded on polaroid film.

RESULTS AND DISCUSSION

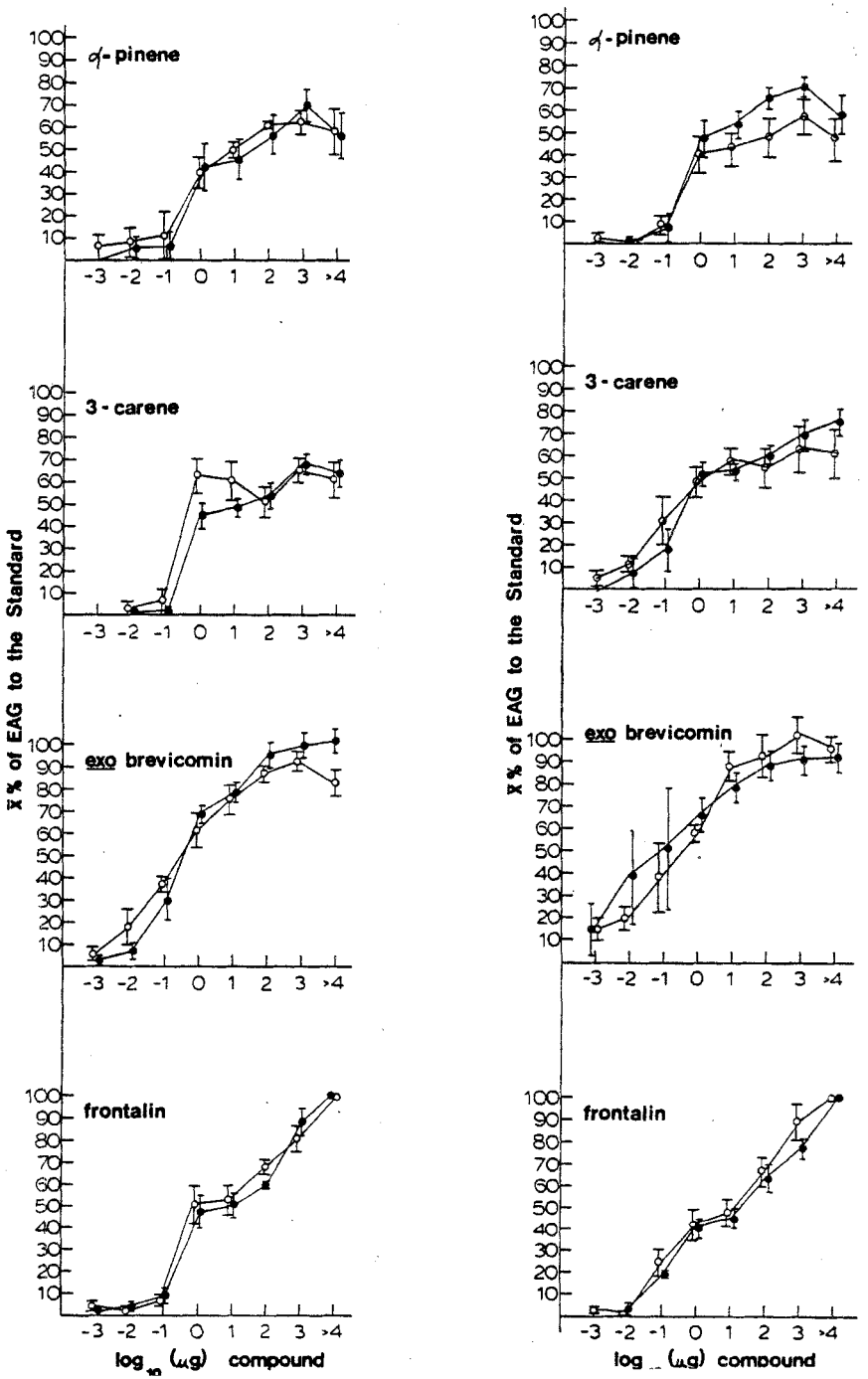
Both males and females of *D. frontalis* and *D. brevicomis* responded at the antennal receptor level to the host tree terpene hydrocarbons 3-carene and α-pinene and the aggregation pheromones *exo* brevicomin and frontalin (Figures 1 and 2). In general, responses increased to stimulation by an increased concentration of each compound.

Receptor Sensitivity

Based upon the thresholds of response, the data show no significant differences in the relative sensitivities to the terpene hydrocarbons and pheromones between the species and the sexes of each species. The slight, apparent differences that did occur in response to a compound at a given concentration were not substantiated by the limits of standard error. The intensities of response by males and females of *D. frontalis* and *D. brevicomis* were considerably less to the terpene hydrocarbons than to the pheromones at the higher concentrations. Since the EAG is considered to be the summation of receptor potentials from the simultaneous firing of several olfactory receptor cells (Boeckh et al., 1965; Schneider, 1969), the intensity of the EAG (increase in response) can be taken as a measure of the relative number of responding receptor cells. The data suggest, therefore, that the

(LEFT) FIG. 1. Mean percent response of *D. frontalis* to pheromones and terpenes. Responses presented as percent of response to the standard (ca. 10^4 μg frontalin). Mean response to standard = ca. 2.09 mV. Data points represent 3 replicates (different beetles) per point for 10^{-1}–10^{-3} μg and 8 replicates for 1–>10^4 μg. Filled circles = male, open circles = female, and vertical bars = standard errors.

(RIGHT) FIG. 2. Mean percent response of *D. brevicomis* to pheromones and terpenes. Responses presented as percent of response to the standard (ca. 10^4 μg frontalin). Mean response to standard = ca. 2.50 mV. Data points represent 1–3 replicates (different beetles) per point for 10^{-1}–10^{-3} μg and 8–10 replicates/point for 1–>10^4 μg. Filled circles = male, open circles = female, and vertical bars = standard errors.


d-pinene
3-carene
exo brevicomin
frontalin
X % of EAG to the Standard
log (µg) compound
-3 -2 -1 0 1 2 3 >4
10 20 30 40 50 60 70 80 90 100

antennae of the beetles possess more receptors for the pheromones than for the terpene hydrocarbons. This may be of adaptive significance to the beetles since the pheromones are not likely to be as prevalent in the environments of the beetles as are terpene hydrocarbons. Yet, pheromones provide a critical link in the line of communication within the species, leading to aggregation on host trees and mating.

These results are probably not because of a difference in evaporation rates between the terpene hydrocarbons and pheromones, which are comparable. Furthermore, it is unlikely that the receptors for the terpene hydrocarbons and pheromones occur in similar number but respond with different intensity. The data show, for example, that in both *D. brevicomis* and *D. frontalis* the intensities of response are similar to α-pinene, 3-carene, and frontalin at the 10^{-3} to 10^{1} μg concentration levels. These results indicate that the receptors for the terpene hydrocarbons respond with an intensity comparable to that of the pheromone receptors. Therefore, differences in intensity of response that occur to higher concentrations do appear to be the result of differences in the number of available receptors.

The differences in the intensities of response to *exo* brevicomin and frontalin at a given concentration are not understood. It is not the result of evaporation rates from the filter paper substrate since frontalin is known to have a higher vapor pressure than *exo* brevicomin, and, therefore, more molecules would reach the antenna. It is probably not because of the presence of different receptors for frontalin and for *exo* brevicomin, as results presented later in this paper indicate. Possibly the pheromone receptors have a greater affinity for *exo* brevicomin than frontalin.

The response of *D. frontalis* males (4) and females (5) at the antennal level to the mixture of frontalin + α-pinene was not different from the response to frontalin alone. In addition, there were no significant differences in response to the mixture between the sexes as determined by an analysis of variance.

Receptor Specificity

When the receptors for frontalin were completely adapted in male or female *D. frontalis*, they failed to respond to stimulation by *exo* brevicomin (Table 1). The reverse effect also resulted. However, when receptors were adapted to either pheromone, response was still elicited by either terpene hydrocarbon, indicating that all receptors on the antenna were not blocked by saturation with one compound. Furthermore, adaptation to one terpene hydrocarbon reduced, but did not eliminate, response to the other terpene.

The results suggest that frontalin and *exo* brevicomin are specific for

TABLE 1. MEAN EAGs OF RESPONSE BY *D. frontalis* TO STIMULUS COMPOUND DURING PERIOD OF ADAPTATION TO PREVIOUS STIMULUS COMPOUND[a]

Previous compound	Stimulus compound	Response (mV) to stimulus compound[b]
frontalin	frontalin	0
exo brevicomin	*exo* brevicomin	0
α-pinene	α-pinene	0
3-carene	3-carene	0
frontalin	*exo* brevicomin	0
exo brevicomin	frontalin	0
3-carene	α-pinene	0.63
α-pinene	3-carene	0.59
α-pinene	frontalin	1.13
frontalin	α-pinene	0.26
3-carene	frontalin	0.58
frontalin	3-carene	0.18
α-pinene	*exo* brevicomin	1.04
exo brevicomin	α-pinene	0.31
3-carene	*exo* brevicomin	0.98
exo brevicomin	3-carene	0.60

[a] Stimulus compound delivered < 10 msec after adaptation to previous compound. Data from 9 replicates of 1 beetle/replicate.

[b] Unadapted response to the compounds: α-pinene = 1.78 mV, 3-carene = 1.80 mV, *exo* brevicomin = 2.31, and frontalin = 2.41.

the same receptors. The relative reduction, but not elimination, of response to a pheromone or terpene hydrocarbon due to adaptation to a different compound suggests that both compounds may share some, but not all, of the same receptors. If that is the case, it provides the insect with greater flexibility for coding incoming peripheral signals to the central nervous system than is the case with receptors that respond to only one compound (Boeckh, 1969).

Behavioral Correlation

In general there are no correlations between the behavioral responses and EAGs of male and female *D. frontalis* and *D. brevicomis* to the pheromones and terpene hydrocarbons tested.

Behaviorally, males of *D. frontalis* and *D. brevicomis* were attracted

in greater numbers than females to frontalin alone and to frontalin + α-pinene (Renwick and Vité, 1969). However, response at the gross antennal receptor level showed no differences in response between the sexes.

Exo brevicomin was found to inhibit the attraction of *D. frontalis* to frontalin + α-pinene (Vité and Renwick, 1971). The attraction of females was reduced 25% over males. The EAGs showed no differences in the responses of males and females to *exo* brevicomin. Furthermore, there were no apparent differences in response to *exo* brevicomin and frontalin (or frontalin + α-pinene) to indicate one as an inhibitor and the other as an attractant. It is clear, however, that since *exo* brevicomin and frontalin appear to stimulate the same receptors, a receptor can receive more than one type of information.

Both laboratory (Browne and Wood, personal communication) and field (Bedard et al., 1969) results of behavioral response of *D. brevicomis* to *exo* brevicomin showed males more responsive than females by ca. 2:1, although responses were at low levels. In field tests, frontalin alone attracted females over males by 1.4:1 (Vité and Pitman, 1970). As with *D. frontalis*, there were no correlations in *D. brevicomis* between the EAGs and behavioral responses to the pheromones. Furthermore, in *D. brevicomis*, *exo* brevicomin, which is an important component with frontalin and myrcene in the attractant mixture for *D. brevicomis* (Vité and Pitman, 1969; Bedard et al., 1970; Wood, 1972), does not elicit different responses from *D. frontalis* as measured through the EAG. Yet, for *D. frontalis*, *exo* brevicomin is an inhibitor.

Behavioral data were not available for response of *D. frontalis* to terpene hydrocarbons alone. In *D. brevicomis*, females were more attracted to turpentine and to myrcene than males in a laboratory bioassay, although in low numbers (Browne and Wood, personal communication). In the field, oleoresin attracted more females than males (Vité and Pitman, 1969). Although only specific terpene hydrocarbons were tested, the EAGs did not show significant differences between male and female *D. brevicomis* in response to 3-carene and α-pinene.

The absence of correlations between EAG and behavioral data makes it clear that for *D. frontalis* and *D. brevicomis*, the EAG is not necessarily a valid indicator of the influence of a compound on the behavior of an insect. In a preliminary report it was suggested that the intensity of response of both *D. frontalis* and *D. brevicomis* to the pheromones and terpene hydrocarbons in undiluted form provided good correlation to the behavioral response of the beetles to the compounds (Payne, 1970). Subsequent findings, however, and the results reported here, make it clear that the EAG does not necessarily provide a reliable correlate to behavioral response.

It is apparent, in any event, that correlation of receptor sensitivity and behavioral response is complex in *Dendroctonus* because of the mixture of compounds (pheromone[s] and host tree terpene hydrocarbons) necessary

to elicit behavioral responses. Pheromones and terpene hydrocarbons alone can be low in activity behaviorally, but become active when both stimulate the olfactory receptors. At the receptor level, however, both the pheromones and host tree terpene hydrocarbons are independently effective.

In contrast to at least one lepidopterous species, *Bombyx mori* L. (Kaissling and Priesner, 1970), stimulation of a specific receptor in *Dentroctonus* does not mean that a given behavioral response will result (Payne, 1974). It is likely that initiation of behavioral response requires the central integration of several lines of information from receptors of differing degrees of specificity.

The lack of differences in receptor sensitivity and the absence of a correlation between the gross receptor level (EAG) and behavioral responses to the pheromones and terpene hydrocarbons emphasize the need for information at the single receptor cell level. Single cell investigations are necessary to elucidate the effects of the pheromone and terpene hydrocarbon receptors in the differentiation of response at the behavioral level. Such investigations are currently underway on *D. frontalis.*

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A FIELD STUDY OF CHEMOTACTIC RESPONSES OF THE MARINE MUD SNAIL, *Nassarius obsoletus*[1]

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Abstract—Chemotactic responses of the salt marsh mud snail *Nassarius obsoletus* were tested in the field, to the introduction of a crushed conspecific and to the sympatric gastropod *Littorina littorea* and the bivalve *Modiolus demissus*. The snails responded with burial and escape to the conspecific stimulus, with strong attraction and feeding to *M. demissus*, and with attraction and feeding to *L. littorea*. In blank tests, the numbers of snails fluctuated only slightly during half-hour observation periods. An alarm substance appears to be liberated from wounded *N. obsoletus* which causes rapid disappearance of conspecific snails in an area of up to 50 cm radius.

Key Words—*Nassarius obsoletus*, *Littorina littorea*, *Modiolus demissus*, alarm pheromone.

INTRODUCTION

After discussions and letters to the editor of an aquarium hobbyist magazine which first described casual observations of intraspecific alarm reactions in aquatic snails (Bröhl, 1940; Kempendorff, 1940*a*, *b*; Weise, 1940), Kempendorff (1942) established that this reaction was caused by a chemical stimulus released from the body of a crushed conspecific. He patterned much of his concepts after the then just published accounts of alarm reactions in fish (von Frisch, 1938) which occur under almost identical circumstances. This early work was considerably expanded by Snyder (1967), who demonstrated that a variety of alarm reactions occur in aquatic gastropod molluscs when

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intraspecific extracts are presented. One of those tested by Snyder was *Nassarius obsoletus*, the subject of the present study.

Alarm responses have been variously described as fright or alarm responses and as escape responses, sometimes interchangeably, and sometimes defining the response by the stimulus that caused it. We will use the terms *alarm* and *escape* only to describe the behavioral responses of an animal, and not to imply which stimulus is causing them. Thus, an alarm response signifies a behavioral change, sometimes characterized by greatly increased locomotor activity and sometimes by "freezing," while specific details vary from species to species; alternatively, in an escape response the animal moves away from the source of (chemical) stimulation. The stimuli can be of intra- and interspecific origin. The most common interspecific chemical stimulus causing alarm and escape responses is the odor of predator(s). In marine invertebrates a rather extensive literature deals with their alarm and escape responses to predatory starfish odor (Bullock, 1953; Kohn, 1961; Feder, 1963; Gore, 1966), while other predator odors, especially from carnivorous marine snails, are known to induce similar responses (Gore, 1966; Snyder and Snyder, 1971). In gastropod molluscs, the most common forms of alarm and escape are: dropping off from a vertical substrate, violent thrashing about with the foot, strong and fast twisting of the shell, fast crawling away from the source, self-burial, and combinations of these. Specific responses take place after specific stimuli.

N. obsoletus, the mud snail, is a common gastropod living on the salt marsh mud flats of the eastern United States and Canada, from New Brunswick to Cape Canaveral, Florida. It feeds primarily on diatoms and other unicellular organisms in the mud, but like the other carnivorous species of *Nassarius*, it is also easily attracted to dead animal material, on which it feeds (Jenner, 1956; Scheltema, 1964). The animal is thus described as a facultative herbivore/carnivore (Brown, 1969). Some of its behavior has been described, including chemically triggered behavior (Crisp, 1969). Social behavior appears in the formation of dense aggregations which can be observed in mid-summer at the end of the egg-laying season (Jenner, 1959).

N. obsoletus can move fast with a long powerful foot, which is also used for self-burial, a process which takes about five seconds and leaves no more than a temporary depression visible in the mud. A long siphon enables the snail to stay buried for long periods while its siphon not only serves a respiratory function, but also keeps the animal informed on the chemistry of its surroundings. An attractant chemical stimulus can thus produce the sudden emergence of dozens of snails from a previously empty-looking mud flat.

The most common molluscs in the area of our study were the mud snail, *N. obsoletus*; the periwinkle, *Littorina littorea*; and the ribbed mussel,

Modiolus demissus. When either the mussel or the periwinkle was crushed and placed on the marsh mud, *N. obsoletus* would emerge in great numbers and start feeding on the crushed molluscs. However, when one individual out of a large group of visible and feeding *N. obsoletus* was crushed and placed on the mud bottom (time: 0; location: center; no water current obvious; water depth 5–15 cm), the following sequence of events generally took place.

0–2 min: immediate (within 2 sec) withdrawal from the immediate center (2-cm radius), followed by the moving away of most snails in 5-cm radius area and many burials on the spot.

2–10 min: subsequent burials of snails in 10-cm radius area and continued moving in near-straight lines in all directions away from the center; in 5–10 min, this area was often almost cleared of visible snails; followed by burials and moving away of snails in 25-cm radius area; some snails, mostly in the 5–10-cm radius band, emerging from the mud, moving further away, and burying themselves again.

10–20 min: a 25-cm radius area is mostly cleared of visible snails, which either move out or bury.

20 min–2 hr: most movement has stopped, a 50-cm radius area can be cleared out, and the appearance is of a bomb explosion; a few snails are often still visible, but motionless.

In order to measure the magnitude of these chemotactic responses more precisely, we carried out a series of experiments using the three crushed molluscs from the marsh as stimuli and including a series of blank tests.

METHODS AND MATERIALS

The field study took place in the summer of 1973 at Little Sippewissett Marsh, a salt marsh in West Falmouth, Massachusetts. Observations were generally made during the afternoon at low tide, when the depth of the water on the mud flats was about 10 cm and when there were no major water currents noticeable as measured by stirring up some soft mud. Water movement was usually restricted to surface movements caused by shore breezes.

To test the chemotactic reactions of *Nassarius obsoletus*, an area containing 4–33 visible snails was selected for each trial. The initial number of visible snails chosen was higher when a decrease was expected and lower when an increase was likely to result. For blank experiments the initial numbers were sometimes chosen high, sometimes low (see Table 1). The test area was delineated by a circular hoop supported by rocks 5 cm from the mud bottom. The hoop, made of 1-inch polyethylene tubing and weighed down with sand inside, covered a surface area of about 0.4 m^2 (radius 35 cm). Inside the hoop a grid was permanently fastened to facilitate recording of snail positions.

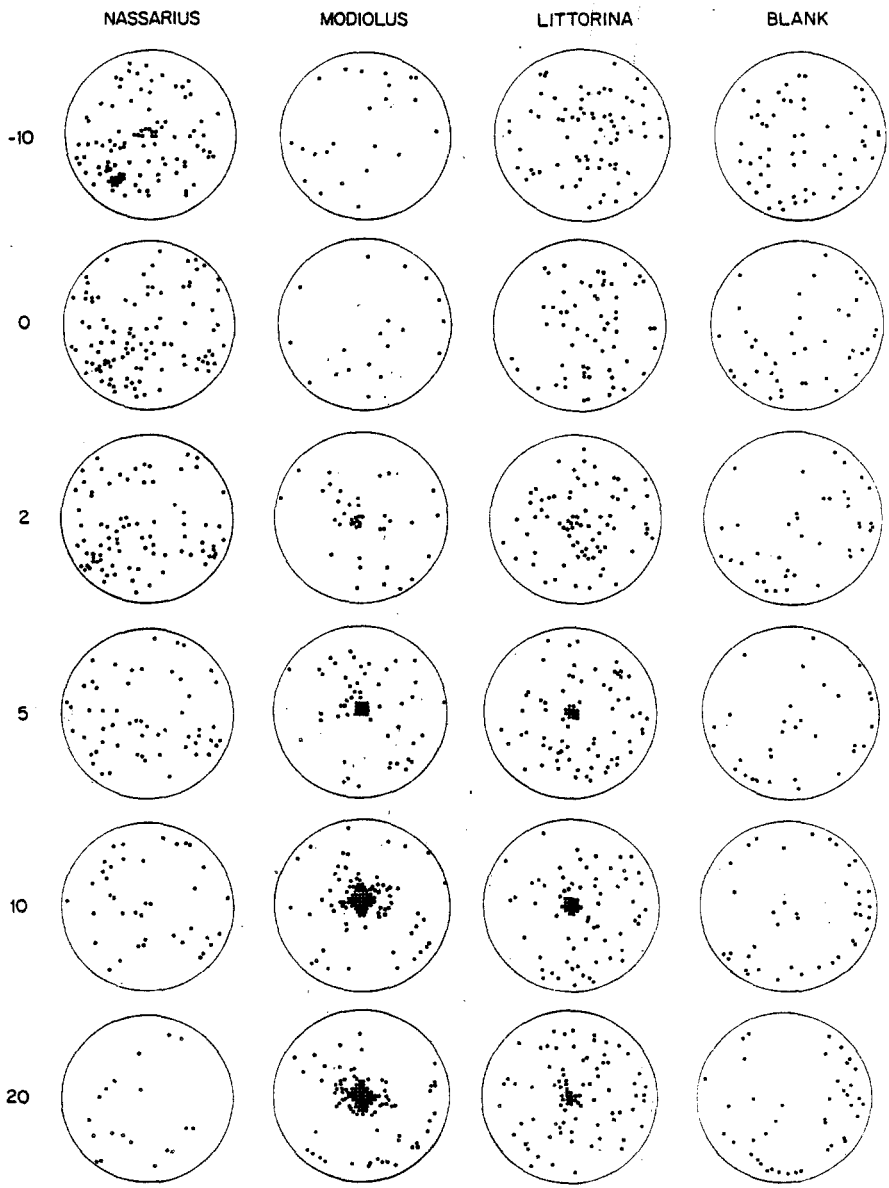

FIG. 1. Responses of the mud snail *N. obsoletus* to four stimuli: crushed *Nassarius obsoletus*, *Modiolus demissus*, *Littorina littorea*, and blank (no stimulus). Dots indicate individual snails. Each circle shows the superimposed sum of 4 replicate trials (no significant difference between trials). The snails were counted at −10, 0, 2, 5, 10, and 20 minutes; stimulus introduction was at time 0 minutes, in the center of the circle.

The positions of all snails in the test area were recorded immediately following the placement of the hoop, and a record was made of individual activities. After 10 min, the positions of the snails were again recorded, after which one of three species of molluscs (wet weight of meat 0.5–1.0 g) was crushed and placed in the center of the test area. The time of stimulus introduction was designated as 0 min. The beginning of the experiment was thus called −10 min.

Further recordings were made at 2, 5, 10, and 20 min after stimulus introduction. The following stimuli were presented: *Nassarius obsoletus* (mud snail), *Littorina littorea* (periwinkle snail), *Modiolus demissus* (ribbed mussel), and blank test in which no stimulus was presented. Each test was duplicated four times on different days and different locations in the marsh. For each testing day, blank experiments preceded attracting stimuli, which

TABLE 1. NUMBER OF VISIBLE SNAILS, *Nassarius obsoletus*, IN OBSERVATION AREA IN 4 REPLICATE EXPERIMENTS UNDER 4 STIMULUS CONDITIONS[a]

	A						B					
Stimulus:	*Nassarius*						*Modiolus*					
Minutes:	−10	0	2	5	10	20	−10	0	2	5	10	20
Trial No.												
I	20	24	24	16	16	12	8	4	11	17	24	7
II	29	29	28	17	6	1	4	8	12	11	15	15
III	33	30	22	18	8	3	6	7	5	11	29	52
IV	20	17	10	5	9	3	5	0	14	28	41	41
Total	102	100	84	56	39	19	23	19	42	67	109	115

	C						D					
Stimulus:	*Littorina*						*Blank*					
Minutes:	−10	0	2	5	10	20	−10	0	2	5	10	20
Trial No.												
I	30	27	39	35	34	32	6	2	4	4	4	3
II	17	11	14	19	26	19	9	15	14	10	11	10
III	14	13	13	14	16	20	25	19	16	13	16	15
IV	12	10	10	13	7	17	16	6	6	5	9	14
Total	73	61	76	81	83	88	56	42	40	32	40	42

[a] A, 1 whole crushed *Nassarius obsoletus* introduced in center of observation area at time 0 minutes; B, 1 whole crushed *Modiolus demissus* similarly introduced; C, 1 whole crushed *Littorina littorea* similarly introduced; D, no stimulus at time 0 minutes.

preceded alarm-producing stimuli. All equipment was cleaned every day before trials to avoid cross-contamination.

The data have been represented in a descriptive (Figure 1), as well as quantitative way (Table 1). Since the initial number of buried snails in the test area could never be determined, and since the snails were free to migrate in and out of the test area during the experiments, we used for representation and analysis only the number of snails visible at each recording time. Visible snails were defined as those that were not wholly or partly buried. They were either stationary on the mud surface, or crawling, or feeding. Buried snails could often still be seen partially or be inferred by a depression left in the mud. Snails in the process of burying were considered buried; those in the process of emerging were counted as visible. We did not discriminate, therefore, between emerging and burying vs. moving in and out of the area. We considered that the biological significance of the chemotactic responses was to increase or decrease the snail density, to feed or to escape respectively, regardless of which of the two methods of appearance and disappearance was used by the snails.

RESULTS

The numbers of visible *N. obsoletus* counted within the test area at 6 observation times in the 4 trials for the different stimuli are listed in Table 1. The superimposed replicates of responses to each stimulus are pictured in Figure 1. It is shown in Table 1 and Figure 1 that the numbers of visible *N. obsoletus* decrease when a crushed conspecific serves as the stimulus, that the numbers increase with *Modiolus* as a stimulus, increase only in the center area for *Littorina*, and that fluctuations in density occur when no stimulus is presented.

Using the rank sum test, there was no significant difference in snail density between the 16 tests during the control period from -10 to 0 min. The experimental period from 0 to 10 min showed significant differences ($P<0.03$) between blank control and *Nassarius* and between blank control and *Modiolus* tests. *Littorina* tests were not significantly different from blank control. The same results were obtained from a comparison of the slopes of the curves between 0 and 10 min and from a comparison of the percent change in the numbers of snails at 0 and at 10 min (rank sum test).

DISCUSSION

Overall, the results indicate that a crushed conspecific represents a repelling chemical stimulus to *N. obsoletus*, and that a crushed mussel is

chemically attractant to them. Some irregularities and exceptions, however, deserve further comment. Irregular decreases in *Nassarius* tests (Table 1) are often caused by snails that are moving away, but are still inside the test area. This effect was shown clearly in a more complicated data analysis (not represented here) where snails were counted in three concentric areas within the test area; numbers in the center area decreased sharply as they increased in outer areas. Another cause for irregular decrease, especially at 10 min, is the emerging of previously buried snails, which move farther out and bury again later. In general, our data analysis—which considers as a response only visual disappearance by either burial or leaving the test area—tends to be a conservative measure of response. Snails in the process of moving away from the center are responding, but are not counted in this analysis.

On occasion one or two snails would ignore the chemical stimulus of a crushed *N. obsoletus* and proceed toward the center area, mostly to pass by, but sometimes to feed on the crushed *N. obsoletus*. In some rare cases after as much as 10 minutes of feeding, they would leave and/or bury. Since feeding on *N. obsoletus* was observed several times, we investigated the effects of the hunger state on the alarm reaction, and we will report on this later (Atema and Stenzler, in prep.).

In cases where we introduced crushed *N. obsoletus* on top of a group of snails that were already feeding on *Modiolus demissus*, we observed occasionally that one or two would continue to feed on *M. demissus*. However, when a food stimulus (*M. demissus*) was presented after an initial alarm response to *N. obsoletus*, none of the buried snails emerged to feed. When food was presented 1½ hrs later, some snails emerged, mostly to bury again soon. It is our impression, although we did not test it, that areas used on previous days to test the alarm response contained far fewer visible snails the next day. Thus, the effect would seem to last over 24 hrs and to survive two tides flushing the marsh.

On several occasions, the alarm substance was further distributed over the marsh by fish and crabs, which came to feed on crushed *N. obsoletus*. They often competed for the food and took off in various directions with pieces of it, causing alarm reactions in previously unalarmed snails. We have avoided this in the tests described in Figure 1 and Table 1 by scaring the thieves away.

This same bait-stealing partly caused the irregular decreases in *Modiolus demissus* tests, where the attractant stimulus was sometimes gone before the end of the experimental period. In Table 1, trial 1, we can thus see a decrease between 10 and 20 min. However, here the feeding *N. obsoletus* were a large part of the food disappearance.

It is not certain whether crushed periwinkle (*Littorina littorea*) is an

entirely attractive stimulus or that it also contains elements of repulsion. Figure 1 shows that several snails are feeding on periwinkle, and we always observed a number of snails emerging and approaching. However, initial attraction was often followed by some turning away and not feeding.

The biological significance of the alarm response to intraspecific juices has not been demonstrated by direct observation. Much circumstantial evidence, however, supports the hypothesis that it serves as an antipredator mechanism. Snyder (1967) argues for this idea in his discussion of the alarm response of a great many aquatic gastropod snails. We have tried to determine the natural predators of *N. obsoletus* in a marsh area. Once in the field, we observed a moon snail (*Lunatia heros*) on top of a *N. obsoletus*. In the laboratory, we observed regular predation of moon snails (*L. heros* and *Polinices duplicatus*) and star fish (*Asterias forbesi*) on our snails. The type of response (burial and moving away) would make it an effective defense against visual or tactile predators. The speed of the response would make it effective against slow-moving predators. The chemical nature of the substance and the fact that the substance is released from any wound in the snail's skin or body tissues (Atema and Stenzler, in prep.) make it seem designed as a defense against a sloppy feeder. These descriptions suggest predatory snails, echinoderms, and (perhaps) insect larvae as the possible predators that have been reinforcing the use of an alarm substance in *N. obsoletus*.

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ALL-*trans*-FARNESYL HEXANOATE AND GERANYL OCTANOATE IN THE DUFOUR GLAND SECRETION OF *Andrena* (HYMENOPTERA: APIDAE)[1]

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Abstract—The volatile material which emanates from the Dufour gland of female *Andrena* bees has been examined in 13 species. In 11 of these, all-*trans*-farnesyl hexanoate is the dominating compound, whereas in two species geranyl octanoate is the largest component of the volatile secretion. Either of these two terpene esters were also found as major component in the cephalic secretion of some male *Nomada* bees. Bees of the genus *Nomada* are nest parasites on *Andrena*. The chemical identification was carried out by capillary gas chromatography using a splitter-free intake system, alone and in combination with mass spectrometry. Some farnesyl and geranyl esters were prepared in the course of the work by reacting terpenol and acid with CDI (*N, N'*-carbonyldiimidazole). The secretion is thought to serve as a nest marking.

Key Words—bees, volatile secretions, marking, mass spectrometry, isoprenoids.

INTRODUCTION

Orchids of the genus *Ophrys* L. are pollinated by aculeate Hymenoptera males. This phenomenon has been studied by Kullenberg (1952, 1961, 1973). During these investigations he also noted (1956) that both males and females of the insects give off odoriferous compounds. Many of the *Ophrys* pollinators belong to the genus *Andrena* Fabr. (Apidae). These bees are distributed over the whole world and are of great importance for the pollination of

[1] This report forms Part XII of the series Studies on Natural Odoriferous Compounds.

plants belonging to many different families (Alfken, 1914; Friese, 1923; Linsley and McSwain, 1959; Linsley et al., 1963; Thorp, 1969).

We are currently investigating volatile secretions both from *Ophrys* flowers (Kullenberg and Bergström, 1973) and from glands of *Andrena* (Bergström and Tengö, 1974). The present communication reports on the analyses of the volatile secretion from the Dufour gland in 13 species of *Andrena*. A more complete list of references relevant to this study was given in the preceding communication (Bergström and Tengö, 1974).

METHODS AND MATERIALS

Collection and Taxonomy of the Bees

All bees used in this study were collected on Öland, an island near the Swedish mainland in the southern Baltic Sea. The taxonomical determination in species was made after v.d. Vecht (1928) and the separation in subgenera after Warncke (1968). The nomenclature follows Warncke (1967).

Preparation of Glands and Technique of Chemical Analysis

Glands were prepared directly from fresh animals and either put directly on the precolumn of the gas chromatograph-mass spectrometer or extracted with hexane. The techniques of analysis employed in these studies have been described earlier (Ställberg-Stenhagen 1972; Bergström 1973; Stenhagen et al., 1973). The glass capillary columns used are coated with OV-101 as stationary phase. They are 23 m long, with an inner diameter of 0.25 mm. The temperature of the gas chromatographic oven was programmed by 4°C/min, typically from +30°C to +220°C. In Figure 2 straight-chain, saturated hydrocarbons C_{13} to C_{20} plus C_{22} and C_{24} have been added as references, permitting the calculation of relative retention indices. In the gas chromatograms shown, the temperature increases from right to left.

Deposition of Material

Those specimens from which Dufour glands have been prepared in this study are deposited at the Ecological Station on Öland, together with chromatograms and mass spectra.

RESULTS

Capillary Gas Chromatographic Analysis of the Volatile Dufour Gland Secretion of Andrena

In Table 1, some data regarding the analyses of the biological material have been summarized. Figure 1 shows a typical capillary gas chromatogram

TABLE 1. ANALYSES BY CAPILLARY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY OF *Andrena*

Subgenus	Species	Capillary gas chromatography		Mass spectrometry		Main component[b]
		Whole gland[a]	Extract in hexane[a]	Whole gland[a]	Extract in hexane[a]	
Andrena s. st.	*A. clarkella* (K.)	—	2:1 (1974)	—	2:1 (1974)	G-8
	A. praecox (Scop.)	1:1 (1971) 1:1 (1972)	1:1 (1972) 1:3 (1973)	—	1:5 (1973)	G-8
	A. fucata Smith.	—	1:2 (1973) 1:1 (1974)	—	1:2 (1973)	F-6
Charitandrena Hed.	*A. hattorfiana* (Fabr.)	—	1:1 (1972)	—	1:1 (1972)	F-6
Chrysandrena Hed.	*A. fulvago* (Christ.)	—	2:1 (1972)	1:1 (1972)	—	F-6
Hoplandrena Pér.	*A. carantonica* Pér.	2:1 (1971) 2:1 (1972)	1:1 (1972)	1:1 (1971) 2:1 (1972)	1:1 (1972)	F-6
Melandrena Pér.	*A. vaga* Panz.	—	1:1 (1974)	—	1:1 (1974)	F-6
Poecilandrena Hed.	*A. labiata* Fabr.	—	—	—	1:1 (1974)	F-6
Plastrandrena Hed.	*A. tibialis* (K.)	1:1 (1971)	1:5 (1973)	—	1:1 (1969)[c]	F-6
	A. bimaculata (K.)	—	1:2 (1973)		1:2 (1973)	F-6
	A. ovatula (K.)	—	1:1 (1972) 1:4 (1973)	1:1 (1972)	—	F-6
Taeniandrena Hed.	*A russula* Lep.	—	1:5 (1973)	—	1:5 (1973)	F-6
	A. gelrie v.d. Vecht	—	—	1:3 (1971)	—	F-6

[a] The first figure gives the number of separate analyses. The second figure gives the number of individuals used in each analysis. Years of collection and analysis are given in parentheses.
[b] G-8 means geranyl octanoate and F-6 all-*trans*-farnesyl hexanoate.
[c] The material has been isolated by ampule distillation.

FIG. 1. Capillary gas chromatogram of an extract in hexane of Dufour's gland from *Andrena tibialis*. Temperature is increased from the right to the left in the chromatogram.

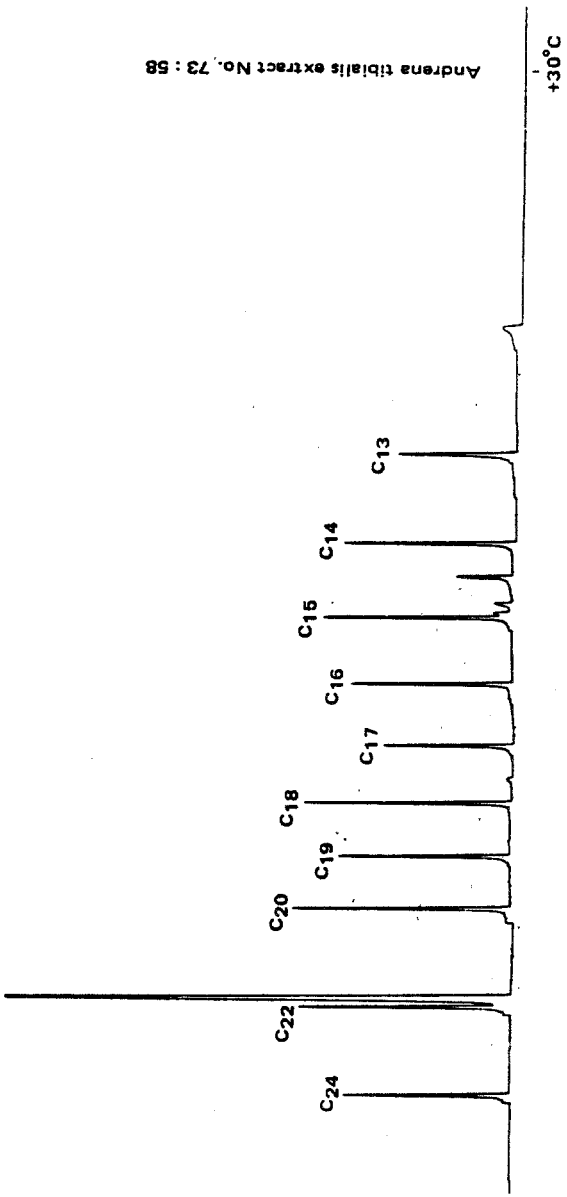

FIG. 2. Compare Figure 1. Reference hydrocarbons added.

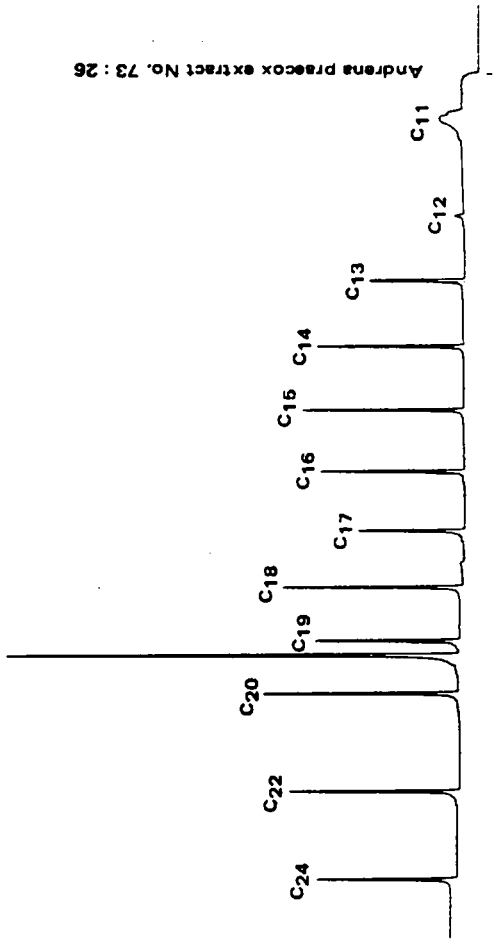

FIG. 3. Capillary gas chromatogram of an extract in hexane of Dufour's gland from *Andrena praecox* with reference hydrocarbons added.

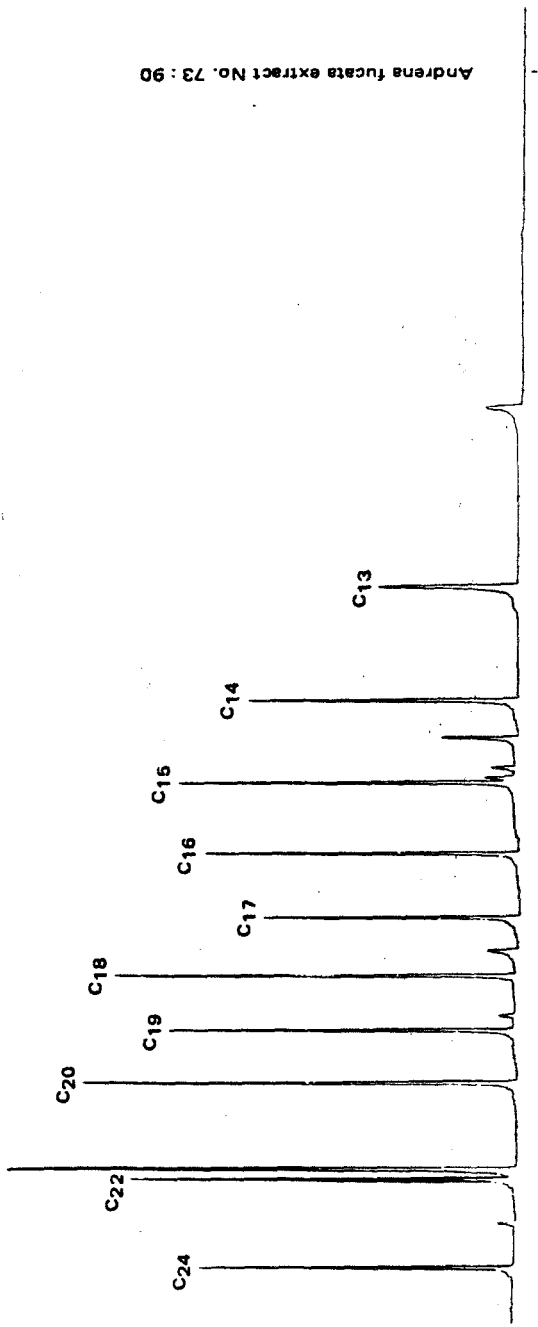

FIG. 4. Capillary gas chromatogram of an extract of Dufour's gland from *Andrena fucata*.

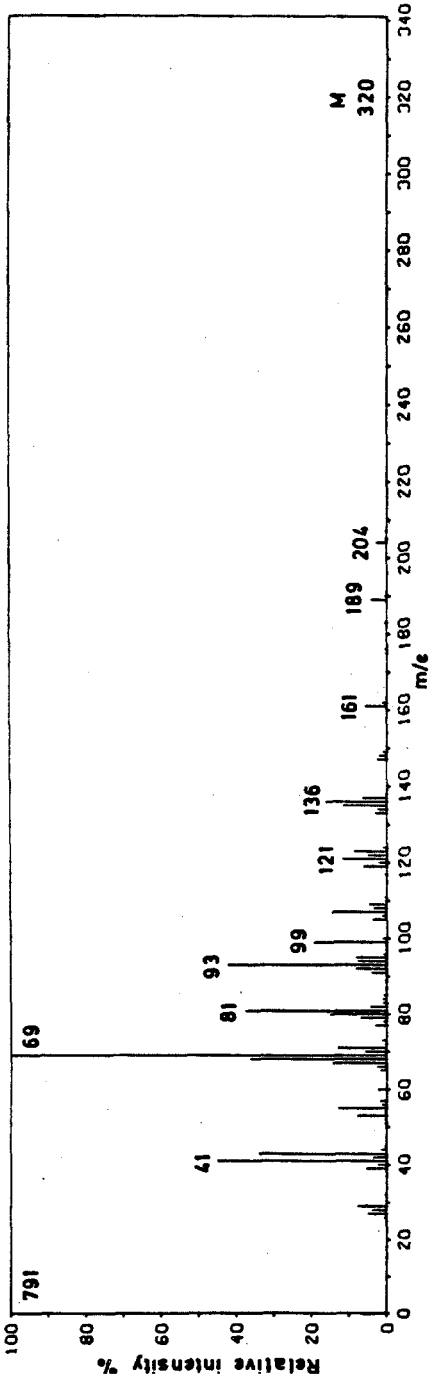

FIG. 5. Mass spectra of the main component of the volatile Dufour gland secretion from *Andrena fucata*.

of a portion of an extract from *A. tibialis* (K.) (a single Dufour gland). The chromatograms show that the volatile secretion is dominated by a single component with the retention index (Kaiser, 1966) 2183.

Chromatograms from all the other species reported here except *A. praecox* (Scop.) and *A. clarkella* (K.) show that they possess a dominating component with the same retention index, with a variation of within ±2 units, as that of *A. tibialis*. In *A. praecox* and in *A. clarkella*, the secretions are dominated (see Figure 3) by a component with the retention index 1929 and 1930, respectively. It may be seen from Figure 4 that the secretion of *A. fucata* Sm. is dominated by a component with a retention index of 2181. This is noteworthy since *A. fucata* is taxonomically grouped with *A. praecox* (subgenus *Andrena* s.st.).

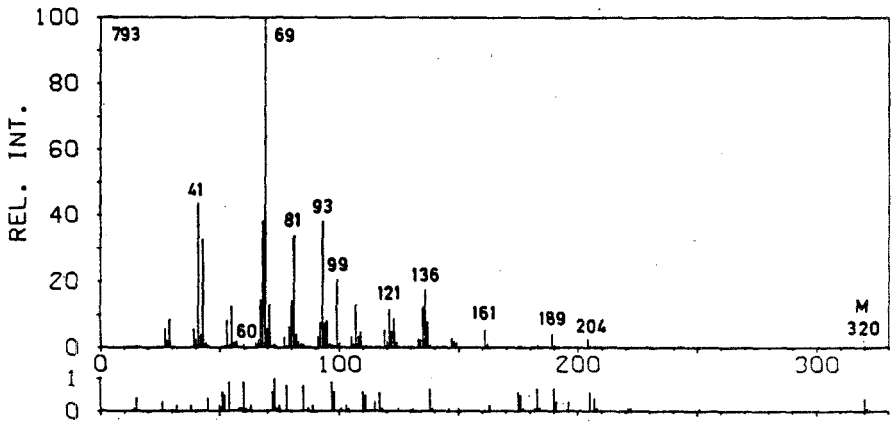

FIG. 6. Mass spectrum of synthetic all-*trans*-farnesyl hexanoate. The lower diagram shows the fragments with a relative intensity of less than 1%, increased by a factor 10. Observe the molecular ion.

Mass Spectrometry of Natural Compounds and Synthetic Isoprenoid Esters

Figures 5 and 7 show mass spectra of the dominating components in *A. fucata* and *A. praecox*, respectively. The spectra are characterized by a fragmentation pattern, typical for an acyclic terpene. The mass spectra of all-*trans*-farnesyl hexanoate and geranyl octanoate, respectively, are given in Figures 6 and 8. These spectra were printed directly via a PDP 15 computer coupled to a LKB 9000 mass spectrometer. The high degree of resemblance between the mass spectra of the natural and synthetic compounds as well as between the retention indices (the retention indices for all-*trans*-farnesyl hexanoate and geranyl octanoate, measured earlier, were found to be 2181 and 1926, respectively) establishes the identity of the main components.

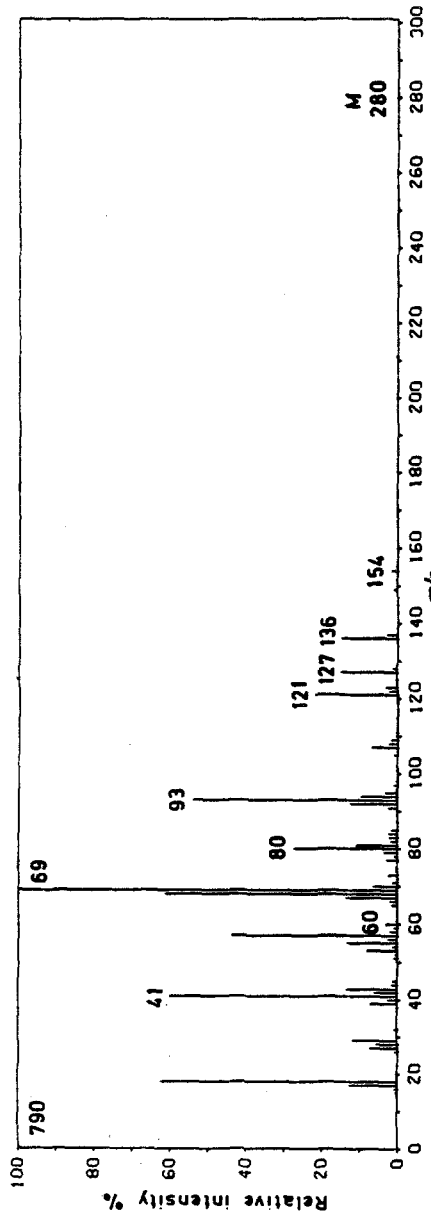

FIG. 7. Mass spectrum of the main component of the volatile Dufour gland secretion from *Andrena praecox*.

Moreover, chromatograms have been run with admixture of the synthetic compounds, producing homogeneous peaks.

The fragments *m/e* 99 and 127, in the mass spectra of farnesyl hexanoate and geranyl octanoate, respectively, correspond to the aliphatic acyl ion. The most intense peak, *m/e* = 69, is due to the isoprenoid ion $(C_5H_9)^+$. A relatively large fragment corresponding to *m/e* = 81 in farnesyl hexanoate and 80 in geranyl octanoate is also characteristic for these compounds. The diagram below the main mass spectra of the synthetic esters gives the fragments with a relative intensity lower than 1%, multiplied by a factor 10. The molecular ion is then revealed.

Three components, identified as isomers of farnesene, are found in small amounts in the Dufour gland secretion of most *Andrena* species. They have a retention index between 1400 and 1500 (see Figures 1, 2, and 4).

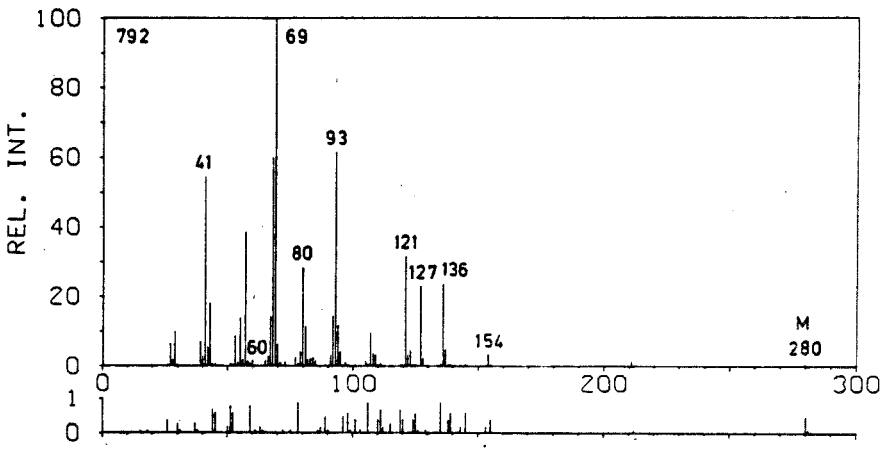

FIG. 8. Mass spectrum of synthetic geranyl octanoate.

Farnesyl and Geranyl Esters in Nomada

The volatile part of the cephalic secretion in males of several species of *Nomada* Scop. has also been investigated. Some of these were found to produce either all-*trans*-farnesyl hexanoate or geranyl octanoate as a major volatile component. Those males of *Nomada*, which were found to produce either farnesyl hexanoate or geranyl octanoate belong to species whose females parasitise *Andrena* bees. The latter have the same isoprenoid ester as main component in the Dufour gland secretion. This remarkable coincidence will be further investigated. Figures 9 and 10 show capillary gas chromatograms of the volatile secretion, emanating from the heads of a single *N. bifida* Thoms. and a single *N. ruficornis* L., respectively. In the latter, a small

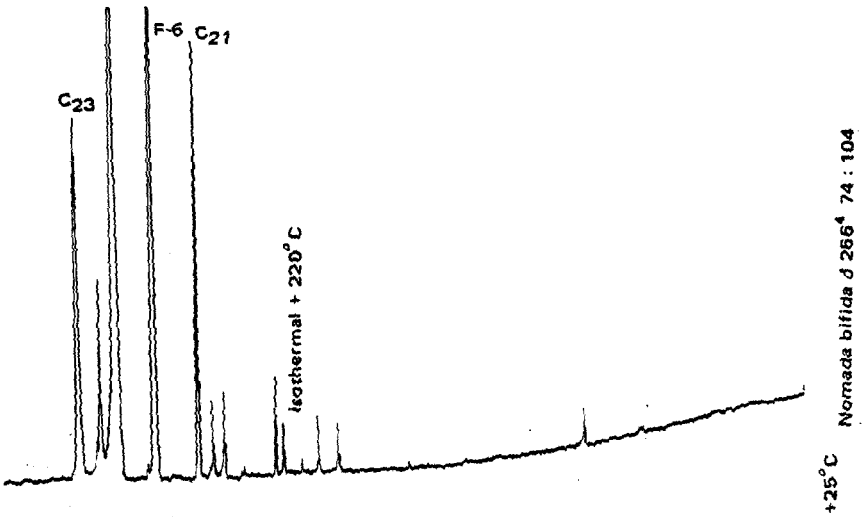

FIG. 9. Capillary gas chromatogram of volatile compounds from one head of *Nomada bifida* male. Temperature is increased from the right to the left in the chromatogram.

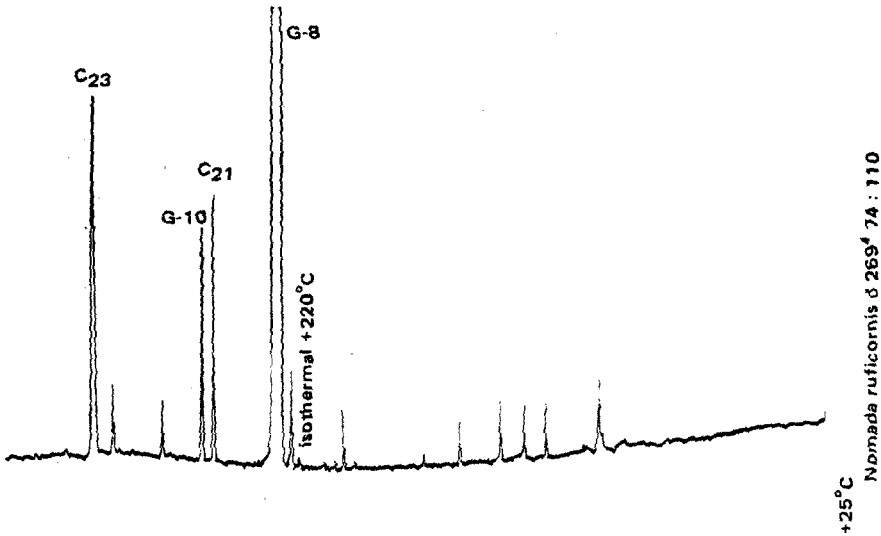

FIG. 10. Capillary gas chromatogram of volatile compounds from one head of *N. ruficornis*, male.

amount of geranyl decanoate (G-10) was also found. Both species also contain heneicosane (C_{21}) and tricosane (C_{23}). The large component between F-6 and C_{23} in *N. bifida* is probably an unsaturated, long-chain alcohol. The amount of this component varies. The gas chromatogram in Figure 9 shows an extraordinary large peak. The small component between F-6 and C_{23} in the same species is a tricosene. A more thorough report of our results from the analyses of the cephalic secretion from *Nomada* is in preparation.

Preparation of Farnesyl and Geranyl Esters

The farnesyl and geranyl esters used in this study were prepared as described earlier by Bergström and Tengö (1974).

DISCUSSION

Together with the earlier reported species of *Andrena* (Bergström and Tengö, 1974), 19 species have now been studied. Of these, 3 have been found to have geranyl octanoate as the main volatile substance, whereas the other 16 secretions are dominated by farnesyl hexanoate. To our knowledge this is the first time that isoprenoid esters with such long fatty acid chains have been found from animal sources. Three of the four species belonging to the *Andrena* s. st. subgenus, have geranyl octanoate as their main volatile compound, whereas the fourth one, *A. fucata,* has farnesyl hexanoate. It is unclear at the present moment whether this fact can be given taxonomical significance.

As shown by Table 1, many species have been collected and analyzed at several different occasions during the years 1969 and 1971–1974. The same gas chromatographic pattern has always been reproduced in the analysis of each species.

The difference in volatility between farnesyl hexanoate (21 carbon atoms) and geranyl octanoate (18 carbon atoms) is noteworthy. In Figure 11, structural formulae for these two compounds are given, together with a diagram showing the relation between retention indices and total number of carbon atoms for farnesyl (F) and geranyl (G) esters. The retention indices refer to an unpolar silicone column, and they express relatively well the corresponding volatility. The possible functional and evolutionary significance of these terpenoid compounds remains an interesting question.

Behavioral tests also remain to be done. Some observations have been made in the field, though, and they point to the fact that the male bees in territorial flight react only weakly toward these substances. It is thought that

these compounds function for nest marking. Butler (1965) has studies this phenomenon in *Andrena flavipes* Panz. He found that soil from the nest sites was attractive to male bees. We have earlier (Bergström and Tengö, 1974) discussed the possible relationship between the volatile esters in the

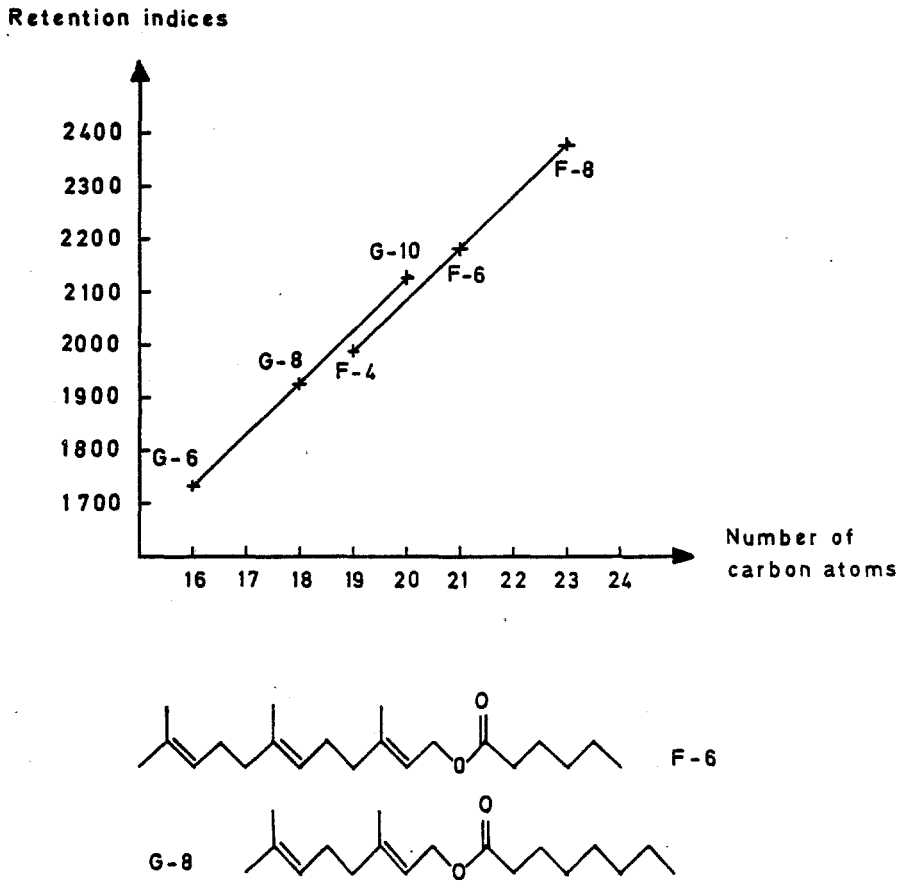

FIG. 11. Retention indices for some farnesyl (F) and geranyl (G) esters. C_n is the total number of carbon atoms. The retention indices are measured relative to straight chain, saturated hydrocarbons. Molecular formulae for all-*trans*-farnesyl hexanoate and geranyl octanoate are given.

Dufour gland and its function for making a hydrophobic coating of the nest cells and galleries.

Analyses of the chemical composition and biological function of glands in the head are in progress in several species of the genera *Andrena*, *Nomada*, and others. Both males and females give off characteristic odors from these glands (Kullenberg, 1956). These odors may function as sex pheromones and/or in species differentiation.

Acknowledgments—This investigation has been financially supported by the Swedish Natural Science Research Council, the Ekhaga Foundation, and the Trygger Foundation. The Axel and Margaret Ax:son Johnson Foundation has also supported the work through grants to the ecological station on Öland. We also want to acknowledge the qualified technical assistance of Miss Monica Åppelgren and Mrs. Inga Åsblom-Groth.

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CHARACTERIZATION AND SYNTHESIS OF WAXES FROM HOMOPTEROUS INSECTS[1]

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Abstract—Wax (mp 102–103°C) taken from the cochineal insect *Dactylopius confusus* was saponified to give 11-oxotriacontanoic acid (1) and 15-oxotetratriacontanol (3) in good yields. The structure of these hydrolysis products follows most directly from examination of the mass spectra of the corresponding methyl ester (2) and acetate (4). These spectra are dominated by McLafferty rearrangements initiated by the ketonic carbonyl groups, and resulting in preferential loss of the unfunctionalized hydrocarbon end of the long-chain esters. The structures are confirmed by synthesis of both hydrolysis products via reaction of di-*n*-nonadecyl cadmium with the appropriate acid chlorides and of the C_{64} wax ester (5) itself by acid-catalyzed condensation of these moieties. Mass spectral examination of the cochineal wax previously characterized as 15-oxotetratriacontanyl 13-oxodotriacontanoate on the basis of purely chemical evidence confirms the assignment, and the Wooly Alder Aphid *Prociphilus tessalatus* is now found to produce the same C_{66} keto-ester.

Key Words—keto-esters, mass spectra, insect waxes, exocrine secretions, defensive substances.

INTRODUCTION

Many insects have integumental glands that secrete waxy materials. The waxes are sometimes produced in large quantity, and they may accumulate

[1] Paper No. 40 of the series *Defense Mechanisms of Arthropods.*

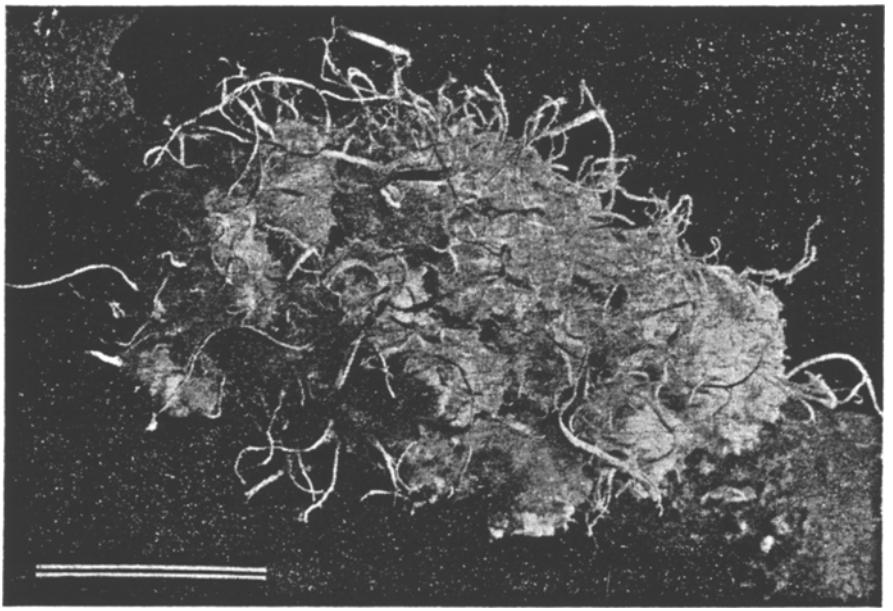


FIG. 1. Small colony of Wooly Alder Aphids (*Prociphilus tesselatus*) on speckled alder (*Alnus rugosa*). The white, loose, waxy covering of the insects is clearly shown. Reference bar = 1 cm.

on the surface of the animals in the form of a brilliantly white, usually powdery or fluffy investiture (Figure 1). As we shall report in a subsequent paper, such investitures are effectively deterrent to predaceous arthropods, which are discouraged from biting into the waxes. Only few waxy secretions of insects have been studied in detail chemically, although it is apparent that complex mixtures are often encountered (Tulloch, 1970). We here report on the structure and synthesis of the wax that loosely covers the body of the cochineal scale insect *Dactylopius confusus* and on the structure of a second wax, previously reported from another cochineal source, which we found to be produced by the Wooly Alder Aphid *Prociphilus tesselatus*.

METHODS AND MATERIALS

A sample of wax from *D. confusus*[5] was isolated by dissolving it from the surface of the insect with benzene and was subsequently purified by

[5] *Dactylopius confusus* Cockerell (*sensu* Ferris, 1955). Four slides with seven specimens of this insect are deposited in the Coccoidea Collection under the label of Dr. D.R. Miller, USNMNH, Beltsville, Maryland 20705. The insects were taken in Tucson, Arizona, on cactus *Opuntia phaeacantha* var. *discata* [Griffiths] Benson and Walkington.

recrystallization from the same solvent. The sample (620 mg, mp 102°–103°C) was saponified with 10% alcoholic potassium hydroxide (reflux, 12 hr). The usual work-up afforded acidic and neutral fractions in high yields (80%). The acidic component was identified as essentially pure 11-oxotriacontanoic acid[6] (**1**, mp 101°–102°C). Esterification of **1** (methanol, sulfuric acid, reflux, 2 hr, 90%) afforded methyl 11-oxotriacontanoate (**2**, mp 71°–74°C), whose spectral data were in accord with the assigned structure: ir (KBr) 5.75 and 5.86 μm; nmr ($CHCl_3$) τ 9.17 (3H, *m*), 8.83 (48H, broad *s*), 7.64 (6H, *m*) and 6.36 (3H, *s*); *m/e* (relative intensity) 480 (3), 310 (22), 295 (14), 228 (100) and 213 (17).[4] Structure **1** was further confirmed by its unequivocal synthesis via the condensation of di-*n*-nonadecyl cadmium (Cason, 1947; Cason et al., 1949) with ω-carbethoxyundecanoyl chloride (Jones, 1947; Chuit, 1926) followed by hydrolysis (10% potassium hydroxide in ethanol, reflux, 2 hr) of the resulting keto-ester (I).

$$[CH_3(CH_2)_{18}]_2Cd + ClOC(CH_2)_9CO_2CH_2CH_3 \xrightarrow[(2)\ KOH]{(1)\ \Phi H,\ H_2O} CH_3(CH_2)_{18}\overset{\overset{O}{\|}}{C}(CH_2)_9CO_2R \qquad (I)$$

1: R = H
2: R = CH_3

The neutral component, isolated after saponification of the wax, was characterized as 15-oxotetratriacontanol (**3**, mp 98°–100°C) on the basis of the following evidence. Acetylation of **3** (acetic anhydride, pyridine, reflux, 5 hr, 80%) afforded the keto-acetate **4** (mp 78°–79°C): ir (KBr) 5.75 and 5.86 μm; nmr ($CHCl_3$) τ 9.17 (3H, *m*), 8.72 (58H, broad *s*), 7.98 (3H, *s*), 7.70 (4H, broad *t*) and 5.95 (2H, *t*); *m/e* (relative intensity) 550 (2), 310 (18), 298 (100), 295 (12), and 277 (20).[7] An independent synthesis of **4** from di-*n*-nonadecyl cadmium and ω-acetoxypentadecanoyl chloride (II) (Chuit and Hausser, 1929) lends additional support for the assigned structure.

$$[CH_3(CH_2)_{18}]_2Cd + ClCO(CH_2)_{14}OCOCH_3 \xrightarrow[(2)\ H_3O^+]{(1)\ \Phi H,\ \Delta} CH_3(CH_2)_{18}\overset{\overset{O}{\|}}{C}(CH_2)_{14}OR \qquad (II)$$

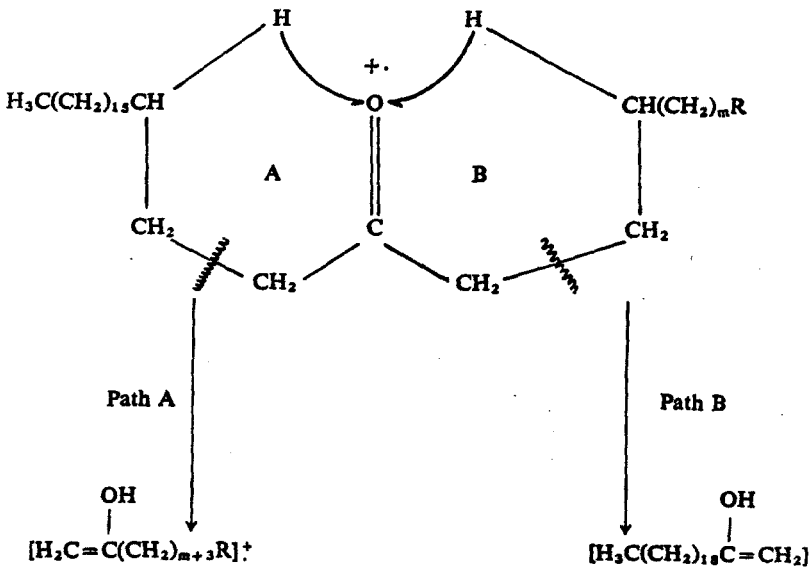
3: R = H
4: R = $COCH_3$

[6] This keto-acid has been isolated previously (Takemoto and Kondo, 1964) from the seeds of *Papaver somniferum*.

[7] Initial data on the wax components obtained by high pressure liquid chromatography followed by chemical ionization mass spectrometry provided the first indication that these were not complex mixtures. This investigation stimulated an in-depth chemical study of waxes from Homopterus insects.

It is interesting to note the diagnostic usefulness of mass spectrometry in elucidating structures **2** and **4**. The presence of the central carbonyl group in these long-chain molecules leads to almost complete suppression of the types of ions usually found in the mass spectra of straight-chain esters (Ryhage and Stenhagen, 1960, 1963). Instead, the major fragments observed result from McLafferty rearrangements initiated by the ketonic carbonyl group. In compounds **2** and **4**, the base peaks correspond to selective γ-hydrogen abstraction, accompanied by β-cleavage as indicated in Path A, Scheme I. The alternative rearrangement possibility (Path B, Scheme I) is clearly less important in both cases.[8] This observation, coupled with examination of the other major fragment ions, allows one to deduce the position of the carbonyl group in the chain. The presence of homologous keto-esters, even in small amounts, is also easily detected without ambiguity by inspection for homologous, even *m/e* fragment ions.

Scheme I



3: R = CO_2CH_3, m = 6: A⟶*m/e* 228 (100), B⟶*m/e* 310 (22)
4: R = $OCOCH_3$, m = 11: A⟶*m/e* 298 (100), B⟶*m/e* 310 (18)

DISCUSSION

Careful inspection of the mass spectra of **2** and **4** (obtained from the natural wax) showed the following results: **2** was found to be homogeneous

[8] The preferential loss of the unfunctionalized hydrocarbon end of these long-chain esters is striking. This selectivity would seem to merit further study.

with no admixture of higher or lower homologs, while **4** contained a trace amount of the next higher even homolog (17-oxohexatriacontanol).

We conclude that this insect wax consists of almost pure 15-oxotetratriacontanyl 11-oxotriacontanoate (**5**). A synthesis of **5** (III) from **1** and **3** (*p*-toluenesulphonic acid, 180°C, 10 mm, 4 hr, 95%) produced an ester (mp 102°–104°C, mixture mp 102°–104°C) whose infrared spectrum was indistinguishable from that of the natural wax.

$$1+3 \longrightarrow \underset{\mathbf{5}}{CH_3(CH_2)_{18}C(CH_2)_9CO(CH_2)_{14}C(CH_2)_{18}CH_3} \qquad \text{(III)}$$

This result takes on special interest in view of our earlier work on a wax obtained from a commercial source of cochineal insect (labeled as *Coccus cacti*, without reference to geographic origin) (Chibnall et al., 1934). In that study we concluded on the basis of extensive degradative experiments that the wax consisted largely of an ester of the same C_{34} keto-alcohol (**3**) and a C_{32} keto-acid (13-oxodotriacontanoic acid, **6**). We have now subjected these original wax samples to hydrolysis, derivatization, and mass spectral analysis, as described above, and were able to confirm the previously assigned structure. In addition, we have synthesized this C_{66} wax (**7**) and find that its infrared spectrum in the 1450–1300 cm^{-1} region clearly differentiates it from that isolated from *D. confusus*.

$$\underset{\mathbf{(6)}}{CH_3(CH_2)_{18}C(CH_2)_{11}CO_2H} \qquad \underset{\mathbf{(7)}}{CH_3(CH_2)_{18}C(CH_2)_{11}CO(CH_2)_{14}C(CH_2)_{18}CH_3}$$

Finally, we have examined the benzene-soluble wax obtained from the Wooly Alder Aphid *P. tesselatus* (Figure 1).[9] This material also hydrolyzed to give alcohol **3** and acid **6** and is therefore identical to the cochineal wax **7**.

Acknowledgments—We thank Dr. William L. Nutting and Dr. Douglass R. Miller for respectively collecting and identifying the *Dactylopius*, Dr. Charles T. Mason for identifying the *Opuntia*, and Dr. Louise M. Russell for identifying the *Prociphilus*. We also thank Dr. John D. Henion and Dr. Patrick J. Arpino for assistance with obtaining mass spectra and HPLC examination of the wax hydrolysis products. The Huyck Preserve, Rensselaerville, New York, kindly offered the use of its field facilities for the study of *Prociphilus* and for the collection of its wax. The partial support of this research by the National Institutes of Health (Grant Nos. AI-12020 and AI-02908) and by the National Science Foundation (Grant No. BMS-74-15084) is acknowledged with pleasure.

[9] *Prociphilus tesselatus* (Fitch), taken in Rensselaerville, New York, on alder *Alnus rugosa* [Du Roi] Spren.

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VOLATILE COMPOUNDS FROM THE TARSAL SCENT GLAND OF REINDEER (*Rangifer tarandus*)

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Abstract—Volatile compounds from the tarsal scent gland of reindeer (*Rangifer tarandus*) were isolated by a precolumn technique and analyzed on a combined gas chromatograph–mass spectrometer. Saturated aldehydes and alcohols were found in both male and female tarsal scent gland.

Key Words—reindeer, skin glands, tarsal gland, volatile compounds.

INTRODUCTION

Rapid progress has been made in recent years in attaining a better understanding of the biological function of mammalian skin glands, but little is known at present about the chemical signals they emit.

The first chemical and ethological investigation was that of Brownlee et al. (1969), who isolated *cis*-4-hydroxydodec-6-enoic acid lactone from the tarsal scent gland of the male black-tailed deer (*Odocoileus hemionus columbianus*). The lactone was shown to be active in individual recognition (Müller-Schwarze, 1971).

Many other mammals possess cutaneous scent glands, which play an important role in social and territorial organization (Lederer, 1950; Mykytowycz, 1972; Eisenberg and Kleiman, 1972). The tarsal and interdigital glands of reindeer (*Rangifer tarandus*) are the most important skin glands (Quay, 1955). Reindeer biology has been intensively studied in Sweden by Espmark, and it has been found that olfactory as well as visual and acoustic signals play an important role in communication among members of the herd

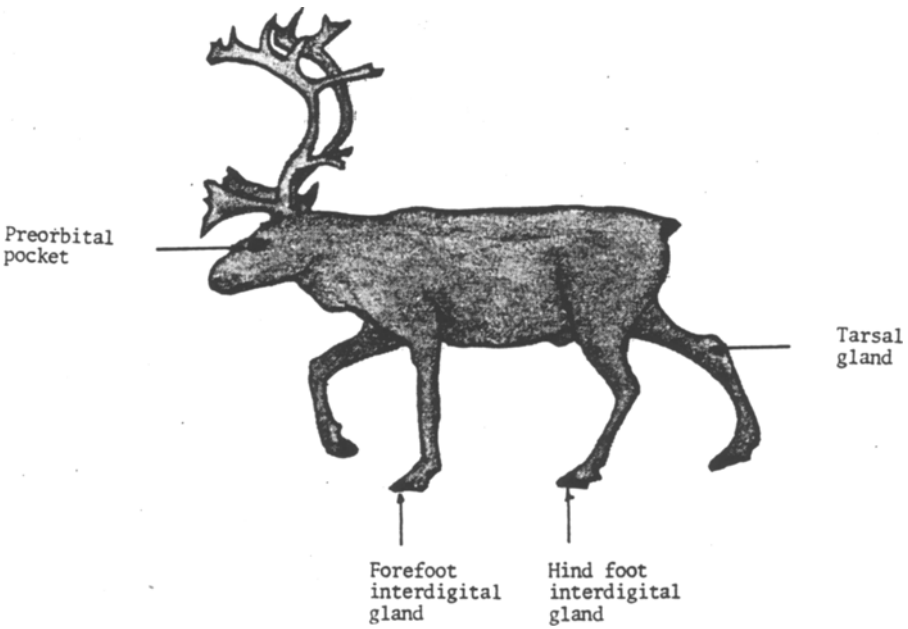

FIG. 1. Location of the skin gland areas in reindeer.

(Quay, 1955; Espmark, 1964, 1971*a*, 1971*b*). The tarsal gland of reindeer is located on the medial side of the tarsal joint and is easily observed because of a tuft of light-colored, stiff hairs (Figure 1).

The cytochemistry and histology of the skin gland areas have been studied by Quay (1955), who found a striking difference between the glands, indicating that they have different functions.

In this paper we wish to report the preliminary results of chemical investigations of volatile compounds from the tarsal scent gland of reindeer.

EXPERIMENTAL SYSTEMS

Gas Chromatographic System

A Pye-Unicam model 64 gas chromatograph equipped with a flame ionization detector (FID) was used. It was fitted with a glass column (2.7 m, inside diameter [ID] 0.40 cm) filled with 3% OV-17 on acid-washed, dimethyl dichlorosilane-treated Chromosorb W (100–120 mesh). The carrier gas was nitrogen at a flow-rate of 40 ml/min. The column-oven was temperature-programmed from 80° to 200°C at a rate of 5°/min, the detector temperature was 250°C, and the injector temperature was 250°C.

Gas Chromatographic–Mass Spectrometric System

A LKB 9000 mass spectrometer equipped with a Pye model 84 gas chromatograph and a split to a FID was used. The carrier gas was helium at a flow-rate of 35 ml/min, 27 ml/min to the separator, and 8 ml/min to the FID.

The column and operating conditions for the gas chromatograph were as above, and the temperature on the connection between it and the mass spectrometer was 250°C. Operating conditions for the mass spectrometer were: separator temperature, 250°C; ion source temperature, 270°C; and electron energy, 70 eV.

METHODS AND MATERIALS

The tarsal glands were collected at the annual slaughter at Klimpfjäll in northern Sweden in September, 1973. The glands were excised within 30 minutes of death, frozen in liquid nitrogen, and then stored at −20°C in the dark until analyzed. In order to analyze a single tarsal gland without solvent extraction, we used a modified precolumn heater (Figure 2) (Karlsen, 1972; Ställberg-Stenhagen, 1972). The hair tuft from a single tarsal gland was shaved off and placed in the inner glass tube to which the gas inlet tube was connected. The metal cylinder was electrically heated to 100°C and a stream of pure nitrogen passed through the inner glass tube at a flow-rate of 30 ml/min. The volatile compounds driven off through the needle were adsorbed at the beginning of the column, kept at room temperature. After 30 minutes the precolumn heater was removed, the carrier gas was adjusted to 40 ml/min, and the column was heated from room temperature to 80°C, and held there for 2 minutes. Thereafter the programming of the column heating was started.

In order to collect larger amounts of the sample for mass spectrometric analysis, the method was modified and the volatile compounds were led into a

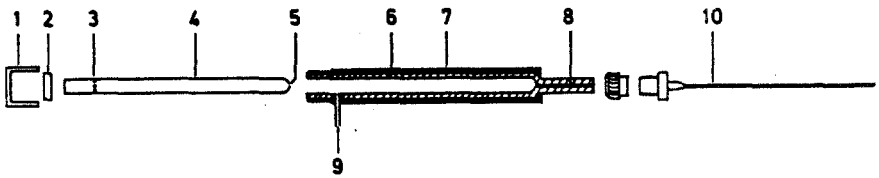

FIG. 2. Precolumn heater for sampling of volatile material for gas chromatography. 1 = brass screw cap; 2 = septum; 3 = gas inlet tube holes; 4 = inner glass tube; 5 = gas outlet hole; 6 = outer glass tube; 7 = brass cylinder; 8 = glass capillary; 9 = gas inlet tube; 10 = injection needle.

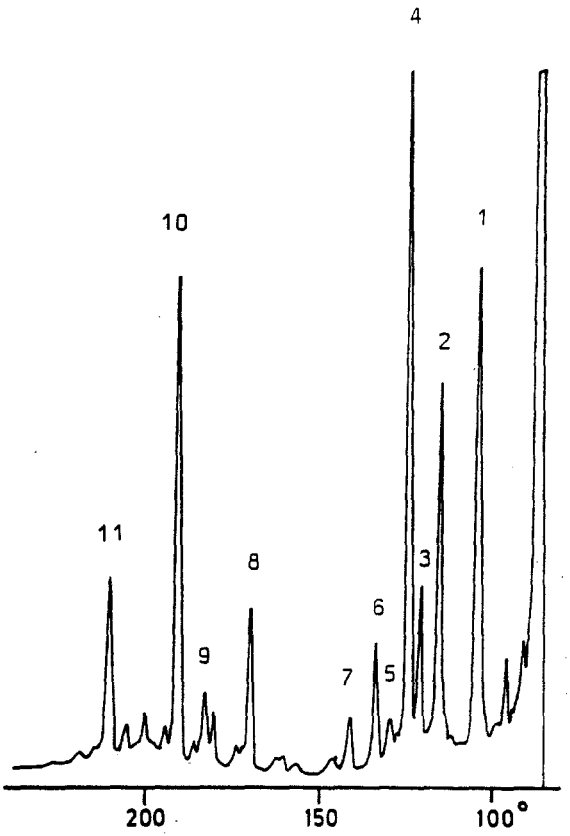

FIG. 3. Gas chromatogram of 10 male tarsal scent glands.

glass tube containing 10 ml of dichloromethane (Merck) cooled to +4°C. Because of the freezing of water in the needle, the dichloromethane could not be cooled below zero. Hair tufts from 10 tarsal glands were treated in this way for 24 hours. The water (0.1 ml) was separated and the organic layer dried over $MgSO_4$. After evaporation of the solvent at 24–30°C under reduced pressure, the sample was analyzed by GC-MS (Figure 3).

RESULTS

By combined GC-MS the following compounds were identified in both male and female tarsal scent (numerals in parentheses refer to Figure 3). *Aldehydes*: *n*-heptanal (1), *n*-octanal (2), *n*-nonanal (4), and *n*-decanal (6);

Alcohols: *n*-dodecanol (8), *n*-tetradecanol (10), and *n*-hexadecanol (11); *Hydrocarbons*: *n*-heptadecane (9).

The structures were verified by comparison with mass spectra of authentic samples and from GC-retention times on several column packings.

High molecular weight alcohols have also been found in human hair fat. Alcohols having an even number of carbon atoms were present in significantly greater quantity than the odd homologue (Brown and Young, 1954).

The data further indicate the presence of a homologous series of unsaturated hydrocarbons in the scent. Thus, peaks 3, 5, and 7 in Figure 3 are believed to be dienes with 11, 12, and 13 carbon atoms respectively. The positions of the double bonds have not yet been established.

Gas chromatograms of single tarsal glands from male and female reindeer, obtained without solvent extraction, are shown in Figure 4. These chromatograms were found to be very reproducible with respect to the retention times of the different compounds, and a comparison of the volatile

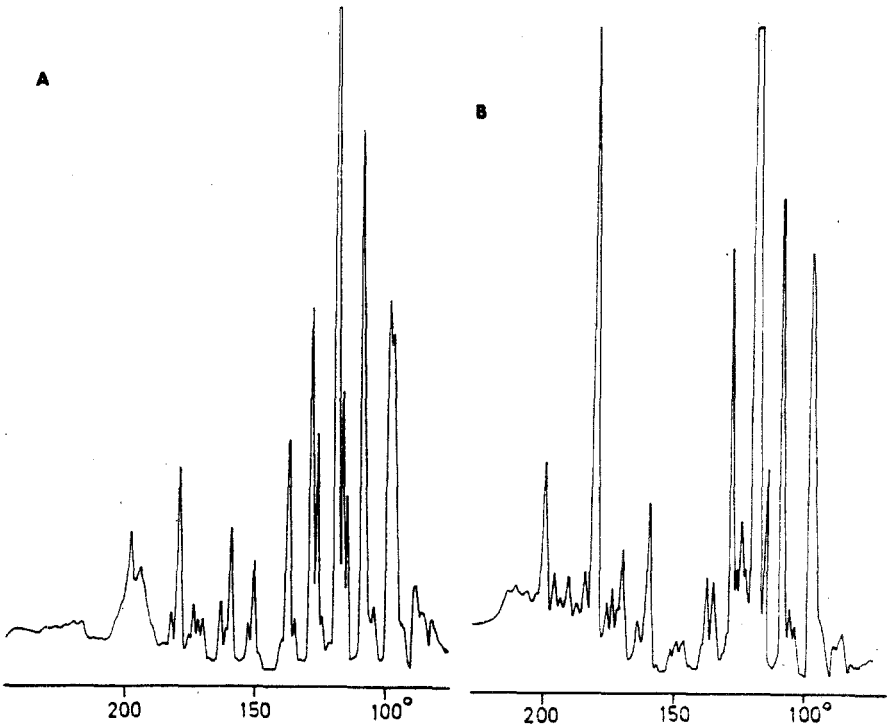

FIG. 4. Gas chromatograms of male (A) and female (B) tarsal scent isolated by a precolumn technique.

compounds from individual tarsal glands was therefore possible. In our investigation we observed a slight difference between gas chromatograms from individual tarsal glands. This was not because of a sexual difference, but was, rather, an individual variation. The total amount of secretion, on the other hand, was found to vary with sex. Female tarsal glands contained larger amounts of the secretion than male, which is in agreement with earlier investigations (Quay, 1955).

The amount of *n*-nonanal (Figure 4, peak 4)—the major component in both sexes—varied from 1 to 10 μg per gland. On the contrary, the very major components of tarsal gland secretion from another season (April, 1973) were identified as valeric and isovaleric acids. These glands also contained a markedly larger amount of the secretion than the "Klimpfjäll" material. This indicates that content and amount of secretion may vary with the season. It was not possible to reproduce this observation because of the small number of glands collected. Further studies in this area are in progress.

Short-chain fatty acids have also been found in the anal gland secretion of the red fox (Albone and Fox, 1971) and in the subauricular scent of the male pronghorn (Müller-Schwarze et al., 1974).

Behavioral studies on reindeer confronted with synthetic compounds of tarsal scent are in progress, and the results of this investigation will be published later on. We also intend to make subsequent studies of tarsal glands collected in different seasons in order to elucidate further the variations found.

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TERMINATION OF AGGREGATION BY THE EUROPEAN ELM BARK BEETLE, *Scolytus multistriatus*[1]

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Abstract—The attractiveness of European elm bark beetle virgin females boring in elm logs declined within two days after the introduction of males. However, in the laboratory and in the field the number of beetles attracted to female-infested bolts was not depressed by the presence of separate logs infested with males or both sexes. It is concluded that the decline in attractiveness following cohabitation of the sexes results from termination of the production of attractant pheromone by mated females rather than the production of anti-attractants by either sex.

Key Words—aggregation attractant, pheromone, *Scolytus multistriatus*, attraction termination.

INTRODUCTION

First observed in the United States in Cambridge, Massachusetts, in 1909 (Chapman, 1910) but probably introduced some years before (Collins, 1938), the European elm bark beetle, *Scolytus multistriatus* Marsham, is now found in 45 of the contiguous states (Barger and Hock, 1971). Prior to the introduction of the Dutch elm disease-causing fungus, *Ceratocystis ulmi* (Buisman)

[1] From a thesis, "Factors affecting attraction of *Scolytus multistriatus* Marsham (Coleoptera: Scolytidae) to female pheromone", submitted by the senior author in partial fulfillment of the Master of Science degree. This study was supported by grant NE-01 (Research Foundation Account # 10-382A) from the U.S. Forest Service.

C. Moreau, *S. multistriatus* was not of great economic significance. However, as the principal vector of the fungus in this country it rapidly became a serious pest.

Early researchers observed that the European elm bark beetle aggregates on certain elm trees or host material (Felt, 1935; Martin, 1935). Evidence that a chemical attractant is responsible for this phenomenon was first presented by Meyer and Norris (1967) who showed that the attractiveness of elm bolts increased following attack by female beetles and attributed this attraction to host volatiles released by mining of the inner bark. However, Peacock et al. (1971) concluded that the greatly enhanced attraction was the result of a pheromone emitted by attacking virgin females; logs that contained males or both sexes attracted far fewer beetles than those that held only virgin females. Furthermore, laboratory bioassays (Peacock et al., 1973) demonstrated that frass produced by virgin females was attractive to both sexes. Attraction increased until the fourth day following initiation of boring and remained high through the tenth day. Attractiveness of frass from an infestation of both sexes peaked on the second day then dropped to a low level. Frass from males was minimally attractive throughout the 10-day period of the test.

From these data is seems clear either that pheromone production ceased after mating or that a masking agent was produced by the male or the mated female. The determination of which of these alternatives is operative was an objective of a program which has now isolated, identified, and synthesized the aggregating pheromone of *S. multistriatus* (Pearce et al., 1975.) The knowledge that an antiattractant is responsible would permit efficient chemical separation of the attractants and antiattractants produced by beetles regardless of sex, rather than the system used, i.e., collection of pheromone produced by several hundred thousand tediously separated virgin females. In addition, an antiattractant per se might be useful in realizing our long-term objective of reducing Dutch elm disease through control of *S. multistriatus*.

This paper presents laboratory and field tests directed at describing the degradation of attraction. Specific objectives were to document the duration of attraction of female-infested logs following the introduction of males and to determine whether the decline of attraction resulted from masking or cessation of pheromone production.

METHODS AND MATERIALS

The olfactometer developed by Moeck (1970) was used for laboratory bioassays conducted in a darkened room maintained at 21°C and 81%

relative humidity. Air forced through a 4.78 liter jar containing the test log was conveyed in a fine jet along the runway of the olfactometer at 300–400 ml/min, directed perpendicular to a beam of light from a microscope lamp. 1–5 beetles were released at the side of the olfactometer opposite the lamp and were induced to cross the stream of air by their positive phototaxis. Beetles that turned toward the source of air and walked upstream and those that circled in the stream of air were considered to have responded positively. Beetles were conditioned prior to bioassays by holding them for 2 days on slightly moistened paper towelling in petri dishes stored in the semidarkness of the bioassay room.[4]

Field assays were conducted in Liverpool, New York, in a natural stand of American elm (*Ulmus americana* L.) heavily infested with *S. multistriatus*. 5 treatments were used to test for pheromone masking after males join females (Table 2). In each case, small (20–25 cm × 8–13 cm), freshly cut elm bolts served as host material. Females were induced to bore into bolts 1 day before exposure in the field, but males were added immediately before the tests. Bolts were put in fiberglass screen bags to prevent movement of the beetles. Each treatment was caged separately in a 30-cm × 30-cm cylinder made of 6 mm (¼ in. mesh) hardware cloth elevated 1 m above the ground on a plywood platform. Another square of plywood served as a top for the cylinder which, along with the base, was coated with Stickem Special®. Treatments were replicated twice in cages 5 m apart in two parallel lines. A first series of treatments exposed from June 8 to June 22, 1972, was replaced by a second set of beetle-infested logs that was in the field from June 23 to July 11.

The duration of attractiveness of females after they are joined by males was examined in another field study from August 7 until September 10. Methods used to trap beetles were similar to those described above. Each of two parallel lines included 2 traps that held bolts infested with virgin females and 6 traps that bore bolts with males and females at various ages of infestation. Female beetles were allowed to enter the bolt the day before it was placed in the field whereas males were added when the logs were positioned. A series of 6 logs of different infestation ages was established in each line of traps by adding a freshly attacked bolt every 2 days. Thereafter, continuous representation of this series of bolts bearing both sexes was maintained by removing the oldest log and adding a freshly attacked one every second day. A similar regime was followed every fifth day for female-infested logs.

Beetles used to infest host material and those employed in laboratory bioassays were reared from naturally infested elm logs; sexes were separated using the dense brush of setae on the frons of the male as the criterion.

[4] In more recent tests beetles have been conditioned by holding them for 12 hours on dry paper under a fluorescent lamp.

RESULTS AND DISCUSSION

In laboratory bioassays both sexes responded strongly to females in bolts or to males and females in separate bolts held in the same container; response to males in bolts was weak and response to males and females in the same bolt was almost nil (Table 1). Field tests, for the most part, corroborated these findings (Table 2). Traps that contained a log infested by virgin females, whether alone or combined with a bolt infested by either male beetles or both sexes, consistently caught more beetles than those that lacked virgin females.

Comparison of the response to treatments 2 and 4 (Table 2) indicates that an antiattractant is not emitted after mating; if such a substance were elaborated, attraction to the virgin females in treatment 4 should have been reduced by the presence of mating beetles. However, during the first 4 days of the test daily catches at treatment 4 were substantially greater than those at treatments 1 and 3. Thereafter the response to these treatments was approximately equal. It seems that the second bolt with both sexes enhanced the attractiveness of treatment 4 until the females in this bolt were mated.

The strong response to treatment 2 in line A of series I was an anomoly. An aggregate of only 36 beetles was caught on the 3 other traps that contained treatment 2. However, 521 (67%) of the 778 beetles taken on this trap were caught in a 1-hr period during which one of us (E.W.E.) was tending the traps. The weather had initially been cool (ca. 19°C) and drizzly. While beetles were being removed from sticky traps, the sky partially cleared and the precipitation stopped. Beetles were observed landing in some of the traps

TABLE 1. LABORATORY RESPONSE OF PEDESTRIAN *Scolytus multistriatus* TO INFESTED AND UNINFESTED ELM LOGS IN MOECK OLFACTOMETER

Treatment	Percent positive response by[a]	
	Males	Females
50 females in bolt	71.4	75.5
50 males in bolt	45.7	8.0
50 males, 50 females in the same bolt	0.0	3.2
50 males, 50 females in separate bolts[b]	68.6	71.4
Uninfested bolt	24.0	4.0

[a] 25–45 beetles per test.
[b] Bolts stored separately until test begun.

TABLE 2. FIELD RESPONSE OF *Scolytus multistriatus* TO COMBINATIONS OF FEMALE AND MALE BEETLES BORING IN ELM BOLTS, LIVERPOOL, NEW YORK, JUNE 8 TO JULY 11, 1972

		Treatment				
		1 (♀)[b]	2 (♀+♂)	3 (♀)+(♂)	4 (♀+♂)+(♀)	5 (♂)
Series[a]	Line	Number of responding beetles trapped				
I	A	562	778[c]	525	794	55
	B	280	24	463	309	24
II	A	60	3	154	47	7
	B	53	9	71	71	14
Totals		955	814	1213	1221	100

[a] Series I exposed from June 8 to 22, 1972; series II from June 23 to July 11, 1972.

[b] The sexes within a set of parentheses were in the same bolt. In treatment 2, for example, males and females were in the same bolt, while in treatment 3 the sexes were contained in separate bolts.

[c] The large catch on this trap reflects the "anomalous" collection of 521 beetles in a 1-hour period; see text for discussion.

being tended. All traps were rechecked and found to have captured some beetles, but only the catch on the trap in question was extraordinary. Most of these (ca. 75%) were concentrated on one side as if a compact swarm had encountered the trap. Such swarms of *S. multistriatus* have been observed by A.C. Lincoln[5] (personal communication) and one of us (G.N.L.).

The influence of males on the attractiveness of females in bolts is further documented by our second field test (Figure 1). 2 days after introduction of the males, bolts containing both sexes attracted considerably fewer beetles than those bearing virgin females. Attraction to bolts containing mating beetles continued to decline after the fourth day, whereas attractiveness of the virgin females increased until the eighth day. Finally during the 10th and 11th days the attractiveness of virgin females had fallen to approximately the peak level of attraction to mated beetles. Peacock et al. (1973) reported rapid decline of attractiveness of frass when males were added during the 6th day after infestation with virgin females. Frass from a parallel infestation of virgin females remained attractive after 10 days.

It is unlikely that a change in the quality or quantity of volatile chemical

[5] Formerly U.S. Forest Service, Delaware, Ohio; currently with Environmental Protection Administration.

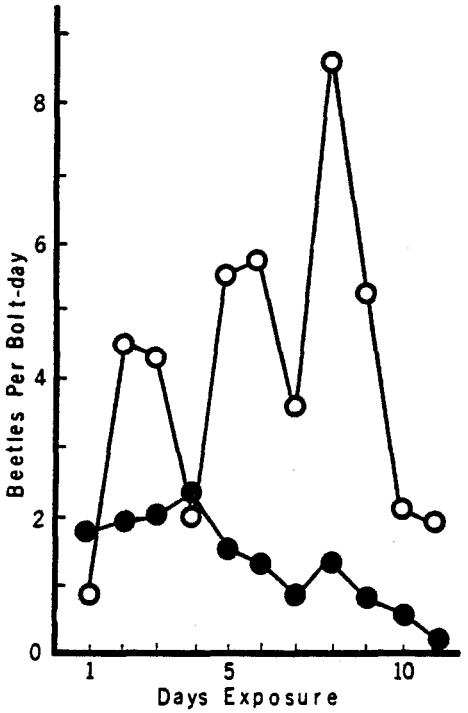

FIG. 1. Response of field populations of *Scolytus multistriatus* to virgin females (open circles) and females with males (solid circles) in elm bolts. The horizontal axis represents the infestation age—i.e., the number of days since introduction of beetles to bolts. The vertical axis indicates the mean catch on traps containing bolts of the various infestation ages. After the first day of the experiment bolts of different infestation ages were present simultaneously; by the eleventh day all infestation ages were present.

attractants of the host could account for these differences. We conclude from these tests that insemination of all the available virgins occurs rapidly following introduction of the highly polygamous males (Bartels and Lanier, 1974), and that females cease production of pheromone soon after they are mated. It was therefore imperative that the sexes be separated and that only virgin females be used to mass-produce attractant pheromones from boring beetles. Furthermore, the employment of beetle-produced antiattractant appears to be nonviable as a strategy for control of *S. multistriatus*.

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the manuscript by Dr. Douglas C. Allen of the College of Environmental Science and Forestry and Dr. John W. Peacock of the Northeastern Forest Experiment Station, Delaware, Ohio.

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SENSITIVITY OF WOMEN TO MUSK ODOR: NO MENSTRUAL VARIATION

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Abstract—It has been reported that the sensitivity of women to the musky odor of pentadecalactone exhibits cyclic variation, with a marked peak at about the time of ovulation. This claim was carefully reinvestigated, but was found to be untrue in the conditions of our experiments. Possible reasons for the discrepancy are discussed.

Key Words—menstrual cycle, musky odor, odor thresholds, pentadecalactone, women.

INTRODUCTION

Le Magnen (1952) claimed that the sensitivity of women to the odor of the macrocyclic musk pentadecalactone increases substantially, and sometimes enormously, near ovulation. His results received qualified support from Vierling and Rock (1967) who found two peaks of sensitivity, one just before ovulation, and one in the luteal phase. This phenomenon was recently cited by Comfort (1973) in his critique of our finding that odor-blindness to the same compound is inherited as a simple recessive character (Whissell-Buechy and Amoore, 1973). Nevertheless, Le Magnen's phenomenon could not be confirmed by Kloek (1961) or by Henkin (quoted by Vierling and Rock, 1967). We have reinvestigated the reported cyclic sensitivity of women to musk odor, but with improved olfactometric technique we find only negligible random variations.

METHODS AND MATERIALS

We determined the olfactory thresholds to fresh aqueous binary dilutions of pentadecalactone (Thibetolide®, Givaudan Corp., Clifton, New Jersey). Each binary dilution step is half the concentration of the one before, starting from step 0 for the saturated solution (1.1 ppm, w/v). Our 3-flask test procedure has been described (Whissell-Buechy and Amoore, 1973). The subject has to distinguish 1 odorous flask from 2 blanks; this test is done twice at each dilution step. The threshold step numbers recorded in this paper are the highest consecutive dilution correctly chosen. The established normal threshold range extends from step 7 to step 13, mean about step 10 (0.001 ppm). In the course of a prolonged and intensive search for Le Magnen's phenomenon, we did occasionally observe some extraordinarily high "sensitivities," recalling the 10^{-11} ppm (about step 36) noted for one subject by Le Magnen (1952, Figure 4). 2 women (ages 26 and 28) twice achieved scores in the range of steps 22 to 24. These peaks occurred at no common point in the menstrual cycle. Comparable high scores (steps 19–22) were achieved by a 5-year-old girl, by a 55-year-old woman, and by a man, which ruled out ovulation as the cause of the phenomenon. The peaks were traced to a *nonmusky* and much stronger odor, which on these occasions accumulated in the diluted pentadecalactone if the flasks remained unopened for 20 min or more. The off-odor was reminiscent of a bacterial culture. We found that taking quasiaseptic precautions during the preparation of the test flasks completely prevented any further off-odors and phenomenal peaks.

In the following experiment the glass-stoppered 125-ml Erlenmeyer flasks were replaced with plain flasks, capped with inverted disposable 1-oz polypropylene medicine cups. After each daily use the flasks were washed successively with ethanol, trisodium phosphate, and deionized water, then dried in a forced-air oven overnight at 105°C. Directly after cooling they were rinsed with odorless sterilized water and capped to keep out dust. During the threshold tests each subject opened and sniffed the entire series of flasks from step 4 through step 16. With an average interval of 6 min between subjects there was no build-up of foreign odor, and nobody ever achieved a higher threshold sensitivity than step 15.

RESULTS

Our volunteers were 22 women (ages 17–39) and 5 men (ages 27–43) employed at the Western Regional Research Laboratory. Persons specifically anosmic to pentadecalactone were omitted. The women recorded their oral temperatures daily on arising, and noted onset of menses. The average cycle

TABLE 1. DAILY MEASUREMENTS OF OLFACTORY THRESHOLDS TO PENTADECALACTONE FOR 22 WOMEN AND 5 MEN

			August 1973 calendar day of test																		Menses[a]		
Subject	Sex	Pill	7	8	9	10	13	14	15	16	17	20	21	22	23	24	27	28	29	30	31	Before	During	After
1	F	–[b]	8	9	7	8	9	9	11	8[d]	6	8	7	9	7	8	8	9	5	8	9	8/1	8/31	
2	F	–[b]	9	9	9	11	9	8	8	8	7	7	9	9	7		10	8	8	8	9	7/13	8/9	9/6
(3)[e]	F	–[c]	5	5				8	6	5	6	8		6			8	6	5	7	8		8/17	9/16
(4)	F	–[c]			7	10	6		8	8	6	7		6	5	8	10		8	7	10			9/4
(5)	F	–[b]			11	15	13	11	13	13	11	11	12	10	13	10	13	9	11	11	11	7/23	8/20	9/19
(6)	F	–			5	8	8	8	5	6	6	6			4	7	6	6	10	9	10	7/3	8/8	9/8
(7)	F	–[b]	6	11	4	7	6	8	10	10	9		8	10	9	7	8	6	7	11	10	8/4		9/2
8	F	–[b]	9			8	9	11				7		9	9		9	10	7	8	10	7/26	8/22	9/14
(9)	F	–	7	7	9	10	5	10	7	5	7	6	8	7	4	6	6	8	5	7	6	7/20	8/17	9/9
(10)	F	–[b]	11	13	9	11	11	8	10	9	11											8/3	8/30	
(11)	F	–[b]	8		11	9	10		9	9	12	10	10	11	11	12	13	11	10	12	11	8/3	8/26	9/19
(12)	F	–[b]	12	11	6	9	9	11	10	11	9	10	10	11	11	9	11	11	10	11	12	7/28	8/25	9/21
(13)	F	–[c]	9				8	7	6	8	7	8	8	7	9	6	6	8	5	7		8/1	8/30	
(14)	F	–[b]	<4		7	7	7	6	7	6	4	4		6			6	6	7	9	7	7/14	8/13	9/12
1	F	+	12	11	9	13	8	12	10	8	8		9	10	9	8	11	11	7	11	11	7/29	8/26	9/23
2	F	+			10	9	9			7	8	5	9	9	11	8	12	7	10	10	12	7/26	8/22	9/19
3	F	+	8	11	10	10	10	10	10	11	11	9	9	10	8	10	12	12	10	12	10	8/6		9/3
4	F	+	6	9	8	8	6	8	7	8	6	7	6	6	6	5	9	7	8	8	7	7/28	8/26	9/22
5	F	+	10	14	11	13	10	11	10	6	9	11	9	11	9	7	6	8	11	10	9	7/24	8/21	9/18
(6)	F	+[c]			8	8	8	8		8				9	8	9					9		8/24	9/17
(7)	F	+			7			7	4	9	9	10		7		7		8	4	8			8/10	9/4
8	F	+	11		12	13	12	11	11	9	9	9	9	11		11	11	11	11	11	12	7/30	8/28	9/23
1	M		10	10	10	12	9	12	11	12	12		11	13	10	10	10	11	10	12	11			
2	M		8	10	10	11	9	10	10	13	10	8	9			9	9	9	9	10	7			
3	M			13	12	12	12	11	15	9	8	9	9	9	10	10	11	9	7	11	8			
4	M		9	9	7	9	7	8	7	9	9	10	9	9	10	8	9	8	7	12	11			
5	M		9	14	12	12	10	11	10	8	6	8			12	11	11	13	11	11	11			

[a] First day of menses, before, during or after period of tests.
[b] Ovulation occurred during period of tests, according to temperature chart.
[c] Subject did not keep adequate chart.
[d] Underlined figures indicate that the nose was partially obstructed; these values excluded from all statistical analyses.
[e] Parentheses indicate subject not used in one-way trend analysis because data missing in one or more cycle phases.

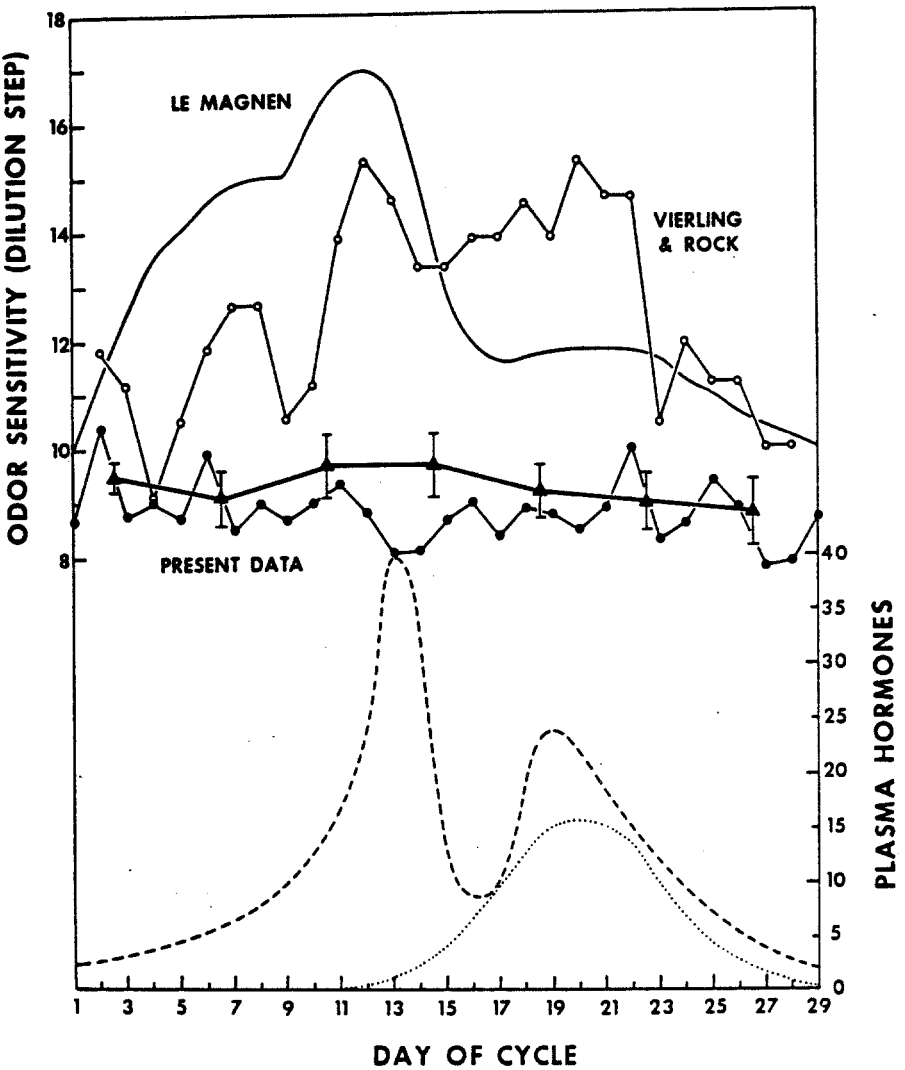

FIG. 1. Sensitivity of women to the odor of musk during the menstrual cycle. Day 1 is the first day of the menses. Data of Le Magnen (1952) recalculated from his Figure 1. Data of Vierling and Rock (1967) recalculated from their Figure 1. Triangles show our present data, corrected as described (vertical lines indicate standard error). Data on estradiol (dashed curve, ng/100 ml) adapted from Baird and Guevara cited by Loraine and Bell (1971), Figure 82. Data on progesterone (dotted curve, ng/ml) adapted from Johansson, cited by Loraine and Bell (1971), Figure 88.

was 29.0 days. Olfactory thresholds were measured between 9:30 AM and noon daily, Monday through Friday, during 4 weeks. All the available data are recorded in Table 1, except for the initial training session on Monday, August 6, 1973. Gaps in the table indicate absentees. The subjects did not wear perfume, and refrained from eating, drinking, or smoking for 15 min before the test. If the subject complained of partial nasal obstruction, due to allergy or infection, the datum was underlined in Table 1, and was rejected from statistical analysis. None of the women showed in her daily thresholds any meaningful trends in terms of hormonal relationships. 8 women were taking progestational agents and 14 were not, but this factor had no significant effect on sensitivity.

A 2-way trend analysis (Winer, 1971) by calendar day for the 373 acceptable threshold determinations showed small linear and cubic trends (of unknown origin) which were parallel for males and females. The mean for all females was 8.80 binary dilution steps (*SD* 2.08). The male mean was 10.16 steps (*SD* 1.66). The data were then rearranged by day of cycle derived by counting back from the first day of the following menses (Vierling and Rock, 1967). The averaged threshold for each cycle day is shown by solid circles in Figure 1. The points, representing on average 10 determinations (mean *SE* 0.65), appear randomly distributed about a horizontal or slightly downward sloping line.

Since no two daily averages, shown by the solid circles in Figure 1, contained thresholds on the same set of individuals, the data were divided for statistical analysis into seven 4-day intervals corresponding roughly with 7 cycle phases: menstrual, early follicular, late follicular, ovulatory, early luteal, midluteal, and late luteal. After grouping, there remained 9 subjects with no missing data. Again no trends were visible for any individual subject.

TABLE 2. ONE-WAY TREND ANALYSIS WITH REPEATED MEASURES (Winer, 1971)

Source of variation	*SS*	*df*	*MS*	*F*	α
Between women	76.8252	8			
Within women	69.0070	54			
Cycle effects	6.4516	6	1.08	0.83	–
Linear trend		1	2.75	2.11	>0.10
Quadratic trend		1	1.51	1.16	>0.25
Cubic trend		1	0.03	0.02	–
Other trends		3	0.72	0.56	–
Residual (error)	62.5554	48	1.30		
Total	145.8322	62			

3 of these women were not taking the pill, and temperature curves indicated that they had ovulated. A 2-way trend analysis showed no significant differences between ovulators and nonovulators, so the data were pooled.

It happened that 6 of the 9 women entered the experiment in the first half of their cycles. This incidental synchronization could have caused a systematic bias, because of the small but significant calendar-day effect noted above. A daily correction factor was taken to be the average deviation of the male group from their overall mean (10.16 steps). This correction was applied to adjust each woman's threshold for the same calendar day. The corrected female means are shown by triangles in Fig. 1 (average *SE* 0.51). The results of a 1-way trend analysis with repeated measures (Winer, 1971) using the corrected data are shown in Table 2. No *F* test has a probability below 0.10. (The uncorrected data also showed no significant trends, $F > 0.05$.)

We tested 2 other women (ages 28 and 38) who were known to be specific anosmics for the odor of pentadecalactone. The younger used birth-control pills and the older did not. During 6 weeks of testing every day they never achieved a sensitivity higher than step 3, nor was there any correlation with their cycles. We had an opportunity to test a patient (aged 18) having untreated Turner's syndrome. Despite evidence for almost total absence of estrogens, her threshold for pentadecalactone (step 8) was close to the normal mean.

DISCUSSION

Figure 1 demonstrates the differences between our data and those of Le Magnen (1952) and of Vierling and Rock (1967). We feel that the present data are the most reliable because we omitted specific anosmics, used a true olfactory discrimination test, avoided odorous artifacts, controlled for calendar-day effects, and analyzed appropriately using repeated measures on the same subjects over the entire cycle. Trend analysis confirms the impression that any fluctuations of threshold are random. Although all three investigations lacked daily plasma hormonal measurements, these are available from the literature (Loraine and Bell, 1971) and have been added to Figure 1.

We also disagree with an earlier claim by Le Magnen (1948) that men are much less sensitive than women to the odor of pentadecalactone. Koelega and Köster (1974) support Le Magnen's findings, and in a private communication Köster has suggested that our divergent results could be due to differences in the purity of the pentadecalactone samples employed. Accordingly, we have repeated our measurement of the olfactory threshold histogram (Whissell-Buechy and Amoore, 1973) but using Köster's sample (Exaltolide®, Firmenich et Cie, Geneva, Switzerland). After omitting 7 specific anosmics (3♂ and 4♀), there remained 23 men (threshold 7.78 steps)

and 24 women, whose threshold of 8.17 steps was not significantly different ($SED = 0.50$ step).

We remain at a loss to explain the discrepancy between our results and those of Le Magnen (1948, 1952). Evidently Le Magnen's phenomenon did not occur in our sample population under our experimental conditions. We, therefore, doubt that this phenomenon could have been a perturbing factor in our family data collected by similar experimental methods. This result serves to nullify Comfort's (1973) chief criticism of our earlier conclusion that pentadecalactone anosmia has a simple recessive inheritance pattern (Whissell-Buechy and Amoore, 1973).

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SPECIFIC ANOSMIA TO 1-PYRROLINE: THE SPERMOUS PRIMARY ODOR

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Abstract—About 20% of human subjects are specifically anosmic to the odor of the heterocyclic Schiff's base 1-pyrroline. Odor threshold measurements on 33 nitrogenous bases were made with panels of specific anosmics and normal observers. The anosmia proved to be very selective, and was encountered only with 1-pyrroline and its homolog 1-piperideine. The odor of 1-pyrroline resembles very closely that of human semen, and possibly represents a vestigial pheromone. It is suggested that this specific anosmia corresponds with the absence of a new olfactory primary, the "spermous" odor. The same odor occurs as an impurity in several organic reagents and can be detected in a variety of biological materials.

Key Words—odor thresholds, primary odor, 1-pyrroline, Schiff's base, semen, specific anosmia, "spermous" odor.

INTRODUCTION

Although the human sense of smell may be of minor importance compared with our dominant senses of vision and hearing, it is still capable of registering and analyzing a rich variety of sensations. The mechanism by which this is achieved remains largely obscure, but has prompted numerous attempts at odor classification (Harper, Bate-Smith, and Land, 1968). Such schemes usually rest on the assumption that there is a limited number of basic or "primary" odors from which all other "complex" odors could be produced by permutation. The central problem, then, is to identify each primary odor in an unambiguous experimental manner. One of us (J.E.A.) has for several years

been developing a concept first clearly stated by Guillot (1948) who suggested that the human primary odors might be indicated by the various forms of specific anosmia. In this condition a person of otherwise normal olfactory acuity finds some particular odor that escapes his attention, while it remains obvious to other people.

The first primary odor to be analyzed in detail was the sweatlike smell of isobutyric acid, to which about 2% of people are anosmic. By measuring the olfactory detection thresholds of 10 such specific anosmics to a variety of related compounds, it was established that the corresponding primary odor is associated especially with the C_4 to C_7 carboxylic acids. The most pronounced anosmic defect was observed with isovaleric acid, which thus emerged as the epitomization of the "sweaty" primary odor (Amoore, 1967, 1970). This primary has since received some support from organoleptic measurements and a study of olfactory fatigue (Amoore, Venstrom, and Nutting, 1972). Nevertheless, we have felt for some time that it would be reassuring to examine another type of specific anosmia, and to learn whether it would yield to the same method of experimental analysis.

We are indebted to John Anderson of Proctor and Gamble in Cincinnati, Ohio, for directing our attention in July 1968 to a newly observed type of odor blindness that appeared favorable for study. He found that, out of a selected panel of 24 well-trained subjects, there were 2 people who did not find commercial putrescine offensive. When it was dilute, they did not smell it at all, although the others could still perceive it. A related observation was made in this laboratory in December 1970 while testing the odor quality of 1-pyrroline. Among a panel of 20 experienced observers, most of whom found the odor very obvious, 2 individuals could not smell it. The odor of 1-pyrroline is practically identical to that of putrescine, and both resemble almost exactly the odor of human semen. In fact, the same people failed to smell either 1-pyrroline or putrescine. The relatively common occurrence of this type of specific anosmia suggested that here would be a good prospect for establishing a second well-defined primary odor.

METHODS AND MATERIALS

Our general experimental procedures for selecting specific anosmics and for measuring their degree of anosmia have been described in detail elsewhere (Amoore, Venstrom, and Davis, 1968). The odor threshold measurements (2/5 tests) are now conducted here in a special room with controlled temperature (22°C) and relative humidity (50%). Most of the odorants were bases with pK_a (half ionization point) in the range of 9–11. They were presented in the test flasks as serial binary-step dilutions in buffers adjusted to the

pK_a of the base (upper pK_a for diamines). Concentrations of all solutions are given as ppm of the free base (v/v except where noted). The buffer, usually $KHCO_3/K_2CO_3$, was increased in strength to 0.1 M in order to counteract absorption of atmospheric or pulmonary CO_2, which would have lowered the pH and reduced the volatility of the bases. Odorants with pK_a 6–8 (and pyrrole) were tested in 0.1 M KH_2PO_4/K_2HPO_4 buffer. Pyridine was diluted in 0.01 M potassium phthalate buffer.

Most of the odorants were commercially available reagents. The bases were freed from neutral impurities by preparing an aqueous solution of the sulfate at pH 7.0 and repeatedly extracting in a separatory funnel with odorless mineral oil. Further extraction at pH 2–3 removed acidic impurities. When the pH was raised again to 7, there was sometimes a residual foreign odor which was removed by stirring ½ hr with activated coconut charcoal, then filtering. The solution was finally titrated with 10 M KOH to raise the pH to the (upper) pK_a. From the amount of KOH required, the actual concentration of free base remaining in the solution was calculated. Serial dilutions in the appropriate buffer were made from this stock solution. When water solubility and/or pK_a values were unavailable in the literature, they were determined by appropriate equilibration and potentiometric titration against standardized H_2SO_4.

Several of the commerical bases contained *basic* impurities that were not removed by the mineral-oil-extraction procedure. Although the impurity amounted to only 0.1–1.0%, the contaminating odor was so powerful that it caused entirely erroneous threshold determinations and/or anosmia values. This problem occurred with putrescine, pyrrolidine, and 3-pyrroline (the probable impurity in each case was 1-pyrroline), piperidine (may contain 1-piperideine), and dimethylamine (likely impurity trimethylamine). These basic impurities were eliminated by recrystallizing the hydrochlorides 3 times from water and/or ethanol. The final recrystallization for putrescine and pyrrolidine was performed within an hour of starting the threshold tests, because even their crystalline hydrochlorides are liable to oxidize and regenerate the impurity.

Pyrrole was freshly vacuum redistilled. Trimethyleneimine was synthesized by Dr. J.W. Corse of this Laboratory and purified by gas chromatography. It is unstable at acidic pH. 1-Pyrroline was generated in solution by the method of Jakoby and Fredericks (1959) from DL-ornithine HCl and *N*-bromosuccinimide. The reaction mixture was saturated with $NaHCO_3$ and repeatedly extracted with ether. After drying, the ethereal extract was submitted to preparative gas chromatography on a 2-m long × 0.64-cm OD aluminum column packed with Chromosorb P coated with 25% Amine 220 and 5% KOH. The early ether peak was discarded, and the 1-pyrroline peak was collected in a capillary tube cooled in dry ice (yield about 1 μl per

injection). 1-Piperideine was made by the same procedure using instead DL-lysine HCl as starting material. This compound, although known in solution, had not previously been obtained in the free state. Both 1-pyrroline and 1-piperideine are somewhat unstable at alkaline pH.

RESULTS

The initial phase of this study was conducted with putrescine, before we realized that its usual semenlike odor is entirely due to an impurity. This odor was unchanged in strength or character by the mineral-oil-extraction procedure. The average odor detection threshold (μ) determined with normal observers was at dilution step 16.00, which corresponds with 15.3 ppm free putrescine (Figure 1). The standard deviation (σ) was ± 2.16 steps. Next, using concentration step 12 (244 ppm, approximately 2σ above the normal threshold), we screened 222 laboratory personnel and found 43 specific anosmics (about 20% of our population). The detection thresholds for the anosmics were determined with a higher concentration series. Nobody was found with threshold at step 11, so the normal observers and specific anosmic in this sample of people segregated into 2 distinct groups.

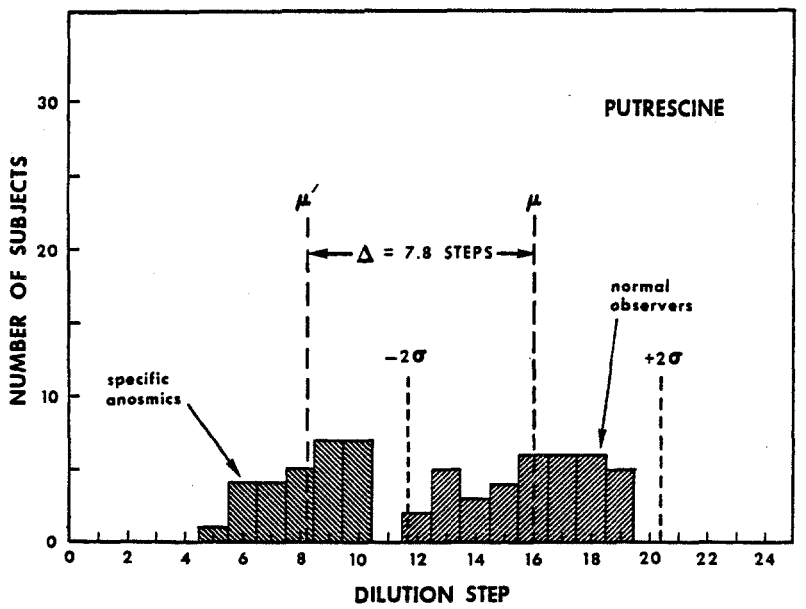

FIG. 1. Olfactory thresholds to (impure) putrescine. Each binary dilution step represents a halving of the concentration, starting from 0 for the undiluted base.

The mean threshold for the specific anosmics to putrescine was at step 8.21 (3380 ppm). The difference in threshold (Δ) between the normals and anosmics was 7.79 steps. On the average, the anosmics were 220 times less sensitive than the normals to the odor of (impure) putrescine. For all subsequent tests on various odorants in this series we used a panel of 29 putrescine anosmics and compared their average threshold with that obtained from a panel of 41 normal observers. These "normal" people were, by definition, neither general anosmics nor putrescine anosmics, although several of them were known to have other specific anosmias. Furthermore, some of the putrescine anosmics had more than this one anosmia, but the use of relatively large panels of subjects tends to minimize any errors that might be introduced by the presence of non-relevant anosmias.

When, instead of oil-extracted putrescine sulfate, we later used freshly recrystallized putrescine dihydrochloride as the test odorant, the results were quite different. The normal detection threshold was initially at about 9200 ppm, or 600 times higher. Subsequently, the apparent threshold concentration of the putrescine solution slowly decreased, at about half a step per hour, until after 48 hr the threshold was the same as that for the original impure putrescine. The most likely explanation is that some of the putrescine oxidizes and cyclizes to form 1-pyrroline. This reaction is well documented in the presence of the enzyme diamine oxidase (Tabor and Tabor, 1964; Figure 2) but has not apparently been suspected of occurring spontaneously by atmospheric oxidation of undiluted putrescine. 1-Pyrroline has a very low threshold (see below), and it may be estimated that the original putrescine need only contain 0.1% of 1-pyrroline to account for the observed normal detection threshold. After repeated recrystallization of putrescine dihydrochloride, the 1-pyrroline impurity must have been reduced to about 2 ppm, but gradually returned to its former level.

In order to "map out" the chemical extent of this type of specific anosmia, we tested a total of 33 adequately purified odorants on our 2

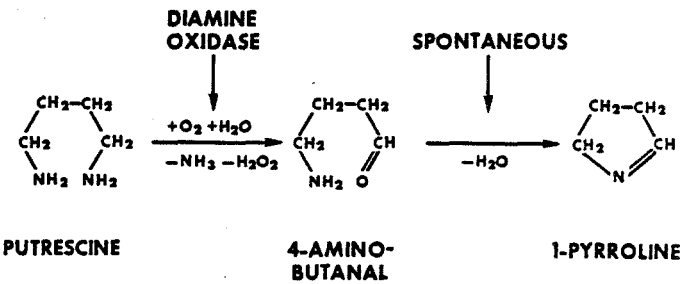

FIG. 2. Oxidation of putrescine to 1-pyrroline.

TABLE 1. SPECIFIC ANOSMIA TO 1-PYRROLINE AND OTHER NITROGENOUS BASES

Odorous compound	Solubility in water ppm[a]	pK_a	Thresholds in water, ppm		Anosmics' defect, steps[b]
			Normal	Anosmic	
Primary amines					
Ethylamine (g)[c]	∞_{16}	10.67	95.1	220	1.21
n-Propylamine	∞	10.58	90.1	200	1.15
n-Butylamine	∞	10.60	50.0	126	1.33
n-Pentylamine	∞	10.64	15.9	37.1	1.22
n-Hexylamine	14,300	10.64	3.67	8.72	1.25
n-Heptylamine	4,180	10.66	13.8	35.0	1.34
n-Octylamine	1,410	10.65	2.64	5.51	1.06
Secondary amines					
Dimethylamine (g)	552,000	10.72	17.6	31.0	0.82
Methylethylamine	∞	10.96	9.02	33.0	1.87
Diethylamine	∞	10.98	12.1	38.1	1.66
Ethyl-*n*-propylamine	∞	11.14	17.4	51.7	1.57
Di-*n*-propylamine	54,000	10.91	22.4	55.0	1.30
Tertiary amines					
Trimethylamine (g)	$410{,}000_{19}$	9.74	0.000367	0.00106	1.53
N-Methylpyrrolidine	∞	10.53	0.0114	0.0434	1.93
Cyclic amines					
Cyclopropylamine	∞	9.36	38.9	63.7	0.71
Cyclobutylamine	∞	10.29	32.5	114.0	1.81
Cyclopentylamine	∞	10.49	190	640	1.75
Cyclohexylamine	∞	10.62	28.5	112	1.98
Cycloheptylamine	25,000	10.89	65.8	122	0.89
Cyclic imines					
Trimethyleneimine	∞	9.78	21.9	72.1	1.72
Pyrrolidine	∞	11.01	20.2	64.6	1.68
Piperidine	∞	11.00	65.8	181	1.46
Hexamethyleneimine	∞	10.90	46.9	159	1.76
Heptamethyleneimine	30,600	11.08	82.8	246	1.57
Unsaturated heterocyclics					
1-Pyrroline	∞	6.70	0.0220	1.61	6.19
1-Piperideine	∞	7.93	0.302	~10	~5.0
3-Pyrroline	∞	10.46	62.8	101	0.69
3-Piperideine	∞	10.43	226	353	0.64
Aromatic heterocyclics					
Pyrrole	60,000	—	49.6	108	1.13
Pyridine	∞	5.25	4.33	9.02	1.06
Aliphatic diamines					
Ethylenediamine	∞	9.98	18,300	44,200	1.27
Trimethylenediamine	∞	10.35	12,400	39,500	1.67
Putrescine	∞	10.65	~9,200	>15,000	?
Putrescine (impure)	∞	10.65	15.3	3,380	7.79
Other primary odors					
Isovaleric acid	51,200	4.77	0.142	0.168	0.24
ω-Pentadecalactone (s)	1.1	—	0.00398	0.00733	0.88

[a] At 25°C except where indicated.
[b] Anosmics' defect is the logarithm on base 2 of the ratio between the anosmic and normal thesholds. Average standard error 0.53 step (range 0.35–0.80).
[c] (g) = gas; (s) = solid, concentrations as w/v. All other compounds are liquids at 25°C, and their concentrations are v/v. (Putrescine, mp 27°C, was treated as a liquid.)

panels of observers, normal and anosmic. The detailed results are given in Table 1. The compounds are all nitrogenous bases selected by a trial-and-error procedure in order to determine what molecular features are associated with this variety of anosmia.

The primary and secondary amines, cyclic amines, and cyclic imines all exhibit rather undistinguished normal thresholds in the range 2–200 ppm. The 2 tertiary amines have remarkably low thresholds, of the order of 10,000 times lower than comparable primary and secondary amines. Actually trimethylamine and *N*-methylpyrrolidine also demonstrated a new type of specific anosmia, in that 2 members of our normal panel, and 1 of the 1-pyrroline anosmics, were about 1000 times less sensitive than the average. These tertiary amines may represent a different (fishy) primary odor. None of the 24 compounds tested in these 5 homologous series registered more than 2 steps of anosmic defect with our panels of observers. Evidently the 1-pyrroline anosmics have little or no difficulty in perceiving any of these saturated amines.

Also conspicuous for its low threshold is the unsaturated heterocyclic 1-pyrroline itself, with a normal threshold at 0.022 ppm. The panel of specific anosmics was 73 times less sensitive to this compound. The anosmic defect was 6.19 binary steps, which is a lesser deficiency than was noted for impure putrescine (7.79 steps). Paradoxically, the gas chromatographically isolated 1-pyrroline seemed not to be as pure, from the olfactory standpoint,

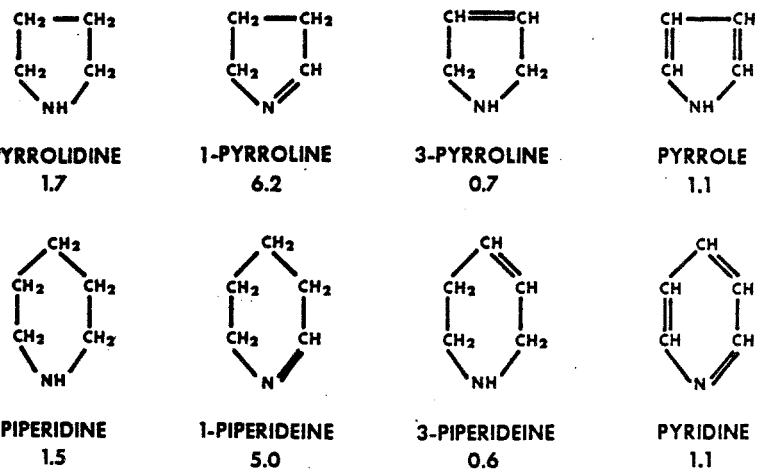

FIG. 3. Heterocyclic compounds related to 1-pyrroline. The numbers represent the measured anosmic defect. (2-Pyrroline and 2-piperideine are unknown.)

as the trace of 1-pyrroline arising spontaneously from putrescine solutions. Among all other pure compounds tested, only the next higher homolog, 1-piperideine, registered a large anosmic defect with this panel, about 5 steps. Its threshold is 13 times higher, however, which leaves 1-pyrroline as the best example known for demonstrating this variety of specific anosmia.

The chemical specificity of the anosmia is notable (Figure 3). When properly purified the corresponding saturated heterocyclics pyrrolidine and piperidine showed no appreciable anosmia, nor did the fully aromatic rings pyrrole and pyridine. Even the shift of the double bond to the 3 position, as in 3-pyrroline and 3-piperideine, removed the compounds from this anosmic class, and raised their thresholds about 1000-fold compared with their 1 isomers. The molecular sizes and shapes of these 5- and 6-membered rings are very similar indeed. We are forced to conclude that the key molecular feature is the —N=CH— functional group (Schiff's base). We do not know if the cyclic shape is essential.

The purified lower aliphatic diamines have very feeble odors (thresholds about 10,000 ppm). Only in the common impure form does putrescine have the semenlike odor, and hence exhibit the anosmic deficiency. (The odor of semen is also present in the higher homologs 1,5-diaminopentane through 1,8-diaminooctane, and in the amino alcohols 4-aminobutanol and 5-aminopentanol. Their usual odors, however, are almost certainly due to oxidation and cyclization to form traces of 1-pyrroline or its homologs.)

At the end of Table 1 are added 2 compounds that represent other primary dodors. Isovaleric acid is the best-known example for the sweaty primary odor (Amoore, 1967), and pentadecalactone must be close to the musky primary odor (Whissell-Buechy and Amoore, 1973). After omitting the scores of the few specific anosmics to these compounds among our panelists, the 1-pyrroline anosmics registered negligible olfactory defects (less than 1 step). This indicates that nearly all the 1-pyrroline anosmics are very specific in their defect, and are normal in their ability to detect other primary odors.

DISCUSSION

The experimental evidence presented in this paper demonstrates a clear-cut and rather common biochemical defect in the human olfactory mechanism, specific anosmia to certain cyclic Schiff's bases. The specificity of the defect is illustrated in Figure 4, which shows the anosmic defect as a function of carbon chain length in 3 homologous series. The primary amines and cyclic imines show substantially horizontal lines with anosmic defects between 1 and 2 steps. Two other series, secondary amines and cyclic amines, are

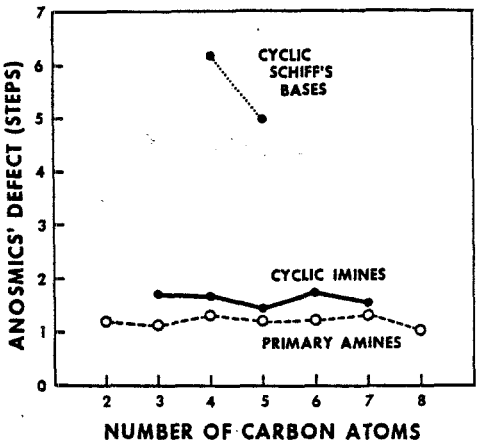

FIG. 4. Specific anosmia to Schiff's bases.

omitted for clarity, but fall into much the same range. We have noticed that our panels of specific anosmics have never quite achieved the same threshold as the normal panel, even for odorants which seem to be totally unrelated to their specific anosmia. This is probably the result of a bias in our method of screening for specific anosmics, which would tend to select persons of less than average general olfactory acuity. The correction factor seems to be about 1 step. Many of these amines and imines scored significantly above 1 step defect but still below 2 steps. We do not know whether this difference is due to residual pyrrolinelike impurities, or whether the receptor mechanism for 1-pyrroline plays a minor part in detecting other nitrogenous bases at high concentration.

The cyclic Schiff's bases are outstanding in the degree of anosmic defect they reveal, 5–6 steps, and they also have distinctly low normal thresholds. Another factor which increases their olfactory significance is that they are very weak bases. The pK_a of 1-pyrroline is only 6.7 compared with pK_a values in the range of 10–11 for most of the bases we examined. Stronger bases like pyrrolidine are fully ionized at neutral pH, and, hence, practically nonvolatile and odorless. 1-Pyrroline, however, is only half ionized at pH 6.7, and therefore will be volatile from most biological materials in which it occurs.

Following Guillot (1948) and the rationale previously advanced (Amoore, 1967; 1970), we believe that the 1-pyrroline anosmia represents a lack of ability to perceive the corresponding primary odor. The odor of 1-pyrroline has been described as being like corn (Yoshikawa et al., 1965). Our (normal) panelists have often described it as like bleach (sodium hypochlorite). Each of these comparisons can be misleading, however. The corn (*Zea mays*) should

be boiled on the cob, and then left in the refrigerator 24 hr to develop the 1-pyrroline odor. Bleach should be applied to the hands and then washed off, leaving the 1-pyrroline odor on the skin. In the interests of descriptive accuracy and possible biological significance (see below) we are adopting the name "spermous" for the new primary odor epitomized by 1-pyrroline.

The exact nature of the biochemical defect is still unknown. It has been shown for another type of odor blindness (to the musky odor of pentadecalactone) that the defect is inherited as a simple Mendelian recessive character (Whissell-Buechy and Amoore, 1973). Probably some functional protein is inherited defectively, but it remains an open question whether this protein is the bearer of the initial odorant receptor site, or lies at some higher level of the olfactory pathway.

The possible occurrence of impurities should evidently be born in mind in any research which seeks to relate odor thresholds and/or qualities to molecular structure. Mizuno, McMeans, and Chipault (1966) commented that the odor of putrescine is not of a putrid character, but rather similar to that of ammonia, although considerably weaker. Dudley, Rosenheim, and Starling (1926) and Wrede (1926) observed that when the polyamine spermine is thoroughly purified it loses its characteristic odor, but the mildest chemical oxidation yields the volatile base 1-(3-aminopropyl)-3-pyrroline, to which was ascribed the semenlike odor (Wrede, Fanselow, and Strack, 1926). The same compound was synthesized by a different route by Parker et al. (1963). A sample was supplied by Dr. William Shive. We found that the pure compound possesses no trace of the semen odor, only a very weak fishy smell.

We suspect that the semenlike odor of oxidized spermine is the result of a second oxidative break in the spermine chain producing 4-aminobutanal, which promptly cyclizes to 1-pyrroline. In fact 1-pyrroline is a possible oxidation product, directly or indirectly, from at least 8 common metabolites (Figure 5). Considering the wide distribution of diamine oxidase (Tabor and Tabor, 1964) and the susceptibility of some of these compounds to direct atmospheric oxidation (Wrede, 1926) we anticipate that 1-pyrroline may be a frequent contributor to the odor of biological materials. We shall mention

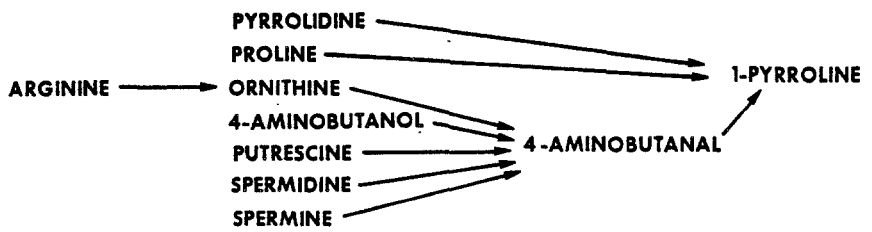

FIG. 5. Oxidation of various precursors to 1-pyrroline.

some possibilities, even though confirmation by chemical analysis is generally lacking.

The spermous odor is obvious not only in semen but in the sweat of the male pubic area. Human semen contains appreciable quantities of spermine, spermidine, and putrescine, together with diamine oxidase (Williams-Ashman and Lockwood, 1970). In the presence of these precursors the formation of 1-pyrroline in semen appears inevitable. Our demonstration of a very specific and sensitive primary odor receptor system in the human nose for the same compound prompts the speculation that 1-pyrroline may be a vestigial male sex pheromone for our species. This principle, though not couched in modern terminology, was noted in 1763 by Haller who referred to the identical odor in Spanish chestnut husks (*Castanea sativa*) as the *odorem aphrodisiacum* (quoted in Harper, Bate-Smith, and Land, 1968). Incidentally, we find no difference between men and women in the occurrence of 1-pyrroline anosmia or in the sensitivities of those who can smell the compound.

As Yoshikawa et al. (1965) have pointed out, 1-pyrroline is likely to arise in food processing by the Strecker degradation of proline or ornithine. One of us (R.G.B.) has indeed identified 1-pyrroline in corn chips by gas chromatography and mass spectrometry. In view of its low threshold, its neutral pK_a, and its somewhat cornlike odor, it probably contributes significantly to the aroma of corn chips. The spermous odor may also be detected by a discriminating nose in cucumbers, bean sprouts, fresh flour, and Italian dry salami. 1-Pyrroline, with its powerful and characteristic primary odor, is being proposed elsewhere as a contribution towards rationalizing synthetic flavors and increasing their safety (Amoore, unpublished).

Once the nose is attuned to it, 1-pyrroline, or at least the identical odor, may be perceived in a variety of situations that may have practical consequences. It is formed promptly when hypochlorite bleach or other mildly oxidizing cleansers contact animal secretions or proteinaceous materials. Among the likely amino acid precursors (Figure 5) only proline reacts without delay. Most people find the odor disagreeable when it is associated with laundering or dishwashing. The same smell can be detected on dental plates if they are not freshly cleaned, and is also noticeable on the toothbrush if the natural teeth are scrubbed without dentifrice. Reinwein (1926) isolated putrescine from sputum, so 1-pyrroline is likely to be formed by oral bacteria, and could be an important contributor to some forms of mouth odor. The semenlike odor is an aid in identifying certain fungi (Gilbert, 1932) and is especially prominent in the stinkhorn *Phallus impudicus*. Here it may contribute to mimicking the odor of carrion for attracting flies to aid in spore dispersal.

From the theoretical standpoint, the spermous odor respresents the second primary odor to be analyzed in detail by the specific anosmia method.

Although the chemical problems were different, the olfactometric analysis proceeded along exactly the same lines as were established for the sweaty primary odor (Amoore, 1967). We feel that this result provides additional support for the anosmia method of identifying the human primary odors. We are therefore encouraged to continue the pursuit of additional primaries, which could eventually number as many as 30, if all the known varieties of specific anosmia represent primary odors (Amoore, 1969; 1970).

Acknowledgments—We are especially grateful to John Anderson, Jacques Chipault, Ralph Lewin, Howard Myers, William Shive, and Esther Whited for a variety of helpful information. Reference to a company or product does not imply endorsement.

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A MECHANISM TO AUTOMATICALLY BAIT ELECTRIC GRID INSECT TRAPS WITH SEX PHEROMONE[1]

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Abstract—A mechanism to automatically bait electric grid traps with prospective sex pheromone samples was developed and used during bioassays of chemical components isolated from the extracts of female tobacco budworms, *Heliothis virescens* (F.). By baiting traps at specified times during the night, this mechanism contributed to the final identification of the sex pheromone.

Key Words—automatic baiting device; *Heliothis virescens*, Lepidoptera: Noctuidae, tobacco budworm sex pheromone.

INTRODUCTION

Often many replications of field-cage bioassays are required to determine the efficacy of methods of extracting sex pheromones of leptidoptera, the relative purity of chemical isolates, and the biological activity of chemical substances. Also, when behaviorial bioassays involve nocturnal species such as the tobacco budworm, *Heliothis virescens* (F.), the traps must be baited at night during the hours of peak activity of the moth (Shorey and Gaston, 1965).

Electric grid traps (Wolf et al., 1972) were used for sequential bioassays to evaluate chemical isolates and purity of the sex pheromones of the tobacco budworm (Hendricks and Tumlinson, 1974) and other noctuids. Since these traps had to be baited from 9:30 PM to 3 AM and since test locations were

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

[2] The technical and graphic assistance of Ms. J.M. Crockett of this laboratory is gratefully acknowledged.

usually remote, a mechanism was designed to bait the electric grid traps at designated times.

METHODS AND MATERIALS

The 7 electric grid traps used in each of 3 bioassay cages (12 × 17 × 3 m) were energized by an electric time switch connected to a single (120 V ac) power supply line. All grids were thus energized or deenergized simultaneously. A clock alarm was set to trigger release of the attractant to be tested (Figure 1), and the alarm was adjusted to any desired time (the clock was checked for accuracy of both time (CDT) and pace). A steel weight (W), 1.2 cm diam × 8.6 cm and weighing 100 g, was positioned in the top of the 1.27-cm-diameter copper tubing barrel (T) and supported by a cotter pin (P). A string (NS) was so oriented that when an alarm key (K) was triggered and rotated at the preset time, the string coiled around the key axis and pulled the cotter pin from the hole (PH); this allowed the weight to drop through the tubing and crush a vial (C) wrapped in filter paper lodged in the bait holder. A bronze rod (WR), 0.3 cm diameter × 44.5 cm, soldered into the weight was used to retrieve the weight after the mechanism was tripped.

The bait holder (Figure 2) was fixed to the bottom of the copper tube barrel. A copper coupling joint was soldered to the bottom of the barrel and sealed with a threaded plug (TP). A hole (1.1 cm) was drilled through joint fitting so an ampoule wrapped in filter paper (FP) could be inserted in line with the weight. Ventilation holes (H, 0.4 cm diameter) were drilled through the copper tubing (T) and joint just above the point of impact of the weight and the sealed ampoule.

Chemicals to be tested were prepared in a solvent system made of ether, hexane, or an ether–pentane mixture (total liquid volume usually did not exceed 0.6 ml) and sealed in the glass ampoule (1 × 6 cm). When the ampoule was crushed, the chemicals were absorbed by the filter paper and volatilized through the ventilation holes.

Baiting of each trap could thus commence at any time set on the respective alarm clocks, and trapping could be discontinued at any time by opening the circuit supplying power (120 V ac) to the trap grids. During some bioassays, the trigger time for the bait was set to activate 15–30 min before the grids were energized to allow adequate time for the solvents to dissipate. (At certain conditions rapid vaporization of solvents may be repellent to insects and result in increased variance in catch.) Hence, the device allowed asynchronous baiting up to 1 h or more before or after the grids were energized. Otherwise, a 120 V ac solenoid wired to the power supply line could replace the clock if baiting and grid current were to occur simultaneously.

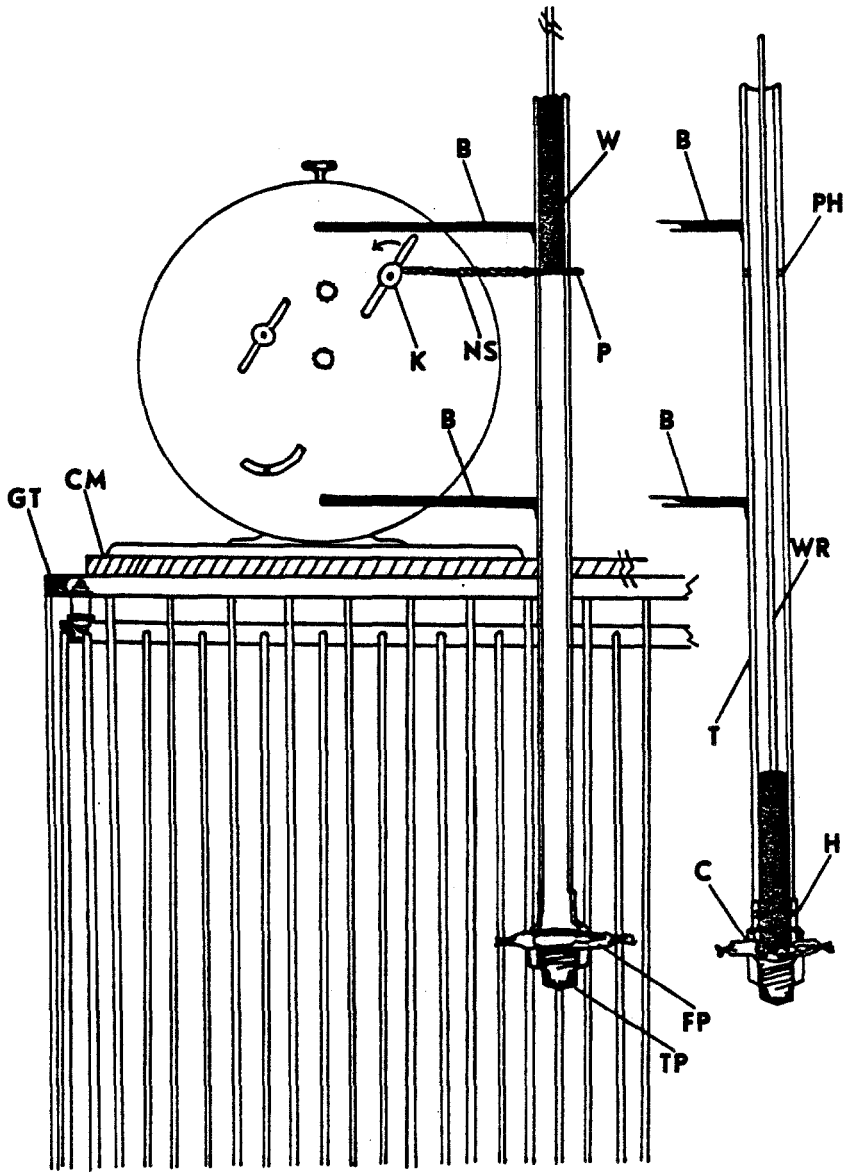

FIG. 1. Trap grid with timed baiting mechanism, both set and tripped. B, brace rod; C, ampoule in paper, crushed; CM, clock mount; FP, filter paper wrapped around sealed ampoule; GT, grid of trap; H, ventilation holes; K, alarm key; NS, nylon string tied to pin; P, cotter pin; PH, pin hold; T, tubing; TP, threaded plug; W, weight; and WR, weight-retrieving rod.

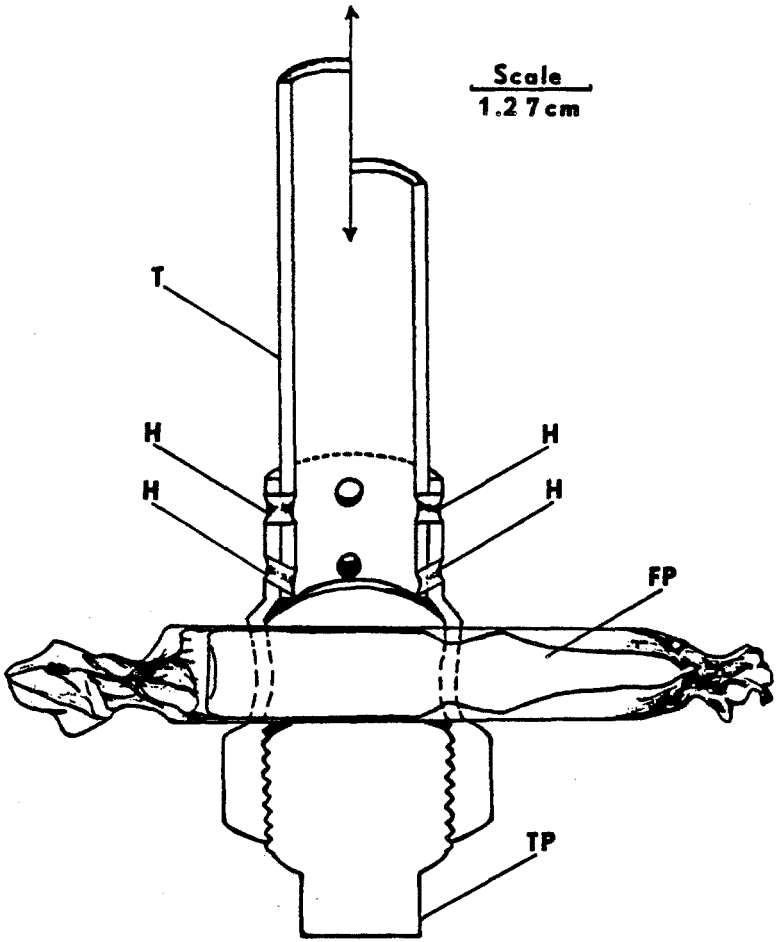

FIG. 2. Terminal of baiting tube. FP, filter paper wrapped around sealed ampoule; TP, threaded plug inserted in the bottom of brass coupling; H, ventilation holds; and T, copper tube barrel. Arrow-line shows path of 100-g weight.

RESULTS AND DISCUSSION

Data were obtained in the 3 field bioassay cages where the baiting mechanisms were used. They were triggered at 10:30 PM and the grid power turned off at 7 AM. Each night the cages were stocked with 1500–4000 laboratory-reared adult male tobacco budworms. Many were caught as a result of random flight into unbaited electric grids (137.3 ♂/trap in all 3 cages, unbaited

check traps). However, the traps baited with 20 female equivalents (FE) of crude extract caught an average of 632.1 ♂, and this was significantly greater by *t* test (0.90 level of confidence) than the number caught in the unbaited traps or in check traps baited with ether (144.3 ♂). Ether checks and 20 FE baits were replicated 9 times; empty trap checks were replicated 3 times. Ventilation of the attractive chemicals from the crushed ampoule was thus adequate and should yield reliable data for evaluation of chemicals attractive to various nocturnal Lepidoptera. The baiting mechanism provides a precise means of baiting the traps and eliminates human error that may increase variance in catches.

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AN ECONOMICAL SYNTHESIS OF THE MAJOR SEX ATTRACTANT OF THE OAK LEAF ROLLER—*cis*-10-TETRADECENYL ACETATE[1]

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Abstract—The major sex attractant of the oak leaf roller (*Archips semiferanus* Walker), *cis*-10-tetradecenyl acetate (I), was synthesized in 55–62% overall yield by a highly economical route from inexpensive azelaic acid (II).

Key Words—synthesis, *cis*-10-tetradecenyl acetate (I), *Archips semiferanus*, oak leaf roller, sex attractant, pheromone.

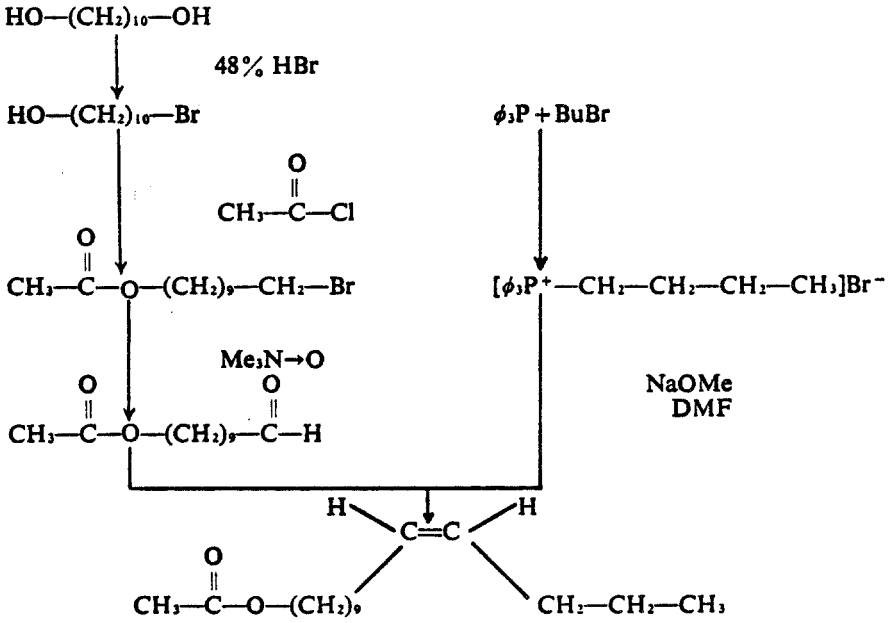
INTRODUCTION

The oak leaf roller moth, *Archips semiferanus* Walker, is a tree-defoliating insect which has destroyed over $70 million worth of timber in the northeastern United States. In Pennsylvania alone, 60–90% of the trees in over 1 million acres of forest land have been destroyed; it has been termed the worst forest-insect disaster in the history of this state.

Efforts to control the pest were initiated with a study of the sexual communication system in the adult moths (Hendry et al., 1973). Over 20 active principles (Hendry et al., 1974*b*; 1975) have been identified in the adult female which attract male moths in field tests (Hendry et al., 1974*a*). The most active component has been shown to be *cis*-10-tetradecenyl acetate (I) (Hendry et al., 1974*c*).

[1] Authorized as Paper No. 4779 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

SCHEME A



(I)

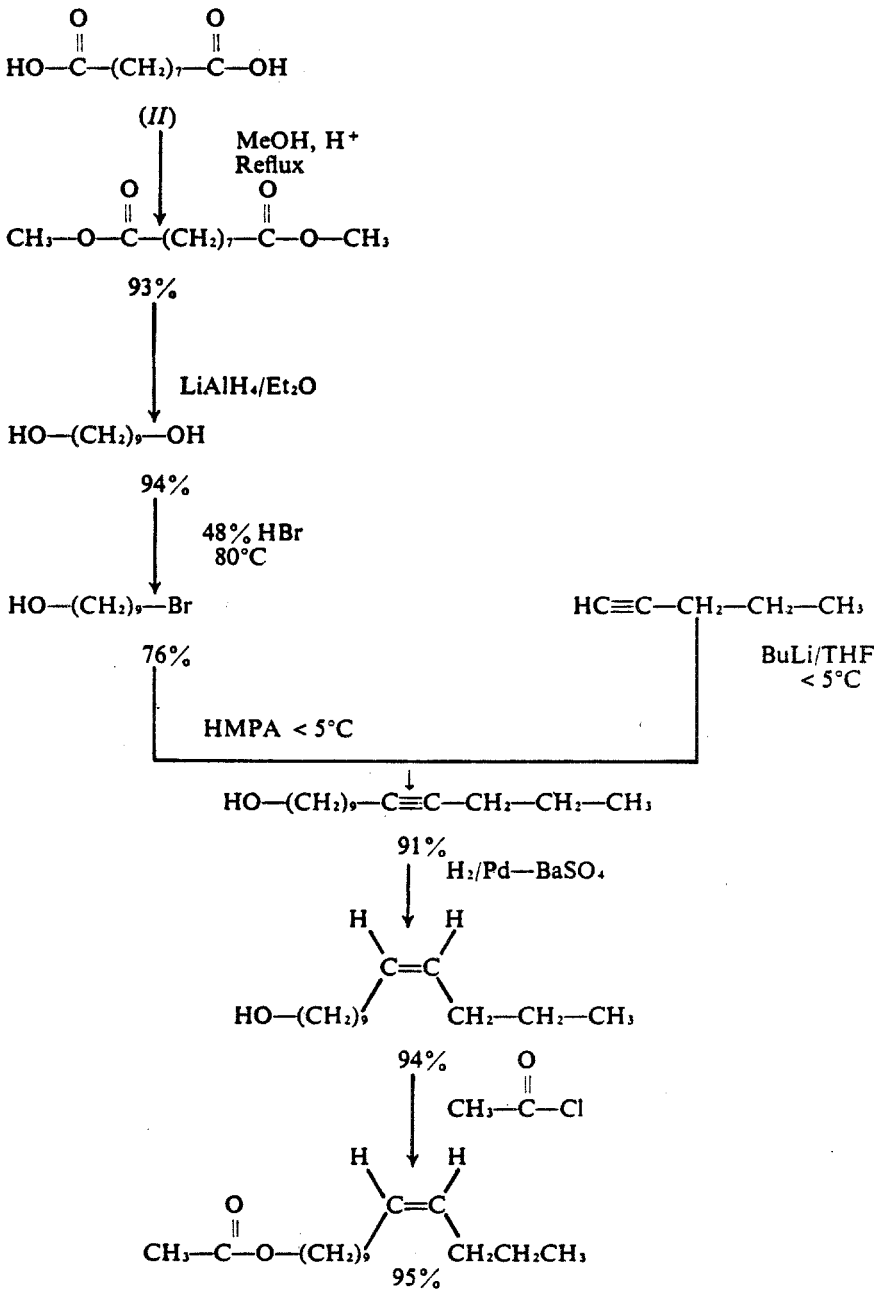
Initial syntheses of compound I were conducted according to scheme A, similar to those previously reported (Hendry et al., 1974*c*). The yield of compound I by this route was poor (< 10%) despite numerous efforts to improve the overall yield. An alternative economical method is reported here (scheme B). The overall yield from inexpensive azelaic acid (II) was 55–62%.

METHODS AND MATERIALS

Instruments

Vapour phase chromatography was performed on a Hewlett-Packard 7610A chromatograph using helium as a carrier gas at 30 ml/min on a 6 mm OD, 2 mm ID, 6-ft glass U-tube column of 10% DEGS on Chromosorb WAW. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A-60. Signals are reported downfield from tetramethylsilane (δ). Infrared (IR) spectra were recorded on a Perkin-Elmer model 257 grating spectrometer and are reported in cm^{-1}. Mass spectra (MS) were run on a Finnigan 3000 GC-MS system with 6000 digital computer.

SCHEME B


HO—C—(CH₂)₇—C—OH
(II)
MeOH, H⁺
Reflux
CH₃—O—C—(CH₂)₇—C—O—CH₃
93%
LiAlH₄/Et₂O
HO—(CH₂)₉—OH
94%
48% HBr
80°C
HO—(CH₂)₉—Br
76%
HMPA < 5°C
HC≡C—CH₂—CH₂—CH₃
BuLi/THF
< 5°C
HO—(CH₂)₉—C≡C—CH₂—CH₂—CH₃
91%
H₂/Pd—BaSO₄
HO—(CH₂)₉
CH₂—CH₂—CH₃
94%
CH₃—C—Cl
CH₃—C—O—(CH₂)₉
CH₂CH₂CH₃
95%

(I)

Dimethyl Azelate (1,9-nonanedioic Acid, Dimethylester)

The diester was prepared by a method similar to that of Vogel (1934) in 93% yield, bp 109°C at 2 mm. Analysis of the diester by IR,[4] NMR,[4] and MS identified the product as dimethyl azelate.

1,9-Nonanediol

Through a modified procedure of Huber (1951; Nystrom and Brown, 1947), the diol was prepared in 94% yield as a clear, colorless oil which crystallized on standing and was identical (IR, NMR)[4] to an authentic sample (Aldrich Chemical Co.), mp 43.5–45.8°C (Huber, 1951).

9-Bromo-1-nonanol

One half kg (3.13 mol) of 1,9-nonanediol was dissolved in 2500 ml of 48% aqueous HBr (22.2 mol) in a 5-liter flask and heated at 80°C (Butenandt et al., 1962). The reaction mixture was extracted continuously with a liquid–liquid extractor using refluxing *n*-hepatane. The heptane in the collection flask was changed 3 times at 24-h intervals. After neutralization of the heptane extracts with saturated $NaHCO_3$ solution and washing once with distilled water, the heptane was evaporated. Fractional distillation of the reaction product yielded 531 g of a clear colorless oil (76% distilled yield), bp 113°C at 0.75 mm.

The crude reaction mixture could also be purified by recrystallization of the solid formed when the heptane extracts were cooled for several hours at 0°C. Recrystallization from hexane gave colorless needles, mp 32.5–33°C (Butenandt et al., 1962; Chuit and Hausser, 1929) in slightly lower yield than the distillation.

IR: γ^{neat} 3350, —OH
NMR: δ^{CCl_4} 3.42 (center of two overlapping triplets, 4H); 2.29 (*s*, 1H) 1.36 (*s*, broad, 14H)
MS: *m/e* 224, 222 (M); 206 (M—H_2O)

10-Tetradecyn-1-ol

A solution of 170 g of freshly distilled 1-pentyne (2.5 mol, Farchan) in 1000 ml of dry THF was treated with 2.5 mol of *n*-butyl lithium in hexane at 0–5°C under a nitrogen atmosphere. After the yellow mixture was stirred for 2 h, a solution of 1 mol (223 g) of 9-bromo-1-nonanol in 750 ml of hexamethylphosphoramide (HMPA, distilled from molecular sieves prior to

[4] These spectra were identical to those in the Sadtler files.

use) was added slowly to the lithium acetylide. The temperature was kept $<5°C$ during the addition. The reaction mixture was then allowed to warm up to room temperature and stirred for 8 h. Ice water (1 liter) was added slowly and the aqueous solution extracted with pentane (4×500 ml). The combined pentane extracts were washed with a saturated NaCl solution, dried over anh. Na_2SO_4, and the solvent was removed leaving 191 g (91%) of the desired product, bp 127°C at 0.5 mm.

IR: γ^{neat} 3350, —OH
NMR: δ^{CCl_4} 3.45 (*m*, 3H); 2.08 (*m*, 4H); 1.71–1.16 (*s*, broad, 16H); 0.96 (*t*, 3H, $J \sim 5$–6 cps)
MS: *m/e* 192 (M—H_2O)

(Z)-10-Tetradecen-1-ol

10-Tetradecyn-1-ol (100 g, 0.476 mol) was hydrogenated (Cram and Allinger, 1956) with 1 g of 5% palladium-on-barium sulfate in 1 liter of absolute methanol to which 1 g of quinoline was added. The solution was kept at 400 psi of H_2 for 30 min with the total uptake of hydrogen being 0.476 mol. After the catalyst was removed, the methanol was evaporated leaving 94.9 g of alkenol (94%), bp 122°C at 0.40 mm.

IR: γ^{neat} 3350, —OH; 1668; 711 (*cis* out of plane bend)
NMR: δ^{CCl_4} 5.29 (*t*, 2H, $J \sim 4$–5 cps); 3.52 (*m*, 3H); 1.96 (*m*, 4H); 1.63–1.15 (*s*, broad, 16H); 0.91 (*t*, 3H, $J \sim 5$ cps)
MS: *m/e* 212 (M); 194 (M—H_2O)

(Z)-10-Tetradecenyl Acetate

To 100 g (0.472 mol) of (Z)-10-tetradecenol in a 500-ml RB flask equipped with dropping funnel, reflux condenser, and magnetic stirrer, 50 ml of acetyl chloride (50% excess) was added dropwise (reaction was highly exothermic). After the addition was completed, the resulting dark red solution was stirred for 1 hr at room temperature. 100 g of ice water was added cautiously followed by 200 ml of diethyl ether. The phases were separated, the ether layer neutralized with $NaHCO_3$, and washed with distilled water. The aqueous layers were combined and washed with three 50-ml portions of ether. The ethereal extracts were dried over anh. Na_2SO_4 and the ether removed by roto evaporation. The product was distilled through a short-path column, bp 125–126.5°C at 0.5 mm, yielding 114 g (95%) of the desired product as a clear, colorless liquid.

IR: $\gamma^{CH_2Cl_2}$ 1729 (C=O); 1634 (C=C); 1233 (C—O); 710 (*cis*-C=C)
NMR: δ^{CCl_4} 5.28 (*m*, 2H); 3.96 (*t*, 2H, $J \sim 6$ cps); 1.96 (*s*, 3H); 1.64–1.12 (*s*, broad, 16H); 0.91 (*t*, 3H, $J \sim 6$ cps)
MS: *m/e* 254 (M); 194 (M—HOAc); 166; 152; 138; 82 (base peak)

An analysis for purity was carried out on a computerized gas chromatograph–mass spectrometer on a 2 mm ID, 150 cm 10% diethylene glycol succinate glass U-tube column (100–120 mesh Chromosorb W) at 135°C; the product was >95% of the *cis* isomer.

Acknowledgments—We are indebted to Mary Elizabeth Anderson who provided gas chromatographic–mass spectral analyses and to Joseph Wichmann who aided in the large-scale synthesis.

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SEX PHEROMONE OF THE FEMALE DERMESTID BEETLE *Trogoderma glabrum* (Herbst)

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Abstract—5 components of the sex pheromone of the female dermestid beetle, *Trogoderma glabrum*, have been isolated and identified: methyl (E)-14-methyl-8-hexadecenoate, (E)-14-methyl-8-hexadecen-1-ol, methyl (Z)-7-hexadecenoate, *n*-hexanoic acid, and 4-hydroxyhexanoic acid lactone (γ-caprolactone). A sixth weakly active compound was isolated but not identified. Each of the identified compounds independently elicited attractive and sexually excitatory responses in *T. glabrum* males.

Key Words—Coleoptera, Dermestidae, isolation, identification, sex pheromone, *Trogoderma glabrum*.

INTRODUCTION

The dermestid beetles include many economically important insect pests that infest nearly all forms of stored products, including grain, meats, dairy products, carpets and clothing. Among the worst pests in this family are the black carpet beetle, *Attagenus megatoma*, and several species of *Trogoderma*, including *T. granarium* (the khapra beetle), *T. inclusum*, *T. glabrum*, and *T. variabile*.

Burkholder and Dicke (1966) have reported evidence of sex pheromones

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for *Attagenus megatoma*, *T. inclusum*, and *T. glabrum*. Subsequently (E,Z)-3,5-tetradecadienoic acid was identified as the principal sex attractant for *A. megatoma* (Silverstein et al., 1967). In 1969, (-)-(Z)-14-methyl-8-hexadecen-1-ol and (-)-methyl-(Z)-14-methyl-8-hexadecenoate were identified as two components of the sex pheromone of *T. inclusum* (Rodin et al., 1969). Vick and coworkers (1970) then observed considerable pheromone interspecificity among 7 *Trogoderma* species and suggested that identical or similar compounds may serve as attractants for these species. Recently the sex pheromone production sites for several *Trogoderma* species have been reported (Hammack, Burkholder, and Ma, 1973). We now report the isolation and identification of the components of the *Trogoderma glabrum* sex pheromone from an extract of virgin females. Fractionation of the extract to isolate the individual components of the pheromone was monitored by a laboratory bioassay.

METHODS AND MATERIALS

Trogoderma glabrum virgin females (30,000 adults), reared at the Stored Products Research Laboratory, U.S. Department of Agriculture, Madison, Wisconsin, were shipped packed in dry ice and were stored at −50°C. Whole insects were extracted with pentane in a Waring blender, and the extract was concentrated by reduced pressure distillation (20 mm Hg, 25°C) to yield an oily residue (about 20 g) that was distilled in a short-path distillation apparatus (0.02 mm Hg, 80°C, 4 h). The distillate was removed from a dry ice–acetone-cooled cold-finger and dissolved in pentane. Ammonia gas was bubbled through this solution for 0.5 min at room temperature. The precipitate was removed by centrifugation, washed with pentane, acidified with dilute HCl, and extracted with diethyl ether; this solution contained the carboxylic acids. The pentane solution of neutral and basic components was extracted with dilute HCl; the HCl solution was neutralized with 5% $NaHCO_3$ and extracted with diethyl ether. This solution containing the amines showed no activity and was not studied further. The pentane solution of the neutral components formed a precipitate on cooling to −6°C. The cold mixture was centrifuged, and the supernatant neutral components were fractionated by gas chromatography on the following columns: column A, 6% Carbowax 20M on Chromosorb G 60/80 mesh, 2.5 m × 6 mm (OD), 60 ml/min He flow rate, held at 149°C for 12 min, then temperature programmed at 2°/min to 230°C and held at 230°C for 72 min; column B, 5% diethylene glycol succinate (DEGS) on Chromosorb G 60/80 mesh, 2.5 m × 6 mm (OD), 60 ml/min He flow rate at 162°C; column C, 5% DEGS on Chromosorb G 80/100 mesh, 6 m × 2.4 mm (OD), 30 ml/min He flow rate at 171°C; column

D, 6% Apiezon L on Chromosorb G 60/80 mesh, 6 m × 6 mm (OD), 60 ml/min He flow rate at 196°C; column E, 5% FFAP on Chromosorb G 80/100, 3 m × 2.4 mm (OD), 30 ml/min He flow rate at 156°C; column F, 4% Carbowax 20M on Chromosorb G 60/80 mesh, 3 m × 2.4 mm (OD), 30 ml/min He flow rate at 139°C.

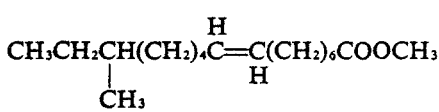
Fractions from the Varian Model 204B gas chromatograph were collected in glass capillary tubes (30 cm × 2 mm OD) in a thermal gradient collector (Brownlee and Silverstein, 1968).

Infrared spectra were obtained on 30-μg samples in spectroquality carbon tetrachloride on a Perkin-Elmer Model 621 grating spectrophotometer with a Barnes Engineering 4-μl cavity cell, a beam condenser, and a variable path-length cell to balance out the solvent. Mass spectra were obtained on 5–10-μg samples on a Hitachi RMU-6 mass spectrometer operated at 70 eV. The samples, collected directly from the gas chromatograph in glass capillaries, were introduced into a modified inlet system. Nuclear magnetic resonance spectra were obtained on 100–200-μg samples in deuterochloroform with tetramethylsilane as an internal standard on Varian HR 100 (computer of averaged transients) and Varian XL 100 (Fourier transform) spectrometers. Microozonolyses were performed on 5–10-μg samples in hexane or carbon disulfide at −65°C (Beroza and Bierl, 1966).

RESULTS AND DISCUSSION

Gas chromatographic fractionation of the supernatant neutrals on column A produced 4 active fractions.

The fraction eluting between 29 and 32 min on column A was further purified on column B. The major component, eluting between 19 and 21 min (about 0.25 mg from 30,000 beetles), was methyl (E)-14-methyl-8-hexadecenoate (I) (Figure 1).



(I)

A strong band in the infrared (IR) spectrum at 1740 cm^{-1} (C=O), coupled with a characteristic 3-band pattern at 1250–1160 cm^{-1} (C—O stretching), suggested a fatty acid methyl ester. A weak band at 3030 cm^{-1} together with a sharp band at 967 cm^{-1} suggested a *trans* double bond. The mass spectrum showed M^+ 282 and diagnostic peaks at *m/e* 253 (M^+—CH_3CH_2), 250 (M^+—CH_3OH), and 74 (McLafferty rearrangement). The

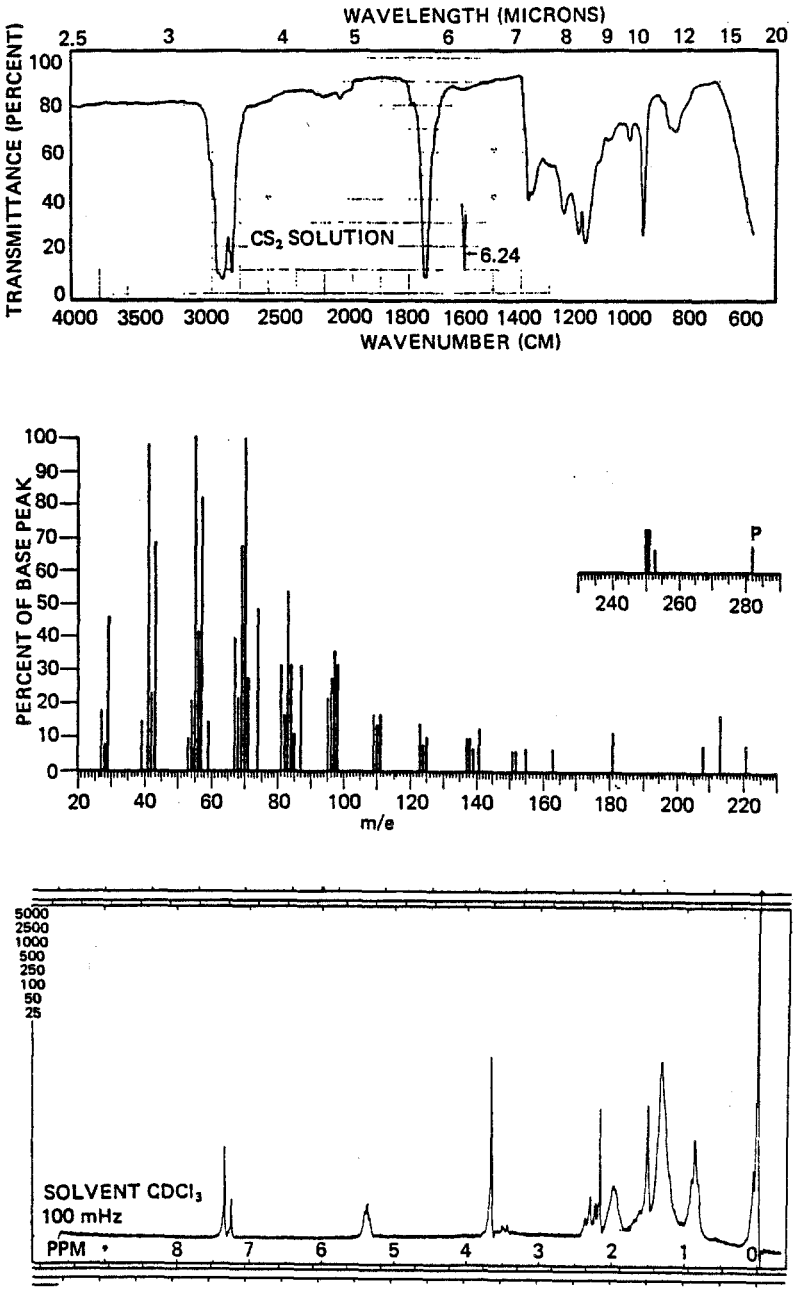


FIG. 1. Infrared, mass, and nuclear magnetic resonance spectra for compound (I).

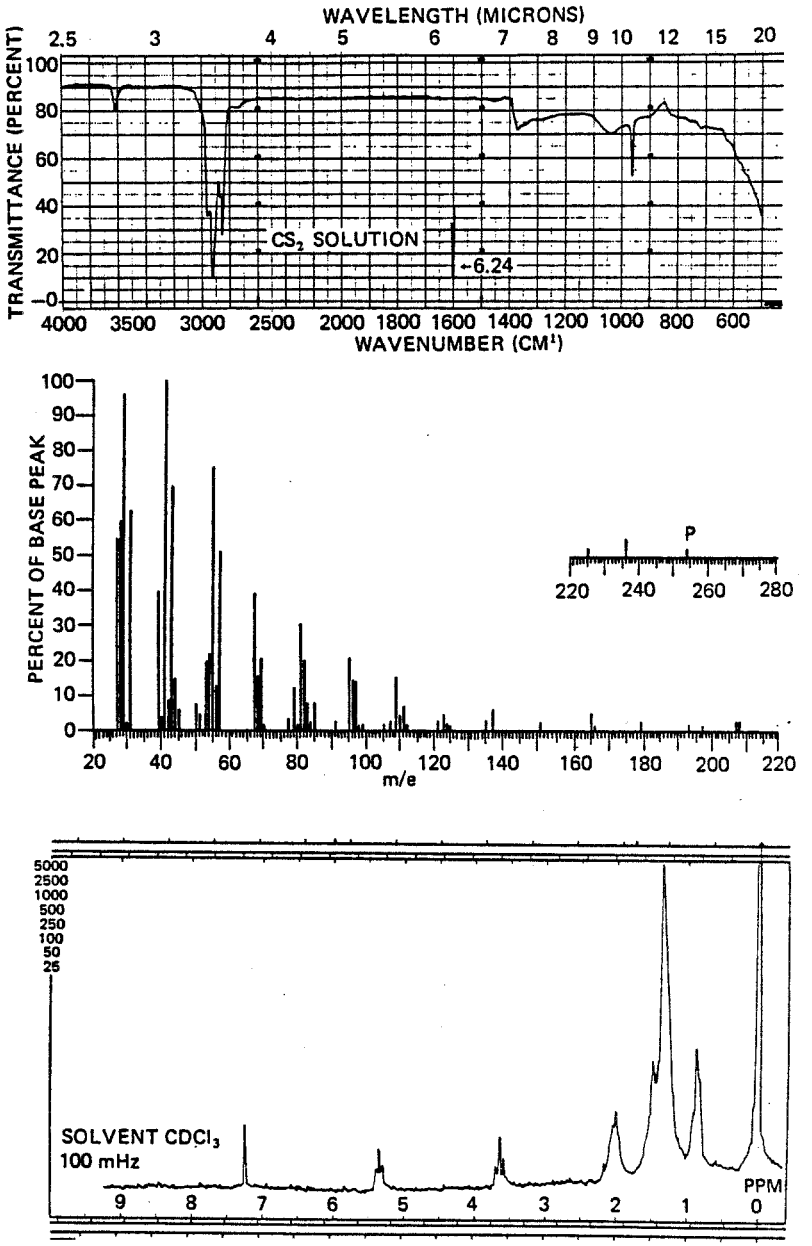


FIG. 2. Infrared, mass, and nuclear magnetic resonance spectra for compound (II).

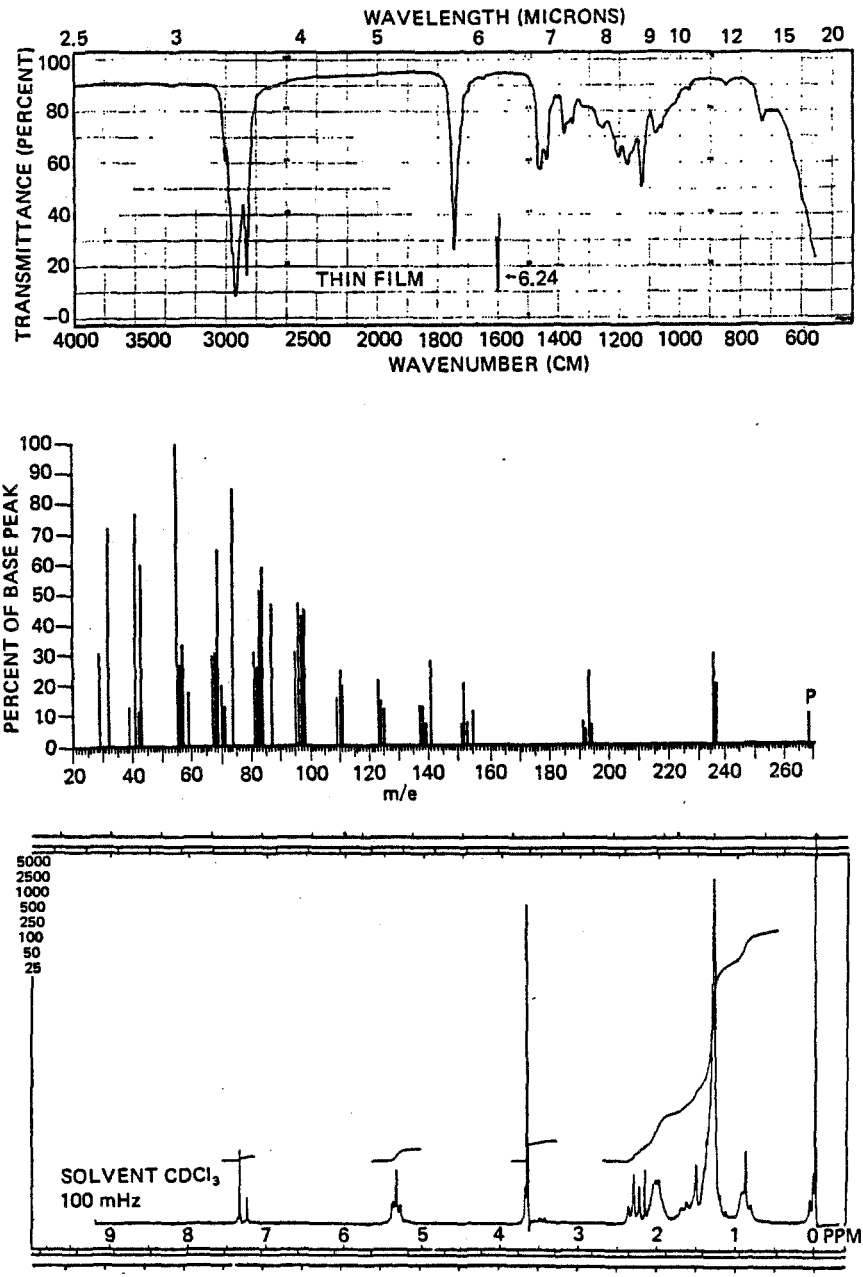


FIG. 3. Infrared, mass, and nuclear magnetic resonance spectra for compound (III).

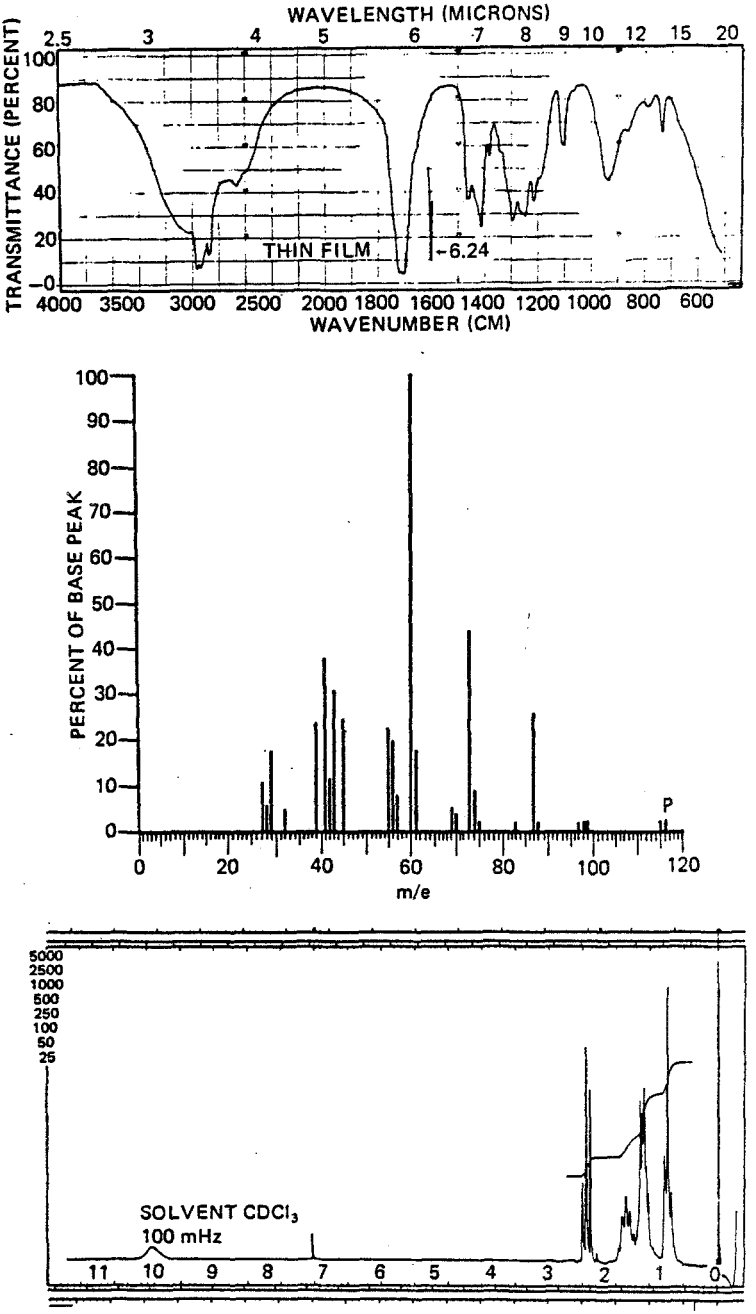


FIG. 4. Infrared, mass, and nuclear magnetic resonance spectra for compound (VI).

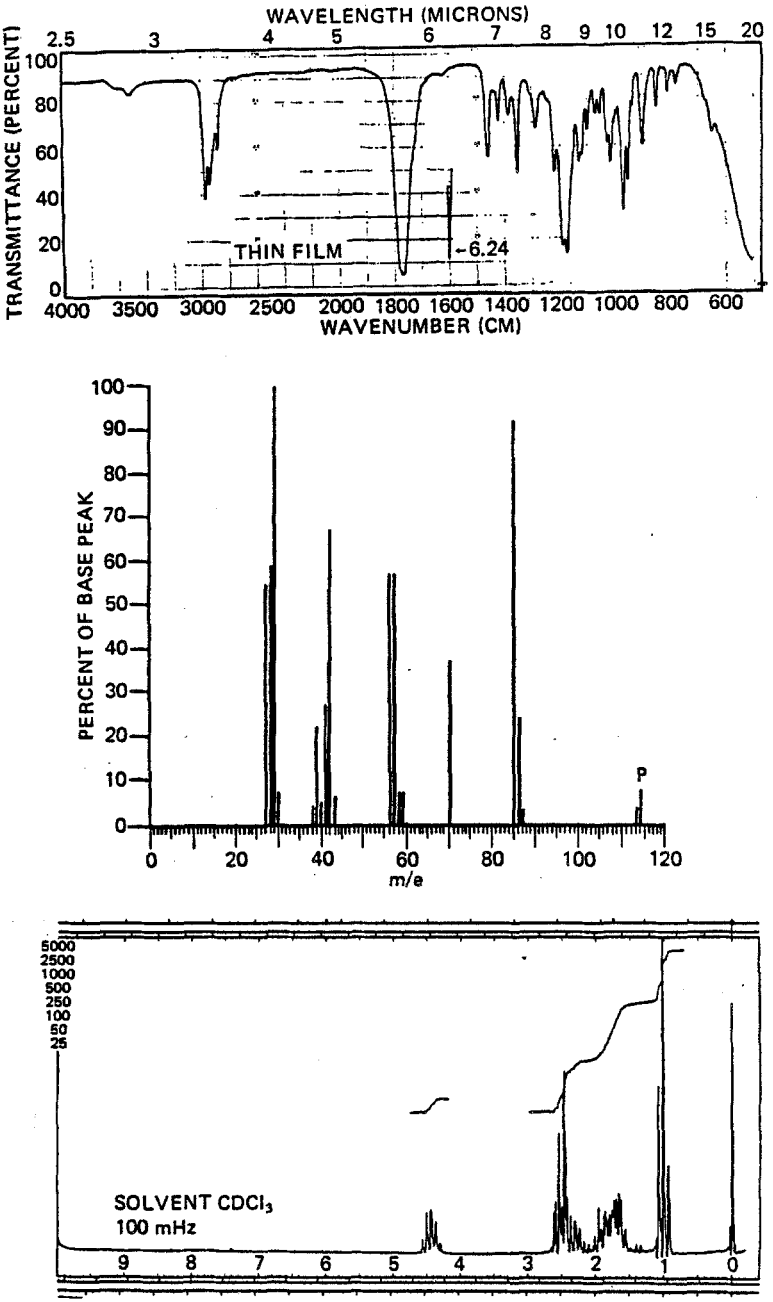


FIG. 5. Infrared, mass, and nuclear magnetic resonance spectra for compound (V).

nuclear magnetic resonance (NMR) spectrum confirmed this with diagnostic peaks at 3.67 ppm (OCH_3), 5.34 ppm (CH=CH), and 2.31 ppm ($—CH_2CO_2—$).

Microozonolysis of 10 μg of (I) in carbon disulfide produced 6-methyloctanal and methyl 8-oxooctanoate.

The major component of the fraction eluting between 36 and 42 minutes on column A (about 0.2 mg from 30,000 beetles) was (E)-14-methyl-8-hexadecen-1-ol (II) (Figure 2). Absorption in the IR spectrum at 3626 cm^{-1}

$$CH_3CH_2CH(CH_3)(CH_2)_4C(H)=C(H)(CH_2)_6CH_2OH$$

(II)

(monomeric O—H stretching) indicated an alcohol, and a sharp band at 965 cm^{-1}, coupled with absorption at 3020 cm^{-1} (olefinic C—H stretching), suggested a *trans* double bond. The mass spectrum showed M^+ 254 and peaks at 236 ($M^+—H_2O$), 225 ($M^+—CH_2CH_3$), 208 ($M^+—H_2O$ and $CH_2=CH_2$), and 31 ($CH_2=\overset{+}{O}H$). These data suggested a branched long-chain primary alcohol. The NMR spectrum supported this with absorption at 5.36 ppm (CH=CH) and 3.65 ppm ($—CH_2O—$). GLC analysis of the methyl ether derivative of (II) (NaH and CH_3I) on an Apiezon L capillary column (15.2 m × 0.5 mm) indicated that only the E isomer was present. (This column, operating at 190°C and 3 ml/min He flow rate, easily separated [$R > 0.95$] E and Z isomers of (I) and (II) [methyl ether derivative].) Microozonolysis of the methyl ether of (II) produced 6-methyloctanal and 9-oxadecanal.

The fraction eluting between 26 and 29 min on column A was further fractionated on column C. The fraction eluting between 26 and 28 minutes on column C was further purified on column D. The component eluting between 69 and 73 minutes (about 0.3 mg from 30,000 beetles) was identified as methyl (Z)-7-hexadecenoate (III) (Figure 3). The IR spectrum of (III)

$$CH_3(CH_2)_7C(H)=C(H)(CH_2)_5COOCH_3$$

(III)

showed absorption at 1748 cm^{-1} (C=O) and characteristic fatty acid methyl ester C—O absorption at 1220–1120 cm^{-1}. Absorption at 3020 cm^{-1} (olefinic C—H stretching) coupled with a lack of absorption at 965 cm^{-1} (i.e., no *trans*-CH=CH) suggested a *cis* double bond. Diagnostic peaks in the mass spectrum included M^+ 268, 236 ($M^+—CH_3OH$), and 74 (McLafferty rearrangement). The NMR spectrum provided confirmation with diagnostic peaks at 3.68 ppm (OCH_3), 5.29 ppm (CH=CH), and 2.26 ppm

(—CH_2CH_2—). Ozonolysis (Beroza and Bierl, 1966) of compound III produced nonanal and methyl 7-oxoheptanoate.

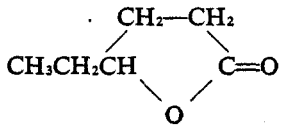
Gas chromatography of the ether solution of the carboxylic acids on column E produced an active component eluting between 8 and 9 minutes (about 5 mg from 30,000 beetles). This components was identified as *n*-hexanoic acid (IV) (Figure 4). A broad band in the IR spectrum at 3600–2400

$$CH_3CH_2CH_2CH_2CH_2COOH$$

(IV)

cm^{-1}, coupled with a band at 1720 cm^{-1} (C=O stretching), suggested a carboxylic acid. The mass spectrum showed M^+ 116 and 60 (—$CH_2C\overset{+}{O}_2H_2$). The NMR spectrum showed absorption at 10.05 ppm (CO_2H), 2.3 ppm (—CH_2—CO_2—), 1.2–1.8 ppm (—$(CH_2)_3$—), and 0.9 ppm (—CH_3).

γ-Caprolactone (V) was isolated from the distillate obtained by the concentration of the crude extract. Gas chromatography of this material on column F produced an active component between 25 and 27 min (about 6 mg from 30,000 beetles). The IR spectrum of compound (V) (Figure 5) showed



(V)

bands at 1770 cm^{-1} (C=O) and 1180 cm^{-1} (C—O). The mass spectrum showed M^+ 114, and prominent peaks at *m/e* 85 (M^+—CH_2CH_3), 56 (M^+—C_2H_5CHO), and 70 (M^+—44). These data suggested a saturated γ-lactone. The NMR spectrum further confirmed this with absorption at 4.30 ppm (—CH—O—), 2.5 ppm (—CH_2—), 1.0 ppm (—CH_3), and 1.6–2.6 ppm (—CH_2—CH_2—).

The fraction that eluted between 48 and 52 min on column A exhibited weak activity, but, due to insufficient material, the active component was not identified.

Biology

The laboratory bioassay apparatus and the responses of *Trogoderma* males to extracts of *T. glabrum* females have been described (Burkholder and Dicke, 1966).

Each of the identified compounds, (I–V), independently evoked an attractive and sexual excitatory response from *T. glabrum* males. No synergistic effects were observed. The compounds are active at the levels at which they are produced by the insect.

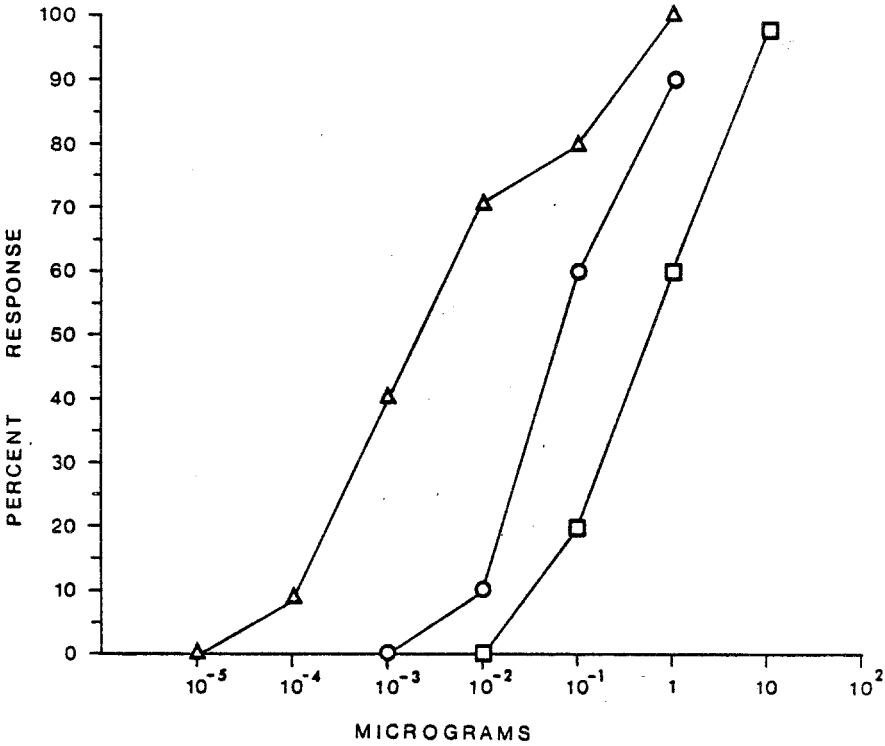


FIG. 6. Response of *T. glabrum* males (8–9 days old) to compounds (I–III). △, (E)-14-methyl-8-hexadecen-1-ol; ○, methyl (Z)-7-hexadecenoate; □, methyl (E)-14-methyl-8-hexadecenoate.

Figure 6 displays the response of *T. glabrum* males (8–9 days old) to different amounts of synthetic samples of compounds (I–III). Hexanoic acid, (IV), and γ-caprolactone, (V), had response thresholds of about 5 μg each, and each compound attracted 10% and 30% of male test insects at 10 μg and 20 μg, respectively. Contributions of individual compounds to the total mating behavior are under study.

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POPULATION-DENSITY-DEPENDENT MATING FREQUENCY AMONG *Plodia interpunctella* (LEPIDOPTERA: PHYCITIDAE) IN THE PRESENCE OF SYNTHETIC SEX PHEROMONE WITH BEHAVIORAL OBSERVATIONS[1,2]

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Abstract—Mating frequency among *Plodia interpunctella* (Hübner) held in enclosed environments was reduced in the presence of the synthetic sex pheromone, (Z,E)-9,12-tetradecadien-1-ol acetate. The effectiveness of any dose of pheromone was markedly increased as population densities were decreased from 10 to 0.1 pairs/m^2 of wall and ceiling surface. Mating behaviors of the insect are described.

Key Words—sex pheromone, *Plodia interpunctella*, density dependent.

INTRODUCTION

The permeation of an insect's environment with synthetic sex pheromones, or with pheromone like materials, will reduce the effectiveness of pheromone-mediated mating communications for certain species (Gaston, Shorey, and Saario, 1967; McLaughlin et al., 1972; Cameron et al., 1974) and so can result in reduced crop damage (Shorey, Kaae, and Gaston, 1974). Females of the Indian meal moth, *Plodia interpunctella* (Hübner), produce the sex pheromone (Z,E)-9,12-tetradecadien-1-ol acetate (ZETA) which excites and attracts

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

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males (Kuwahara et al., 1971; Brady et al., 1971). Females also release (Z,E)-9,12-tetradecadien-1-ol (ZETOH), which aids in species isolation, and perhaps additional sex pheromone materials (Sower, Vick, and Tumlinson, 1974).

We therefore investigated the following hypotheses: (1) that the synthetic sex pheromone ZETA would reduce the mating frequency of Indian meal moths held in an enclosed environment, (2) that the effectiveness of ZETA treatment would be proportional to the dose applied, and (3) that the effectiveness of any dose of ZETA would be dependent on the density of the population of Indian meal moths.

METHODS AND MATERIALS

Moths were reared by using the methods of Silhacek and Miller (1972). Photoperiods for all tests were 14:10, light/dark with photophase intensitive of >150 lux, produced by cool white fluorescent tubes. Scotophase intensities were <1 lux. Light/dark transitions were abrupt unless otherwise stated. The test adults were 1–3 days old except for those that were introduced into experimental environments as pupae.

The ZETA pheromone (ca. 15% of the Z,Z isomer as an impurity) was dispensed by evaporation directly from a surface film. An antioxidant (*N*-phenyl-*N'*-octyl-*P*-phenylenediamine) (Wolf et al., 1972), was mixed with ZETA at 5% by volume unless otherwise specified. Without antioxidant, ZETA exposed to air in a thin film is chemically modified: within 10 days, a much more viscous material that is no longer soluble in diethyl ether or hexane is produced.

Doses for the experimental treatments were regulated by evaporating ZETA from containers with different sized surface areas (10× increments) ranging from 0.05-cm-diameter capillary pipettes to 15-cm-diameter petri dishes. A rate of evaporation at 27°C of ca. 1 mg/day of ZETA was determined by measuring weight loss and by gas chromatographic analysis of residues remaining after specific intervals on 5-cm-diameter petri dishes. Rates of evaporation from the other containers were assumed to be roughly proportional according to the surface areas exposed to the air.

EXPERIMENTAL PROCEDURES, OBERVATIONS, AND RESULTS

Dose/Response Tests

In one series of tests the effects of several doses of ZETA on mating frequency were measured in a 6.1 × 6.1 × 2.4-m moth-tight room. Tempera-

tures were 27±6°C at 73±13% relative humidity. Pupae were separated by sex and 50 pairs/day were placed in 2 opposite corners of the empty room. The emerged adults were sampled at a rate of 30 individuals/day; approximately 6 individuals were randomly selected from each wall and from the ceiling. The population reached equilibrium at 90–100 pairs of adults within a few days, and then was maintained continuously throughout the duration of the tests.

In each room 8 ZETA dispensers were used; these were located 1 m diagonally from the upper right and lower left corner of each wall. Initial doses of ca. 1×10^{-5} mg/m^3 of space each day were released from the 0.05-cm-diameter dispensers. The dose was subsequently increased by about 10× each week. A total of 7 days' data were averaged for each treatment. In the first series the entire range of 6 doses was tested without antioxidant, but the ZETA was renewed at 2–4-day intervals. In a second series the 1×10^{-1} and 1×10^{-2} mg/m^3 per day doses (released from 1.5- and 5-cm-diameter petri dishes) were retested but with 5% antioxidant added. For each treatment with antioxidant added 7 additional days' data were taken. Mating frequencies were the same with and without antioxidant. Control data were taken over 5–7-day periods before, midway, and after each series of tests. Midway through the tests the ZETA applicators were removed for 10 days before control data were taken.

The sampled adult females were dissected to determine spermatophore content so they could be scored as mated or not mated. Test data are reported (Figures 1 and 2) as the percentage reduction in females mated relative to the percentage for the controls as follows:

$$\frac{\%\ \text{♀ mated controls} - \%\ \text{♀ mated treatment}}{\%\ \text{♀ mated controls}} \times 100 = \%\ \text{mating reduction}$$

Figure 1 indicates the relationship between quantities of ZETA released and change in the incidence of mated females sampled from a continuing population. Overall, increased doses decreased mating significantly ($P = 0.05$). Of the control insects, 63±10% *SD* were mated. Most Indian meal moths emerge from the pupae during the last 4 h of the light period, and our samples were collected just before dark. Accordingly, we estimate that about one-third of the control and test female samples were newly emerged and had little opportunity to mate.

Density/Response Tests

The relationship between population density and mating frequency in the presence of ZETA was determined in a 2.8 × 2.3 × 3.1-m-high room and in

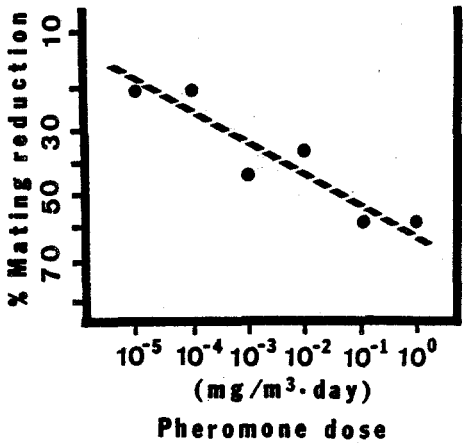

FIG. 1. Reduction of mating relative to controls among *Plodia interpunctella* at several released dosages of (Z,E)-9,12-tetradecadien-l-ol acetate (pheromone). Population densities in the 90 m^3 enclosed environment were ca. 1 pair/m^2 of wall and ceiling surface. Each point indicates averaged data from 7 to 14 days' samples; ca. 12♀ were sampled each day.

four 0.6 × 0.6 × 0.6-m aluminum boxes constructed with 1 acrylic plastic side. Temperatures in the room and boxes were 26±3 and 28±2°C, respectively; and the relative humidity was 70±10 and 60±5%. The room had no air circulating, but filtered air was passed through the boxes (1.5 liter/min) and exhausted into a fume hood. Adult male insects were released into the room and boxes each night 1 h before the scotophase; virgin females were released 20 min later. From 1 to 33 pairs of insects were placed into the boxes and 3–100 pairs into the room each day. Insects were collected, and the females were dissected for spermatophores 22–23 h after release. Control data were taken prior and subsequent to each series of tests in the room and concurrently with the tests in the boxes. The data obtained after each day's tests were considered to constitute 1 replication of any given condition except where only 1 pair of insects was released. When only 1 pair of insects was released, 2 sets of data were combined for each replication. Each data point plotted on Figure 2 was replicated 5 times.

Rates of evaporation into the boxes and rooms were constant and continuous throughout each series of tests. Single pheromone containers of 0.05, 0.5, or 1.5 cm diameter were placed in the boxes and 4.0–5.0-cm-diameter containers, 1 centered on each wall, were placed in the room for the tests.

Population densities are reported (Figure 2) as numbers of pairs of

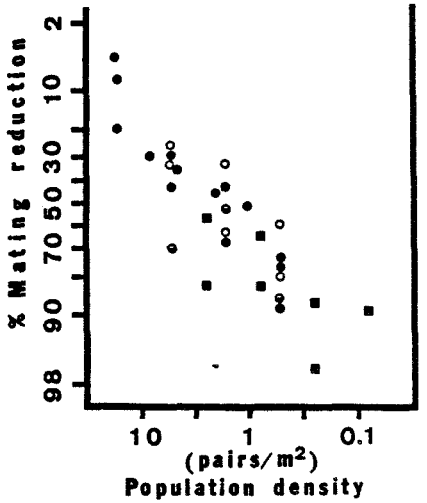

FIG. 2. Reduction of mating relative to controls among *Plodia interpunctella* at indicated population densities in the presence of (Z,E)-9,12-tetradecadien-l-ol acetate (pheromone). Doses of pheromone released/night/m^3 of volume into 0.2 m^3 enclosed environments were: 5×10^{-4} mg (open circles), 5×10^{-2} mg (half-solid circles), and 5×10^{-1} mg (solid circles). Solid squares indicate dosages of 2×10^{-1} mg/m^3/night released into a 30-m^3 enclosed environment. Each point represents the average of 5 replications.

insects/m^2 of wall and ceiling space available. The floor surfaces available were disregarded because calling females were rarely observed on the floor.

A decrease in the population densities markedly decreased the frequency of mating over a 24-h period (Figure 2) in the presence of $>5 \times 10^{-4}$ mg/m^3 ZETA. In the absence of synthetic ZETA, $60 \pm 15\%$ and $83 \pm 18\%$ *SD* of the insects held in the room and boxes, respectively, were mated each day regardless of population densities.

Other Compounds Tested

The Z,Z isomer of ZETA can inhibit the male response to ZETA (Vick and Sower, 1973), and females also release ZETOH. Accordingly, the Z,Z isomer was removed from the ZETA by liquid chromatography,[3] and the effect of the purified material (99%+) was compared with that of ZETA + 15% of Z,Z isomer and that of a mixture of equal parts ZETA and ZETOH, both containing 15% of the Z,Z isomers. These tests were also conducted in boxes at population densities of 2.7 pairs of insects/m^2 of surface at a pheromone dose of 0.5 mg released/m^3/night (10 replications).

[3] We thank K.W. Vick for cleaning the ZETA.

Mixtures of ZETA + Z,Z isomer or ZETA + ZETOH had the same effects on mating frequency as purified ZETA: all such treatments reduced mating by about 30% relative to the controls.

Supplementary Behavioral Observations

Additional information concerning the behavior of the Indian meal moth was obtained by observing insects under the various conditions reported. Also, repeated observations (>100 h) were made of behavior of male, female, and paired insects at diverse population densities in the presence and absence of ZETA in glass containers (0.25–25-liter capacity). Doses of ZETA were comparable to those used in the population density studies. Most of these observations were made during the last hour of the photophase and the first hour of the scotophase.

Finally, the spontaneous activities of 6 individual males were monitored over 72-h periods in the absence of any known variables except photoperiod. These activities were monitored automatically according to the rate of production of CO_2 using the methods of Turner and Charity (1971). These insects were held at $28 \pm 1°C$ and $60 \pm 5\%$ relative humidity in a 17-ml container through which air was passed at a rate of 60 ml/min. Males were introduced into the apparatus within 24 h after their emergence. The light/dark transitions were gradual over a 30-min period.

In the absence of synthetic ZETA, virgin females released into a group of males typically began calling and mated within 2 min. Males newly exposed to calling females exhibited a consistent sequence of activities here separated into *orientation behavior* and *copulation behavior*.

In the *orientation behavior* sequence, males became active, vibrated their wings, and moved about at or near the walls until they located the calling females. The location appeared strongly facilitated by anemotaxis, if moderate air velocities (10–50 cm/sec) prevailed, or a chemokinesis, a persistence of searching in an areas of chemical stimuli, in still air.

Copulation behavior began when the calling females were located. Typically, a male physically contacted the abdomen of the female from the rear with his antennae and legs, moved anterior to the female, and inserted his head and forelegs under the head of the female. When the head contact was secure, the male cast the tip of his abdomen up over the female and into firm contact with the female genitalia and then rotated laterally to a copulatory position posterior to and facing away from the female. Wing vibration by the male was continuous until after he had secured the female's abdomen. The females were not passive during these sequences of behavior: In addition to actively releasing pheromone while calling (Sower and Fish, 1975), a typical female moved in coordination with the movement of the male and,

although infrequently, sometimes approached males to make the initial contact.

We observed that when males were habituated to the presence of synthetic ZETA, the orientation behavior was not stimulated by the introduction of calling females, even when the females were 1–2 cm from the males. Apparently, the synthetic ZETA greatly impaired the ability of females to communicate sexual receptivity to males over a distance >1–2 cm.

Males, whether they had or had not habituated to ZETA, were sometimes observed to spontaneously run or fly. This activity, most prevalent during the scotophase, was therefore monitored in the absence of ZETA by measuring the output of CO_2. The males were active an average of 2 hr/day. However, even in the presence of ZETA, active males sometimes approached and physically contacted other insects. If the other insect was a male, the contact occasionally included a brief (<5 sec) exhibition of copulation behavior. If the other insect was female, copulation behavior almost always occurred and persisted for about 20 sec or until copulation. The habituation of males to ZETA did not appear to inhibit copulation behavior if the females were closely approached (<1–2 cm) by active males.

DISCUSSION AND CONCLUSIONS

The evolutionary development of a distance communication between insects of a given species allows the insects to disperse more widely than would otherwise be possible. Dispersion without efficient mating communication would mean that the replacement of each generation would not be assured. Further, if the ability of a species to communicate over distances were artificially impaired, it follows that higher average population densities would be needed to assure a high frequency of mating.

Our observations suggest that the distance communication between Indian meal moths was virtually eliminated by the synthetic ZETA. Probably the males located females mainly by unsolicited encounter during periods of spontaneous movement. Since we observed that undisturbed Indian meal moths flew an estimated 5–20 cm/sec when they were moving near a wall, the insects can potentially locate other insects in a total area greater than 3 m^2/hr of activity if we assume only a 1-cm distance for effective mating communication in the presence of synthetic ZETA. Female Indian meal moths may release sex pheromone materials which have not yet been tested, and Grant (1974) has shown the presence of a male-produced pheromone that influences the copulatory behavior of the female. Whether the addition of such compounds to the control treatments would increase the obtained effect is not known. The *copulatory behavior*, perhaps mediated by chemical means as well as by visual or other cues, was not affected by ZETA alone.

Control of Indian meal moths in enclosed environments by permeation of the air with ZETA thus appears feasible only when population densities are lower than 0.1 pairs of insects per m^2 of wall surface. However, projections of laboratory data to field environments that are less well controlled should be conservative. We therefore suggest that this density be treated only as an upper limit.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS: I. EVALUATION FOR INCREASING RATES OF PARASITIZATION BY *Trichogramma* spp. IN THE FIELD[1,2,3]

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Abstract—Kairomones for *Trichogramma* spp. were evaluated in the field on soybeans and crimson clover. Blanket (complete coverage) spraying of plots (either a synthetic tricosane or an eluate from a hexane extract of moth scales, depending on the responsiveness of the *Trichogramma* spp. present) resulted in increased parasitization by released and wild *Trichogramma* populations. The increased parasitization resulted for both natural and artificially applied eggs.

Key Words—kairomones, *Trichogramma evanescens*, *Trichogramma achaeae*, biological control, pest management, *Heliothis zea*, *Anticarsia gemmatalis*, pheromones, insect behavior, host finding, parasitoids, behavior chemicals.

INTRODUCTION

Numerous investigations, beginning with an observation by Thorpe and Jones (1937), have demonstrated that the behavior of entomophagous insects is influenced in various ways by chemicals produced by their host or prey. In the studies of entomophagous insects at our laboratory, we have

[1] Hymenoptera: Trichogrammatidae.
[2] In cooperation with University of Georgia College of Agriculture Experiment Station, Coastal Plain Station, Tifton, Georgia.
[3] Mention of a proprietary product does not necessarily imply endorsement by the USDA.

placed a great deal of emphasis on investigations of kairomones as defined by Brown, Eisner, and Wittaker (1970), because we are of the opinion that they may offer considerable potential in the management of insect pests. Lewis and Jones (1971) demonstrated that feces from *Heliothis zea* (Boddie), as well as hexane extracts of the feces, elicited a host-seeking response by *Microplitis croceipes* (Cresson). The active material was later identified by Jones et al. (1971) as 13-methylhentriacontane. Moth scales left by ovipositing moths were determined to be the source of a kairomone that elicited a host-seeking response by *Trichogramma evanescens* Westwood, and the active components were obtained in hexane extracts of the scales (Lewis, Jones and Sparks, 1972). In this same article an initial appraisal of the usefulness of these kairomones in the field for increasing the parasitization by *Trichogramma* was made. The incidence of parasitization of eggs artifically applied on alternating treated and control leaves was compared within an area of release of *T. evanescens* in a 0.5-hectare cotton field. The treatment applied was a hexane extract of *H. zea* moth scales. In this and other related tests, 1.5–2.0 times more parasitization was obtained on the treated leaves than on untreated leaves. Jones et al. (1973) used the same technique to evaluate various fractions extracted from *H. zea* moth scales and determined that tricosane was the most active component.

The reports to be presented in this series will deal with more extended studies of the potential uses of kairomones, their occurrence among various entomophagous insects, and the interrelated behaviors involved.

The studies reported here were designed to determine whether these kairomones, either extracts from moth scales or synthetic tricosane, were active when they were applied in a solid pattern over the leaf surfaces of larger plots, whether they would increase parasitization of naturally deposited eggs that would have natural kairomones present, and whether they could be used to manipulate the behavior of wild *Trichogramma.*

METHODS AND MATERIALS

The hexane extracts of the corn earworm moth scales were formulated according to the procedure described by Jones et al. (1973). The extract formulations and synthetic tricosane were applied with hexane as a carrier solution using the pneumatic spray system described by Nordlund et al. (1974). *Trichogramma* for release were reared according to the procedure used by Lewis and Redlinger (1969). *Heliothis zea* eggs placed in plots to monitor parasitization were applied to the plants with a brush by using Plantgard® as an adhesive according to the procedure described by Nordlund et al. (1974). The procedure for dissecting the eggs was described by Lewis and

Redlinger (1969). The *t* test and analysis of variance were used to determine whether differences between treatments were significant. Arcsin transformations were conducted on percentages prior to analysis.

PROCEDURES AND RESULTS

Experiment 1

Experiment 1 was conducted to determine whether applications of the tricosane in a blanket pattern over an entire plot would result in improved parasitization similar to that obtained in earlier studies by treating single leaves on which eggs were applied (Lewis, Jones, and Sparks, 1972). 12 soybean plots, 6.1 × 7.3 m (8 rows) in size and having 3.5 m of separation on all sides were selected for the study. The plots were arranged in 6 pairs with 1 of each pair selected at random for treatment with tricosane at the rate of 395 mg/hectare, and the other plot of each pair served as a control. Eggs of *H. zea* were placed on the plants at a rate of 64/plot. Plastic cups, 0.5 oz in size, each containing 200 adult *T. achaeae* Nagaraja and Nagarkatti[4] were opened and placed in the furrows throughout each plot at the rate of 8 cups/plot. After a 4-h exposure, the mean level of parasitization was 21% in the treated plot and 8% in the control plots. The difference was significant at the 1% level of probability.

Experiment 2

A study was conducted to determine whether the kairomone would function to improve parasitization of naturally deposited eggs that already must contain some amount of the kairomone. 5 pairs (1 treated and 1 control) of plots 7.6 × 3.7 m in size were selected at random through the field. The treated plots were sprayed with tricosane at a rate of 395 mg/hectare. Equal numbers of *T. achaeae* (ca. 800 adults) were released in each of the plots. Sample collections of eggs were made at random in each of the plots ca. 4 h later. Eggs of *H. zea* and the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, were sufficiently abundant so that 40 eggs were available from each plot. Egg samples were predominately *A. gemmatalis*. The eggs were dissected,

[4] Based on the morphological characteristics of the genitalia, the *Trichogramma* colony used for the study fits the description of *T. achaea* as described by Nagaraja and Nagarkatti (1969). This colony (obtained from India) had earlier been accidentally mixed with the *T. evanescens* Westwood used in our previous studies of kairomones, and a loss of the *T. evanescens* colony resulted. Our colony of *T. achaeae* are quite responsive to tricosane. However, since some interchange of genetic material with *T. evanescens* may have occurred, this response is not necessarily representative of the species.

and the percent parasitization in each plot was determined. The parasitization in the treated plots (78%) was significantly higher than in the control plots (58%).

Experiment 3

This experiment and the subsequent experiment were conducted to determine whether the kairomone could be used to increase parasitization by wild *Trichogramma* spp. This appraisal was made in crimson clover. 10 pairs of plots 3.5 × 3.5 m were selected. One of each pair was sprayed with tricosane at a rate of 2965 mg/hectare. 75 eggs were placed in each plot and re-collected after a 24-hr exposure. The parasitization, all the result of naturally occurring *Trichogramma* spp., was significantly higher in the treated plots (14%) than in the control plots (4%).

Experiment 4

The influence of kairomones on parasitization rates of wild parasites on a larger scale was appraised. The study was conducted in a 2.83-hectare soybean field. 8 plots, 15.2 × 38.6 m with 7.6 m between the plots, were selected in a linear pattern across the field. Every second plot was sprayed with a hexane eluate of moth scale extract [this eluate was obtained as described by Jones et al. (1973) and was used in this experiment because a preliminary test indicated that the wild *Trichogramma* in this field were not very responsive to tricosane]. 10 subsample plots consisting of 1 row 3.5 m long were selected in each plot. 40 *H. zea* eggs were placed on each subsample plot, left for 24 h, then re-collected on the first and fifth days after treatment. The mean parasitization for both days was significantly higher in the treated plots (22%) than in the control plots (13%). Assessments of parasitization were not made on the second, third, and fourth days because of adverse weather conditions. There was a long and heavy rain on the second day. Also, there was brief, moderate rainfall ca. 1 hr after treatment and before the first day's evaluation. These data demonstrate that the kairomone treatment is durable and lasts for at least 5 days.

DISCUSSION

The results demonstrate that kairomones can be used, by applying them on the plant surfaces of crops, to increase the rate of parasitization by released or wild *Trichogramma* populations. Increased parasitization resulted for both natural and artificially applied host eggs. These results are highly

encouraging because they are evidence that kairomones offer a variety of possible uses in pest management, both with naturally occurring and released parasites.

Since the distribution of the kairomones does not have to be limited to the immediate vicinity of the host eggs, but functions to increase parasitization when applied in a complete coverage pattern throughout the target area, the possible uses of the materials are enhanced. This fact also indicates that the kairomones function primarily as a releaser to elicit a more intensified search pattern in the areas where they occur rather than as a guidance trail directing the parasite to the egg.

The factor(s) causing the higher rates of parasitization and the various interrelated behavioral patterns could have an important bearing on their utility. Studies on this subject are presently underway and results of these studies will be presented in subsequent reports of this series.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS: II. MECHANISMS CAUSING INCREASE IN RATE OF PARASITIZATION BY *Trichogramma* spp.[1,2,3]

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Abstract—When the effect of the kairomone, tricosane, on parasitization by *Trichogramma achaeae* Nagaraja and Nagarkatti of eggs of *Heliothis zea* (Boddie) was studied in petri dish tests, the greatest percentage parasitization ($\bar{X}$ = 64%) was obtained if the entire filter paper was treated. Treatment of smaller areas (about the eggs) resulted in decreased parasitism. In the greenhouse, highest parasitization ($\bar{X}$ = 71%) by *T. pretiosum* (Riley) of *H. zea* eggs placed on pea seedlings grown in pie pans was obtained if the whole pan was treated; lowest parasitism ($\bar{X}$ = 29%) occurred when the pans were untreated. Parasitization was intermediate ($\bar{X}$ = 52%) in other pans treated only at selected spots. Dissections of *H. zea* eggs collected from kairomone-treated and untreated field plots revealed that eggs of *Trichogramma* spp. were more efficiently distributed (less superparasitism) among host eggs in treated plots. These kairomones increase parasitization of *Trichogramma* spp. by releasing and continuously reinforcing an intensified searching behavior rather than by attracting and guiding the parasite directly to the host.

Key Words—*Trichogramma achaeae*, *Heliothis zea*, biological control, kairomones, pest management, parasitoids, host finding, insect behavior, pheromones, behavior chemicals.

[1] Hymenoptera: Trichogrammatidae.
[2] In cooperation with the University of Georgia College of Agriculture Experiment Station, Coastal Plain Station, Tifton, Georgia.
[3] Mention of a proprietary product in this paper does not constitute an endorsement of this product by the USDA.

INTRODUCTION

The results of the previous report in this series demonstrated that field plot application of tricosane or similar appropriate kairomone(s) resulted in increased rates of parasitization by certain *Trichogramma* spp. (Lewis et al., 1975). It was also demonstrated that the kairomone did not have to be limited to the immediate vicinity of the host eggs, but increased parasitization when applied by blanket spraying of the material throughout the plot. These data suggested that the kairomone functioned primarily as a releaser to elicit a more intensified search pattern in treated areas rather than as a trail substance or attractant which might guide parasites to host eggs. The present studies were designed to elucidate the behavior modification(s) causing increased parasitization.

METHODS AND MATERIALS

Petri Dish Test

Comparative evaluations were made of rates of parasitism by *T. achaeae* Nagaraja and Nagarkatti of *Heliothis zea* (Boddie) eggs in petri dishes with different treatment patterns. The bottom of each 150 × 15-mm petri dish was covered with a piece of 13.8-cm Whatman No. 1 filter paper which was marked with a pencil at 6 different locations in a pattern which would allow maximum separation of the eggs. The kairomone, tricosane, was applied to the paper at the rate of 50 ng/cm^2 using a hypodermic syringe. Each piece of filter paper was treated according to 1 of 4 patterns: circles of 1.2 cm, 2.0 cm, or 3.7 cm in diameter around each designated egg location, or treatment of the entire piece of paper. Eggs were attached to the papers at each of the 6 locations using a spot of rubber cement. Two freshly emerged *T. achaeae* females were introduced into each dish and allowed to search for 45 min, after which the eggs were dissected to determine parasitization according to the method described by Lewis and Redlinger (1969). For each treatment pattern 10–20 replications of this test were conducted.

Greenhouse Studies

A comparison was made in the greenhouse of the parasitization rates by *T. pretiosum* (Riley) of the eggs of *H. zea* placed on plants having different treatment patterns. Crowder pea seedlings grown in 22.8-cm pie pans were used. The 3 treatment patterns evaluated were (1) complete coverage application of the kairomone (blanket treatment), (2) application at restricted locations (partial treatment), and (3) untreated controls. An aerosol chroma-

tograph spray device was used as described by Lewis, Jones, and Sparks (1972) to apply the kairomone. The banket treatment was accomplished by putting 10 pans of peas in a group and spraying them all at once at a rate of 10 μg of tricosane in 1 ml hexane/pan (ca. 26 ng/cm^2). The partial treatment was accomplished by using wrapping paper as a shield. The paper shield was designed so that it could be held over a group of 10 pans. There were 10 groups of 5 holes (3 cm in diam) in the shield arranged so that each group of holes would be directly above 1 of the 10 pans. The 5 holes were evenly spaced over the pan. The area under each hole was marked with a small piece of masking tape. The spraying was done in the same manner as with the blanket spraying, except that the shield allowed the treatment to occur only at the 5 designated locations for each pan. Thus, partially treated pans received the same amount of kairomone per unit of treated area as the blanket-treated pans.

Eggs were applied to the plants by hand, using a camel's hair brush moistened with saliva. 2 eggs were placed in each of 5 treated and 5 untreated locations in the partially treated pans, resulting in a total of 20 eggs/pan. 20 eggs were placed in similar areas of the blanket-treated and control pans. 20 replications (pans) were prepared for each of the 3 treatments, and the pans were arranged in a randomized complete block design with interspacings of ca. 15.2 cm within replications and ca. 0.6 m among replications.

Newly emerged females of *T. pretiosum* (6/pan) were released from 2-dram shell vials placed in the center of each pan.

The eggs were collected after 1 h exposure to the parasites and dissected to determine percent parasitization. Arcsin transformations were made for the percentages, and an analysis of variance was conducted. The means were separated by Duncan's multiple range test.

Distribution of Parasite Eggs, Small Plots

When dissecting host eggs to determine parasitization, a record was kept of the number of parasite eggs per host egg. Occasionally as many as 12 parasite eggs were found in a single host egg, leading us to believe that a significant increase in the percent of parasitization could be obtained simply by achieving a more efficient distribution of the parasite eggs. Subsequent experiments were conducted to determine whether kairomones affect the distribution of parasite eggs.

Plots, 1 row × 3.0 m, were selected in a soybean field. Pairs of plots were arranged on 2 parallel rows separated by 2 interspacing rows (50.8-cm rows). A 1.5-m space was left between plots on the same row. Plots in each pair were designated as treated or control in an alternating pattern. A total of 10 pairs of plots were used.

The treatment consisted of a 1/1000 dilution of a standard moth scale hexane extract eluant (Jones et al. 1973) applied at the rate of 1 ml/30.5 cm of row using a pneumatic sprayer (Nordlund et al. 1974). *H. zea* eggs were applied by hand at the rate of 40 eggs/plot, using Plantgard as an adhesive. Control plots were not sprayed, but eggs were applied in the same manner. All parasitization was by naturally occurring parasites.

The eggs were collected after ca. 20 h and dissected to determine parasitization. The experiment was repeated on the following day. The data for both days were combined to give a total of 10 replications.

The levels of parasitization which would have occurred with a random parasite egg distribution was calculated on the basis of the mean number of stings observed per host egg, as described by Wadley (1967). Based on unpublished personal observations that *Trichogramma* spp. most frequently deposit 2 eggs/sting per *H. zea* egg, the number of stings was considered to be 1/2 the total number of parasite eggs. The ratio of percent random parasitization to percent observed parasitization (R/O) was taken as a measure of the efficiency of the distribution. The lower the R/O, the more efficient the distribution. Significance was determined by a paired t test.

Distribution of Parasite Eggs, Large Plots

Plots 15.2 m long and 40 rows wide were marked in a soybean field using plastic flagging. There were 6.1-m spacings between plots. Treated plots were blanket-sprayed with a 1/1000 dilution of the hexane eluate of the moth scale extract (Jones et al. 1973) at a rate of 2 ml/30.5 cm of row using a pneumatic sprayer (Nordlund et al. 1974). Control plots received no treatment. Eggs were placed on 10 sample subplots, 3.0 m × 1 row, randomly distributed about the plot. The eggs were placed by hand using Plantgard (Nordlund et al. 1974). There were 4 replications of each treatment, and parasitization was evaluated on 3 different days. Eggs were collected after ca. 20 h exposure to the naturally occurring parasites and dissected to determine degree of parasitization. The data were totalled for the 3 days, and the R/O was calculated as above. Significance was determined by a paired t test.

RESULTS

Petri Dish Test

The results of the laboratory test (Table 1) indicated that the heaviest parasitization ($\bar{X} = 64\%$) occurred when the entire piece of filter paper had been treated. Progressively lower rates of parasitization occurred as the area

TABLE 1. PERCENT PARASITIZATION OF *H. zea* EGGS BY *T. achaeae* IN PETRI DISHES WITH DIFFERENT TREATMENT PATTERNS WITH TRICOSANE (±SE)[a]

Day	Diameter of treated circle, 6 locations/dish			Whole dish treatment
	1.2 cm	2.0 cm	3.7 cm	
1	25±12	30± 9	40±12	
2		39±11	49± 8	64±10

[a] 10 replications of each test on each day.

of treatment was decreased, with a mean of 25% parasitization being recorded when the treated areas consisted of circles only 1.2 cm in diameter. The test results demonstrate that the kairomone stimulated a more effective searching behavior rather than acting as an attractant, guiding parasites directly to the host. Treating the entire substrate would be detrimental in the case of an attractant.

Greenhouse Test

The data from this experiment (Table 2) supported those from the petri dish test. Best parasitization ($\bar{X}$ = 71%) was obtained with treatment of the entire pan, while the lowest parasitization ($\bar{X}$ = 29%) occurred in the control pans. Parasitization in the partially treated pans ($\bar{X}$ = 52%) was intermediate between that of the untreated and fully treated pans. Comparison of parasitization of eggs located on the treated and untreated areas of the partially treated pans showed higher parasitism in the treated areas of these pans

TABLE 2. MEAN PERCENT PARASITIZATION OF *H. zea* EGGS BY *T. pretiosum* ON PANS OF PEA SEEDLINGS COMPLETELY, PARTIALLY, OR UNTREATED WITH A MOTH SCALE EXTRACT

Complete	Partial			Control
	Treated areas	Overall	Untreated areas	
71[a]	57[b]	52[a]	47[b]	29[a]

[a] Means are significantly different (P = 0.01).
[b] Means are significantly different (P = 0.05).

($\bar{X} = 57\%$; $\bar{X} = 47\%$). The average parasitization within the treated areas of the partially treated pans was lower than the parasitism recorded for the completely treated pans. However, parasitization in the untreated areas of the partially treated pans was higher than that in the control pans.

These relationships are exactly those that would be expected with a host-seeking stimulant. Treatment of the entire pan should result in the quickest activation of the maximum number of females, and the response should be reinforced and maintained throughout the pan. Parasitism in untreated pans would be expected to be very poor because the parasites receive no stimulation except that derived from random contact with eggs. The number of females activated and the rapidity with which activity occurs in the partially treated pans should be proportional to the amount of treated to untreated area in the pans and the rate at which the wasps come into contact with the treated surfaces. Therefore, fewer parasites are activated and the activation rate is slower in the partially treated than completely treated pans. Also, the stimulated parasites do move into and search the surrounding untreated areas. As a result the level of parasitization in the treated areas is lower than that of fully treated pans, while parasitization in the untreated areas is higher in the control pans.

TABLE 3. EFFECT OF A MOTH SCALE EXTRACT ON THE EGG DISTRIBUTION OF *Trichogramma* spp. AMONG HOST EGGS IN SMALL PLOTS OF SOYBEANS

Replication no.	*R/O* ratio[a]	
	Treated	Control
1	1.20	1.18
2	0.95	1.24
3	1.04	1.25
4	0.92	1.40
5	1.05	1.40
6	1.06	0.90
7	1.92	2.89
8	0.80	1.58
9	0.93	1.00
10	0.89	1.00
Mean[b]	1.08	1.34

[a] *R/O* ratio is the ratio of percent parasitism that would have occurred in a random distribution of parasite eggs to the observed percent parasitism.

[b] Means are significantly different at the 5% level as determined by a paired *t* test.

Distribution of Parasite Eggs, Small Plots

The hexane eluate of the moth scale extract apparently caused an increase in the efficiency of egg distribution by ovipositing females of *Trichogramma* spp. (Table 3). The *R/O* was significantly lower ($P = 0.05$) in the treated plots (1.08) as compared to the control plots (1.34). This is apparently one mechanism by which the increase in parasitization was achieved as a result of the kairomone influence. The presence of the kairomone on the surrounding plant surfaces probably induces the ovipositing females to depart more rapidly from host eggs than they have stung and to go in search of other hosts, thereby reducing superparasitism. On the other hand, the absence of the kairomone from the surrounding substratum would result in the wasps lingering on and repeatedly stinging each host that is found.

Distribution of Parasite Eggs, Large Plots

The results of this test (Table 4) were similar to those obtained in small plots. The calculated *R/O* in the treated plots (1.14) was lower ($P < 0.10$) than that of the control plots (1.27). The hexane eluate of the moth scale extract caused an increase in the parasitization efficiency of *Trichogramma* spp. Again, these results were indicative of an activator, not an attractant.

DISCUSSION

The studies all indicated that the primary process by which these kairomones function to increase parasitization rates of *Trichogramma* spp. is by releasing and maintaining the appropriate motor response in the wasp rather

TABLE 4. EFFECT OF A MOTH SCALE EXTRACT ON THE EGG DISTRIBUTION OF *Trichogramma* spp. IN HOST EGGS, IN LARGE PLOTS OF SOYBEANS

Replication no.	*R/O* ratio	
	Treated	Control
1	1.08	1.26
2	1.17	1.20
3	1.25	1.46
4	1.06	1.16
Mean[a]	1.14	1.27

[a] Means are significantly different at the 10% level as determined by a paired *t* test.

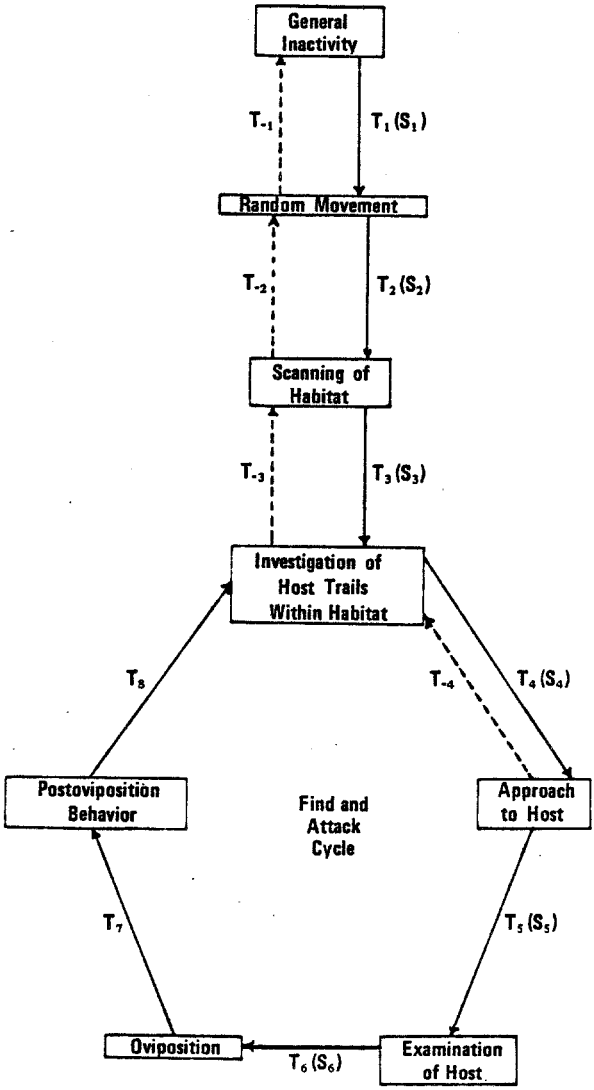

FIG. 1. Basic sequence of host-finding activities by females of parasitic insects: T_1 to T_8 and T_{-1} to T_{-4}—transitions among the indicated behavioral acts. S_{1-6}—stimuli releasing the indicated behavioral patterns. S_2—olfactory, visual, and physical cues associated with host plants on other habitats. S_3—primarily chemical cues from frass, moth scales, and decomposition products associated with the presence of host insects. S_4—olfactory, visual, auditory, and other chemical or visual cues from host insect. S_5 and S_6—olfactory, tactile, auditory, and/or combination of these cues from host individual.

than by attracting and serving as a steering mechanism. Treatment of the entire target area gave the best results. This treatment procedure apparently serves to elicit host-seeking response more quickly from more females and continuously reinforces this behavior throughout the area. The data also demonstrate that a more efficient egg distribution pattern is achieved in the treated areas, apparently as a result of the activation process.

The fact that these kairomones do not function as attractant has important implications for their use in pest-control programs, since such use will be less likely to adversely disrupt natural distribution patterns of the parasites. As they are understood at this point, it does not appear that use of these kairomones would be detrimental to *Trichogramma* populations since they function primarily to intercept and activate otherwise less productive *Trichogramma* females.

Studies of these kairomones have helped in a better interpretation of the overall host-finding sequence of insect parasites in general. Although the sequence of activities to be noted here is fundamentally similar to generalized patterns described in previous reports (Salt, 1935; Flanders, 1953; Doutt, 1964), we shall attempt to analyze the sequence in a manner that better relates the behavioral patterns to the stimuli releasing the responses, so that we may identify key points for parasite manipulation in pest management programs.

A diagram of what we consider to be the basic pathways of host location and selection is presented in Figure 1. The solid lines show the processes that occur if the necessary stimuli are provided at each step. The dotted lines show the alternative processes that occur if the necessary stimuli for the subsequent steps are not present. This diagram is a generalization and includes the basic components. Additional substeps and minor alternative pathways are involved and can be incorporated with the discussion of an individual species.

The transition (T_1) from inactivity to initial random movement is initiated by an innate appetitive drive together with prevailing environmental conditions and the current physiological state of the parasite. The means of locomotion for most parasites is flight, but some walk or run in search of habitats (Wylie, 1972). The parasites make a transition (T_2) from random movement and scan selected habitats by orienting to certain olfactory, visual, or physical cues (S_2) associated with the plants or habitats where hosts occur (Arthur, 1962; Askew, 1961; Nishida, 1956; Read, Feeny and Root, 1970; Smith, 1943; Thorpe and Caudle, 1938; Ullyett, 1953; Varley, 1941; and Wylie, 1958; and others).

Upon detecting the appropriate stimuli indicating inhabitation by the host (S_3), the T_3 transition to a localized search within the habitat is released. Frass, moth scales, decomposition products, or some other cue(s) associated with the presence of hosts in the environment are the stimuli that elicit this response (Ullyett, 1953; Wylie, 1958; Lewis, 1970; Lewis, Jones, and Sparks,

1972; Flanders, 1947; Laing, 1937; Vinson and Lewis, 1965; Doutt, 1959; Mudd and Corbet, 1973; and Hendry, Greany, and Gill, 1973; and others). Two or three substeps are usually involved in the search of trails of the host, all of which apparently are released by these and other sources of stimuli from the host. Actual detection of the host results (T_4) in an orientation toward and approach to the host. Olfactory, visual, auditorial, and other chemical or physical cues (S_4) from the host individual release this behavioral act (Edwards, 1954; Laing, 1937; Lewis, 1970; and others). If the host is concealed, perception of noise, movement, or infrared radiation from the host may be the means of host detection (Labeyrie, 1958; Lathrop and Newton, 1933; Ryan and Rudinsky; 1962; Richerson and Borden, 1972; and others).

Upon reaching the host the female parasite examines it for acceptability for oviposition. Chemical, visual, and tactile cues (S_5 and S_6) have been demonstrated to be involved in releasing these responses (Salt, 1935; Jackson, 1968; Ullyett, 1936; Edwards, 1954; Richerson and Deloach, 1972; Salt, 1958; Simmonds, 1943; Arthur, Hegdekar, and Batsch, 1972; Hegdekar and Arthur, 1973; and others).

After ovipositing, parasites often innately make an intensive investigation of the area adjacent to that host (Laing, 1937; Jackson, 1966; Hokyo and Kiritani, 1966; and others). The female then reverts to search for other hosts within the habitat.

The T_3 and T_{-3} transitions and the associated stimuli (S_3) between scanning of the habitat and the more thorough investigation of host trails within the habitat are the processes of primary interest to us in our studies of kairomones and their use in pest management. In order to induce females of insect parasites to be retained and effectively search in a desired target area, necessary stimuli (S_3) for the T_3 transition must be provided. Contact with the mediator which releases this behavioral pattern must occur periodically to reinforce and maintain the process. Lack of sufficient reinforcement otherwise allows the T_{-3} reversion to general scanning and a loss of the parasite from the target area. Therefore, a sufficient quantity and distribution of the stimuli is important. A blanket distribution throughout the target area is perhaps optimal for the *Trichogramma* species studied.

The ability to manipulate the behavior of parasites at this point of the host-finding sequence is obviously of great potential importance for more effective utilization of parasites in pest-control programs.

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CYCLICAL CHANGES IN VOLATILE ACIDIC METABOLITES OF HUMAN VAGINAL SECRETIONS AND THEIR RELATION TO OVULATION

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Abstract—The major volatile constituents in human vaginal secretions were identified. Data collected during the course of 29 cycles from 9 subjects showed that the nature and abundance of the vaginal organic constituents differed with respect to subject. Only 3 of 9 subjects investigated consistently had C_3–C_5 aliphatic acids; acetic acid was present in all subjects. Lactic acid was the major acidic compound consistently found in the vaginal secretion of all subjects near the time of ovulation. Cyclical changes in the acidic constituents were demonstrated.

Key Words—human odors, human vaginal secretions, lactic acid, small aliphatic acids, GC–MS, metabolic profiling, chemical communication, ovulation detection.

INTRODUCTION

Odors play an important role in communicating conspecific receptivity, reproductive maturation, and dominance status, in addition to influencing estrous synchronization and implantation of the fertilized ovum in many mammals (Bronson, 1970; Epple, 1974). Anecdotal information in the literature (Schneider, 1971; Comfort, 1971), along with scientific reports, has stimulated speculation on the role of odors in human reproductive biology. McClintock (1971) documented the fact that the menstrual cycle of women living in all-female residential groups became synchronous. Michael, Keverne, and Bonsall (1971), as well as Curtis et al. (1971), reported that short-chain

aliphatic acids (C_2–C_5) isolated from the vaginal secretions of female rhesus monkeys act to induce mating by sexually active male rhesus monkeys. This has been used as the starting point for other reports (Waltman et al., 1973; Keverne, 1974) and speculations (Comfort, 1972; Anon., 1971) concerning the role these acidic compounds might play in humans.

Michael (1972) and Michael, Bonsall, and Warner (1974), as well as Waltman et al. (1973), have reported that fatty acids are present in human vaginal secretions; however, discrepancies exist in their reports as to the type and amount of acids present. Michael, Bonsall, and Warner (1974) found larger amounts of aliphatic acids in normally cycling subjects when compared to subjects on birth control pills (type of pills—sequential or combination—not specified). In addition, normally cycling subjects showed increased amounts of acids near midcycle. These amounts decreased in the second half of the cycle. Only gas chromatographic retention times were used to determine the identity of the acids—no confirming mass spectrometric data were reported. Neither Michael, Bonsall, and Warner (1974) nor Waltman et al. (1973) reported on the nature and abundance of other volatile organics which could contribute to vaginal odor. Michael, Bonsall, and Warner (1974) did not report documenting ovulation or correlating the day of ovulation with respect to the amounts of acids produced.

Human vaginal secretions are thought to consist of several components (Moghissi, 1972; Cohen, 1969): (1) vulval secretions from sebaceous, sweat, Bartholin's and Skeen's glands, (2) mucus secretions from the cervix, (3) endometrial and oviductal fluids (Doyle, Ewers, and Sapit, 1960; Hafez and Black, 1969), (4) transudate through the vaginal walls (Masters and Johnson, 1966), and (5) exfoliated cells of the vaginal mucosa. The type and amounts of (2), (3), and (5) are known to be influenced by biochemical processes that are dependent on sex steroid levels; consequently, metabolic by-products of these processes should also vary with sex steroid levels. We used gas chromatography (GC) and combination gas chromatography–mass spectrometry (GC–MS) to investigate changes in the nature and abundance of volatile, chromatographable compounds found in the vagina during the menstrual cycle. Such changes could reflect changes in circulating hormone levels and may thus be diagnostic of the fertile period (Ferrin, Thomas, and Johnson, 1972; Marshall, 1973; Hartman, 1962).

With the current speculation concerning the possible role of odor in human reproductive biology, more detailed information is needed to establish the nature of the organic volatile constituents of the normal human vagina. Because of the importance placed on the small aliphatic acids by others (see above) and the apparent discrepancies thus far in the literature concerning the presence of these acids in vaginal secretions, more detailed analyses are desired. In addition, the structure and quantity of other organic materials,

as well as their possible cyclical changes in concentration, are of importance since all would contribute to the overall vaginal odor profile being produced at various times in the menstrual cycle. Previously, no thorough study of human vaginal secretions has been reported which employs both serum radioimmunoassays to document ovulation (Moghissi, Syner, and Evans, 1972) and highly sensitive analytical instrumentation such as GC and GC–MS to elucidate the structure and amounts of organic materials found there.

METHODS AND MATERIALS

We employed precleaned tampons from which organic contaminants had been removed by extracting with chloroform–methanol (85:15, v/v) (3 days) and diethylether (2 days) to collect the secretions. The tampons were then autoclaved, dried in vacuo, weighed, placed back in their original holders (using nylon gloves), and stored in glass bottles. Use of plastics of any type was completely avoided during the entire procedure to eliminate contamination by plasticizers. Secretions were collected using two procedures. In the first (procedure A), subjects inserted the tampons before going to sleep and removed them the next morning. In the collection procedure currently being used (procedure B) subjects wear the tampon for 6 h during hours of normal daily activity. After the end of the menses (1 or 2 days), samples were collected each night (or day) for 10 or 12 consecutive nights, and every other night thereafter until the start of the next menses.

With both procedures a total of 29 cycles from 9 different subjects were investigated. All subjects were healthy and had regular menstrual cycles. Secretions were collected using procedure A for 21 of the 29 cycles. Ovulation was documented in 5 of these cycles with radioimmunoassays for circulating levels of total estrogens, progesterone, and luteinizing hormone (LH) (Wu and Lundy, 1971; Devilla et al., 1972; Midgley and Jaffe, 1966). In humans, ovulation is preceded by a large rise in serum estrogens, which in turn is thought to trigger the release of LH from the anterior lobe of the pituitary resulting in a sharp rise in serum levels of this gonadotropin (Ferrin, Thomas, and Johnson, 1972; Moghissi, Syner, and Evans, 1972). Ovulation most likely occurs 12–24 hr after maximum LH levels (Ferrin, Thomas, and Johnson, 1972). In the remaining 24 cycles, the day of maximum LH levels (henceforth day 0) was estimated from basal body temperature (BBT) charts (Döring, 1973; Hartman, 1962). Subjects were asked to record incidents of sexual arousal or coitus and not to use vaginal deodorants or douches. No other restrictions were placed on daily habits or diets.

Heptanoic acid was added to each tampon as an internal standard just before organic materials were extracted from the tampons by a continuous

24-h extraction with dichloromethane. Each extract was concentrated to approximately 250 μl via rotary evaporation at room temperature. Chromatography was performed using internal and coinjected standards on 10-ft × 2-mm, 3% XE-60; and 8-ft × 2-mm, 10% Carbowax 20M glass columns; in addition, a 12-ft × 2-mm, 3% SE-30 stainless steel column was employed for analysis of silylated derivatives. Chromatographic conditions for each of the respective analyses were as follows: XE-60, 60°C, 2 min, 3°/min to 120°C then 8°/min to 230°; Carbowax 20M, 50°C, 4 min, 6°/min to 220°C; SE-30, 75°C, 6 min, 6°/min to 300°C. Combination GC–MS was carried out on selected samples with a Perkin-Elmer 990 gas chromatograph interfaced to a Hitachi/Perkin-Elmer RMU-6L mass spectrometer via a Watson-Biemann separator (Watson and Biemann, 1965). Identifications were confirmed by comparison of mass spectra and GC retention times with those from commercially available samples.

RESULTS

Acetic acid was the only small aliphatic acid consistently present in large amounts throughout each of the 29 cycles studied. The higher aliphatic acids, propionic, iso- and *n*-butyric, isovaleric, and 2-methylbutyric, were confirmed to be present in each cycle recorded from only 3 of the 9 subjects investigated (D, F, and G). Normal valeric acid was tentatively identified in subject D (via GC retention time).

Data from a total of 8 cycles were obtained from these 3 subjects and BBT charts were used to confirm ovulation. Figure 1 shows 5 cycles representative of the way in which total fatty acids varied in these 8 cycles. The top half of Figure 1 shows the total amounts of aliphatic acids produced for subject D plotted vs. the day of the cycle for 3 of her cycles. This subject displayed C_3–C_5 acids in the second half (luteal phase) of 3 cycles and from day 0 through the luteal phase in a fourth cycle. Before day 0, only acetic acid was produced by this subject. However, once ovulation occurred, and progesterone levels began to rise, increases in both acetic and other acids (mentioned above) in the vaginal secretions are rapid.

Data from the first cycle obtained from both subjects F and G are shown in the lower half of Figure 1. These subjects showed C_2–C_5 acids in varying amounts each day in 3 cycles, with maxima occurring within 48 h of day 0. In the 2 cycles not plotted in Figure 1, maximum amounts of acids were found on the following days: subject F, day +1 (398 μg) and +6 (264 μg); subject G, day 0 (818 μg) and day +10 (550 μg). The former cycle from subject F differed from the cycle shown in Figure 1 in that only minute amounts of all acids were produced before day 0. All 4 cycles from F and G

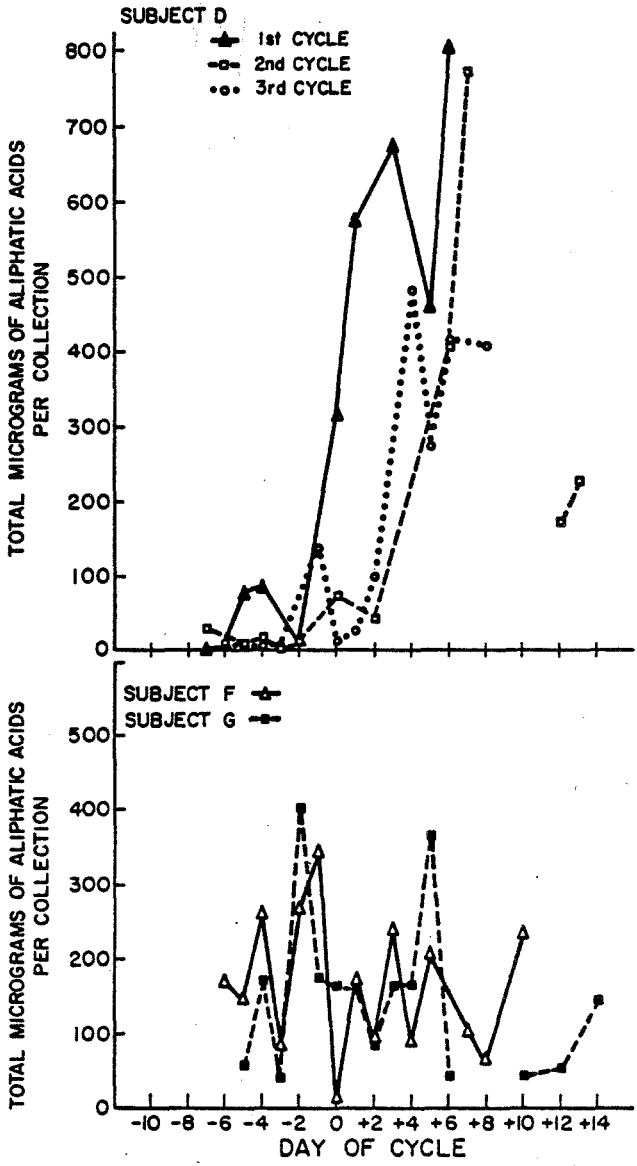


FIG. 1. Changes in the total micrograms of aliphatic (C_2–C_5) acids throughout 3 cycles from subject D and one each from subjects F and G. Before day 0, subject D only produced acetic acid, while after day 0 acetic and the remaining C_3–C_5 acids increase. Subjects F and G produced C_2–C_5 acids each day of their cycles. Maximum production is found within 48 h of day 0; subject G showed a second distinct maximum in the luteal phase (day +5). BBT charts were used to determine day 0 in each case.

displayed a sharp decrease in the production of acids immediately after the midcycle maxima, followed by one or more increases during the luteal phase.

Figure 2 shows the ratios of acids present for 5 days from each of the 5 cycles in Figure 1. In each of these cycles, these were the 5 days of highest acid production. As discussed above, only acetic acid is present before day 0 in cycles II and III from subject D.

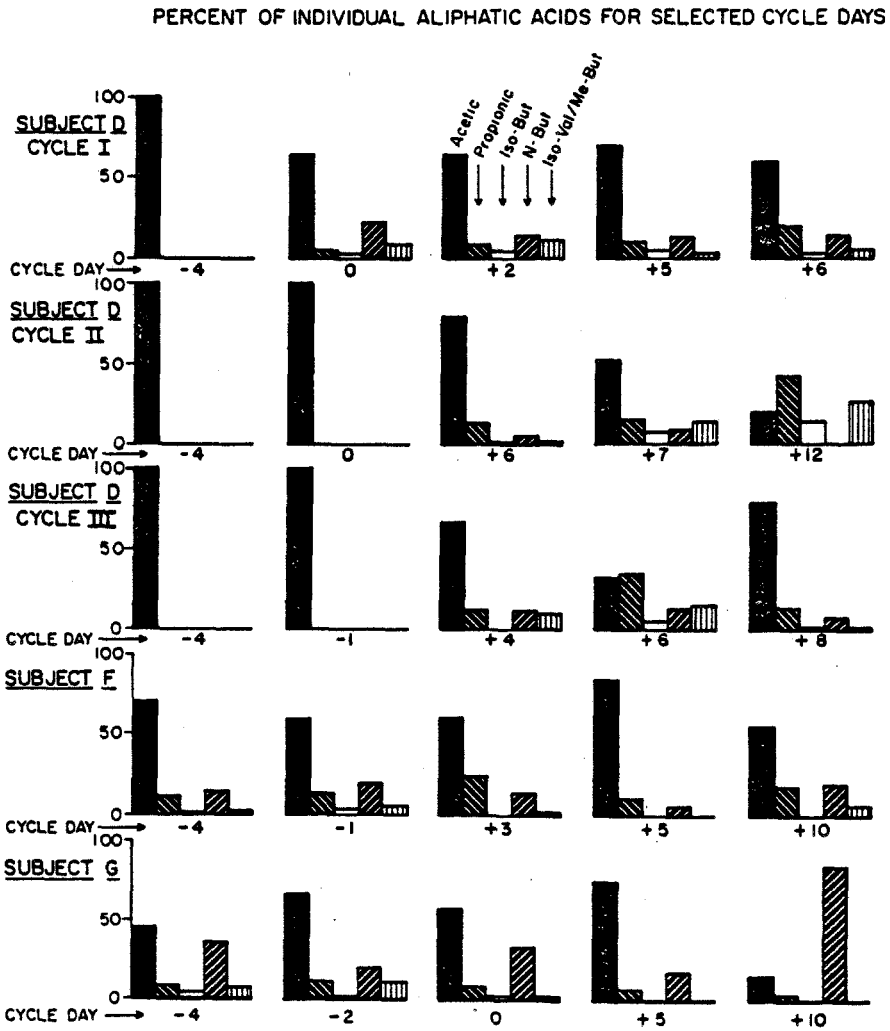


FIG. 2. This shows the ratio of the individual aliphatic acids for 5 selected cycle days. For each given cycle these were the 5 days of highest total fatty acid production.

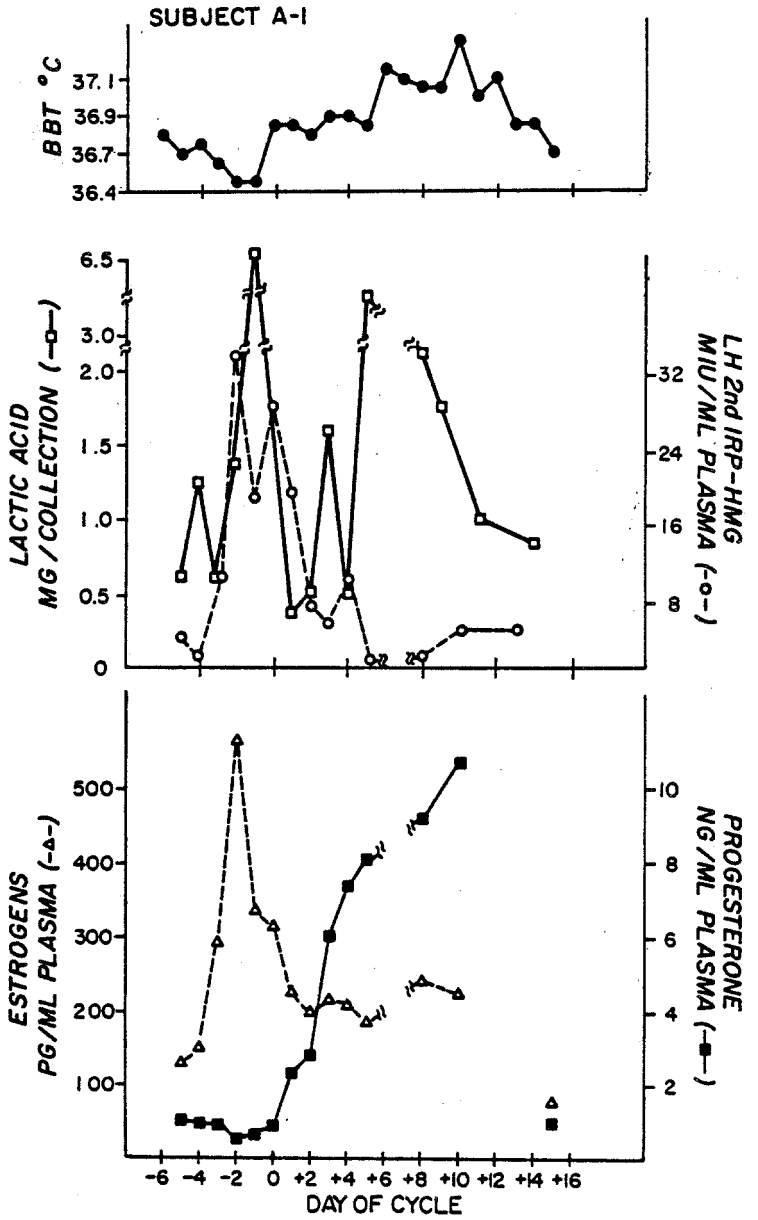
Subjects D, F, and G also tended to produce more of several other compounds than the remaining subjects: 2-piperidone, indole, benzaldehyde, and pyridine. These latter subjects displayed no chromatographic evidence for propionic or isobutyric acids, but did show chromatographic peaks at the correct retention times (on the Carbowax column) for *n*-butyric and isovaleric/2-methylbutyric acids (these coeluted); however, mass spectra taken at various intervals in cycles from the remaining subjects showed these peaks to contain no detectable amounts of acids. Instead of acids, they were found to be (from lower to higher retention time) propylene glycol, ethylene glycol (maybe an artifact) and phenylacetaldehyde/furfuryl alcohol (these coeluted), respectively. Propylene and ethylene glycol were also consistently found in the secretions of subjects D, F, and G.

Lactic acid was present in the vaginal secretions of all subjects. This hydroxy acid was found to be the major acidic constituent in the secretions of all subjects at the time of ovulation. In 26 of the 29 cycles studied, lactic acid reached its highest or second highest values from 4 days before to 2 days after the predicted or determined day of ovulation.

Figures 3 and 4 show 4 cycles where lactic acid production was plotted with circulating levels of estrogens, progresterone, and LH. These documented ovulatory cycles reflect concentration changes of lactic acid seen in a majority of the remaining cycles. A fifth documented cycle from subject C which showed a different pattern than these is discussed below. Although the identity of lactic acid was confirmed as its bistrimethylsilyl derivative, in the majority of the cycles the weight of underivatized lactic acid were determined. Amounts of raw lactic acid were determined for both cycles from subject A. Lactic acid was determined as its bistrimethylsilyl derivative in the cycles shown for subjects B and I. Owing to complications with the latter subject, blood could not be drawn during the days when highest estrogen levels and the LH surge occurred. Day 0 was consequently determined from the thermal shift in the BBT (Döring, 1973; Hartman, 1962). The cycles in the figures all show that the midcycle rise in lactic acid coincides very closely with the midcycle estrogen and LH rises. Although the midcycle rise of lactic acid in subject I in Figure 4 is not as great as those for subjects A and B, it is definitely present.

Each of the cycles from subject A (Figure 3) shows a small rise in lactic acid concentration just before blood estrogens begin to rise. This pre-midcycle rise is much more evident in subjects B and I (Figure 4). These rises occur 1 day before the preovulatory rise in blood estrogens. Of the remaining 25 cycles, 19 showed similar increases in lactic acid occurring 3–6 days before the midcycle lactic acid rise.

All cycles in Figures 3 and 4, as well as 21 of the remaining 25 cycles, show a decrease in lactic acid after midcycle. The second half (luteal phase) of the cycles shows rises in lactic acid which seem to agree with increasing and/or


SUBJECT A-I
BBT °C
37.1
36.9
36.7
36.4
LACTIC ACID
MG/COLLECTION (-o-)
6.5
3.0
2.0
1.5
1.0
0.5
0
LH 2nd IRP-HMG
MIU/ML PLASMA (-o-)
32
24
16
8
ESTROGENS
PG/ML PLASMA (-Δ-)
500
400
300
200
100
PROGESTERONE
NG/ML PLASMA (-■-)
10
8
6
4
2
-6 -4 -2 0 +2 +4 +6 +8 +10 +12 +14 +16
DAY OF CYCLE

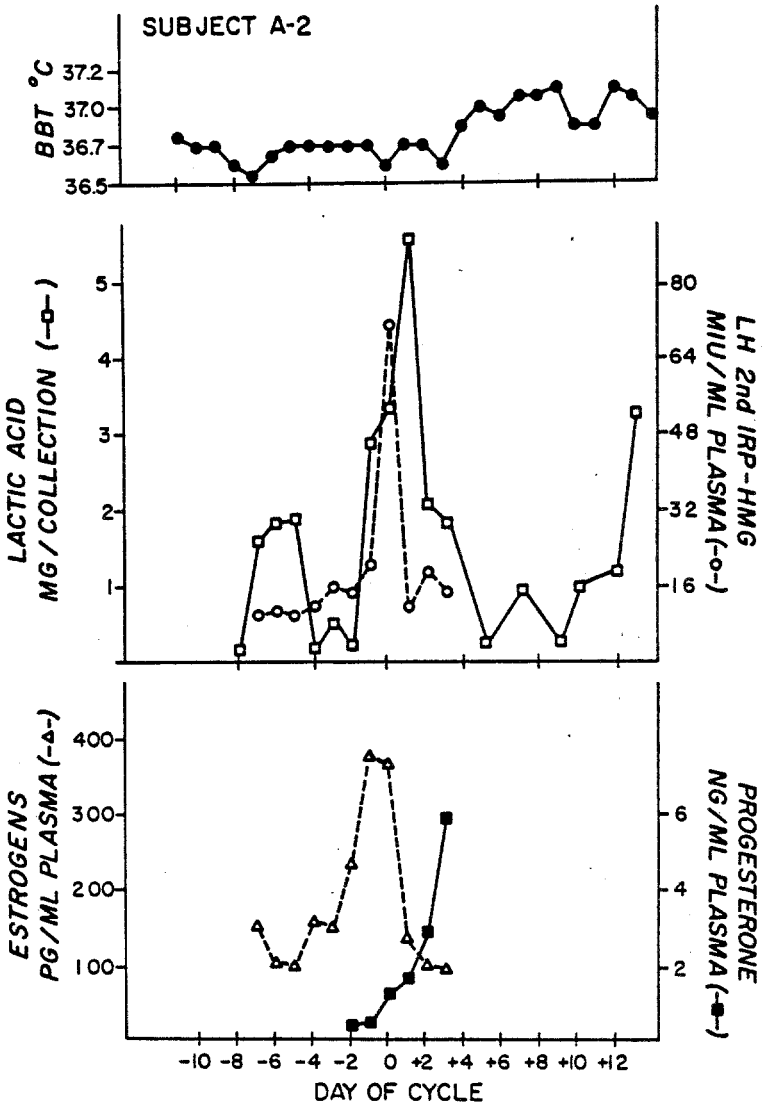


FIG. 3. Changes in BBT, LH, estrogen, progesterone, and lactic acid throughout 2 cycles for subject A (cycle 1 on facing page; cycle 2 above). Blood was not drawn for each day in both of these cycles. However, from both the hormonal and BBT data both cycles are ovulatory. Cycle 1 shows 2 maxima for LH. The second was chosen as the one which triggered ovulation because rising progresterone levels coincided with this maximum (Moghissi, Syner, and Evans, 1972). Preovulatory increases in lactic acid are seen on day −4 in cycle 1 and days −7 through −5 in cycle 2.

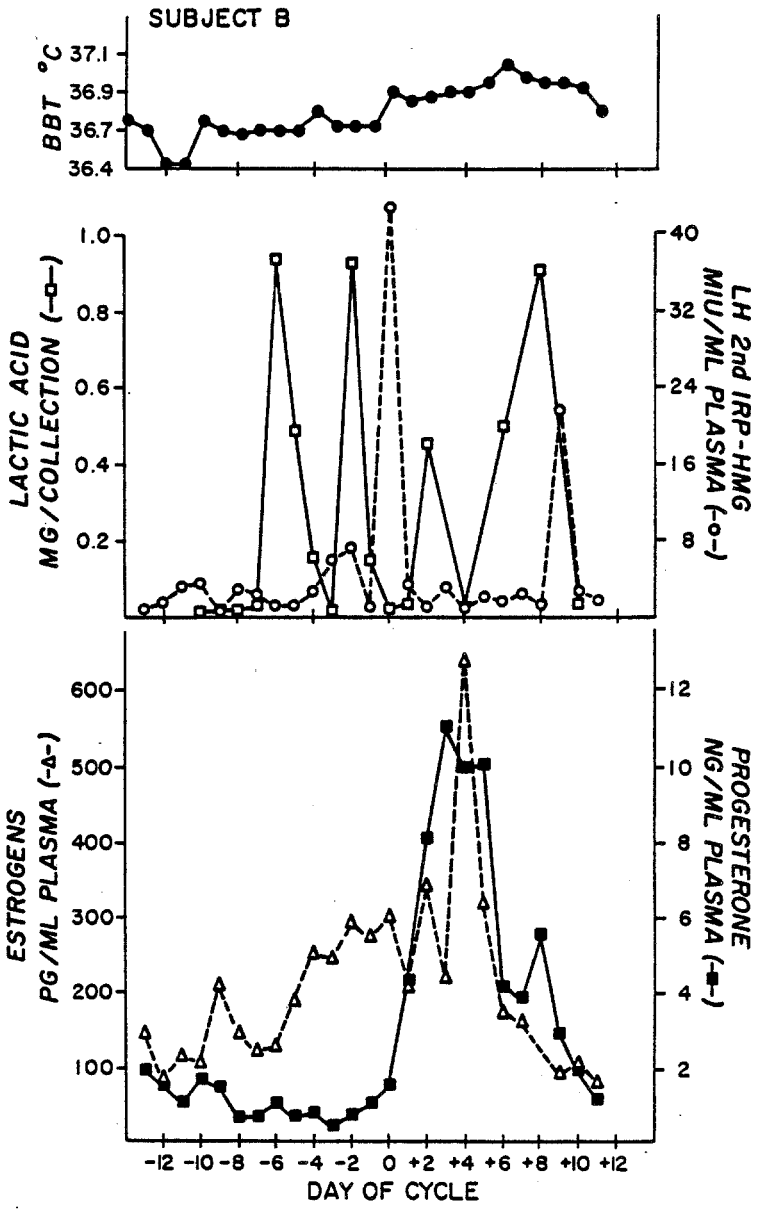
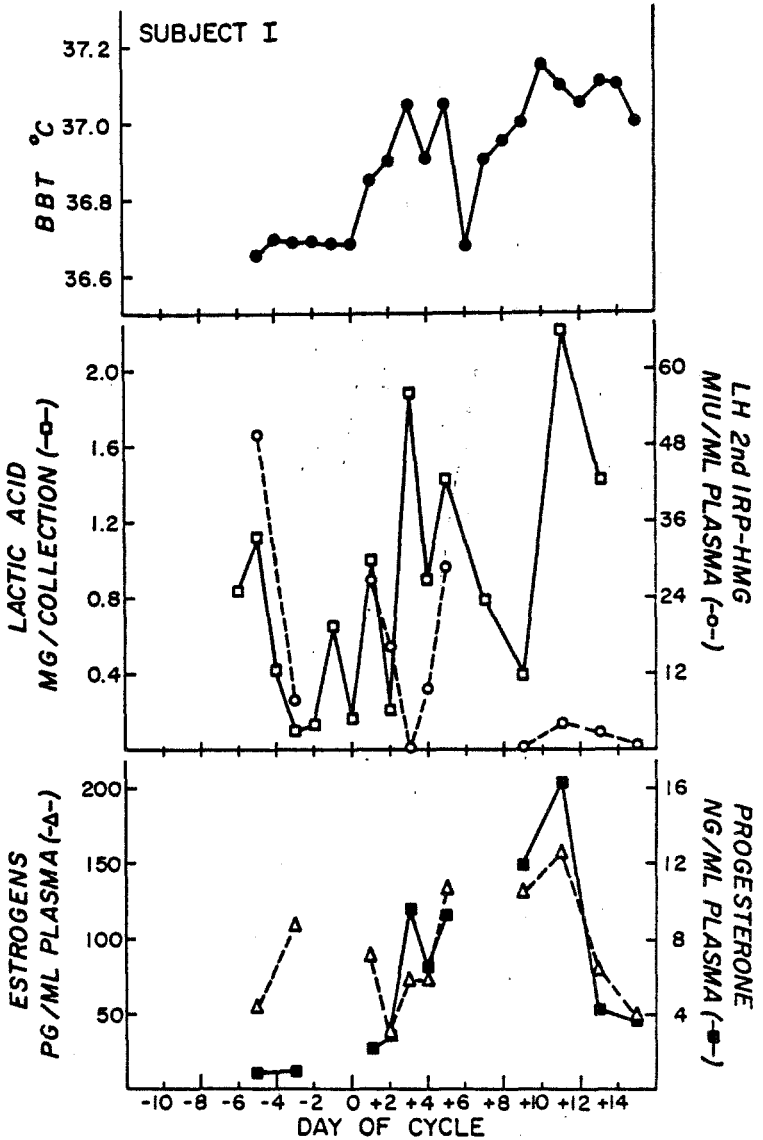


FIG. 4. Changes in BBT, LH, estrogen, progesterone, and lactic acid throughout 1 cycle from subject B (above) and 1 from subject I (facing page). Subject B displays the preovulatory blood estrogen rise on day −6. Blood could not be drawn from Subject I during days −2 through 0 owing to difficulties in obtaining the samples.



However, the rising progesterone levels after the determined day 0, as well as the thermal shift in the BBT clearly indicate that ovulation occurred within this 3-day time span. The start of the preovulatory estrogen rise and the corresponding rise in lactic acid are found on days −4 and −5, respectively. Luteal-phase concentrations of lactic acid are greater than the midcycle rise for this subject.

maximum progesterone levels and the luteal-phase estrogen rise. As discussed above, subjects F and G also showed a drop in aliphatic acid production after midcycle, and D, F, and G all showed an increase in these acids for 1 or more days in the luteal phase.

The amount of both lactic acid and the small aliphatic acids collected did not appear to depend upon the total amount of secretion collected. Secretion weights were recorded in 14 cycles using procedure A, and maximum amounts of all acids coincided with the maximum quantity of secretion in only 2 cycles. Procedure B allows for a standardized duration of collection and therefore a better determination of the amount of all organic materials per secretion weight. Data gathered with this latter collection technique have thus far shown the same changes in the concentration of all the acidic constituents as those gathered with procedure A.

In all subjects who did not produce C_3–C_5 acids, acetic acid varied in a cyclical manner and often paralleled the changes in lactic acid by displaying two concentration maxima: one around midcycle, the second during the luteal phase. Maximum amounts of acetic acid varied from 0.070 mg in subject B to 0.525 mg in subject A. The days on which acid amounts were greatest and the maximum amounts of acid for the cycles in Figures 3 and 4 are the following: A-1, +1 (315 μg) and +7; A-2, +3, and +7 (210 μg); B, 0 and +6 (125 μg); I, −5 and +14 (125 μg).

The following compounds were also unambiguously identified: pyridine, 3-hydroxy-2-butanone, dimethylsulfone, *n*-dodecanol, *n*-tetradecanol, urea (the latter as its bistrimethylsilyl derivative), *n*-hexadecanol, phenol, *o*-cresol, *p*-cresol, squalene, and cholesterol. Tentatively identified from their mass spectra or GC retention times are benzoic acid and *n*-octadecanol. Several subjects seem to produce several of these compounds in greater abundance than others.

DISCUSSION

As noted above, speculation on the possible role of small aliphatic acids in human vaginal secretions has increased since Michael and coworkers (1971) found that these compounds elicited strong behavioral activity in at least one primate species: the rhesus monkey. Our work showed that only 3 of the 9 human females investigated consistently displayed detectable amounts of short-chain aliphatic acids. The presence of higher acids in only 3 subjects suggests that conditions conducive to their production in large amounts are not present in all women. Data from subject D and the second cycle from subject F suggest that large amounts of aliphatic acids were produced only under the influence of increasing or maximum progesterone levels

in these cycles. As discussed above, these 3 subjects also showed increased amounts of several other volatile materials than the other subjects.

In light of these data, it appears that no single type of compound and/or compounds can be assumed to be a major contributor to vaginal volatiles. The secretion is a fairly complex mixture of organic compounds, the type and amount of which differs from subject to subject.

The small aliphatic acids, lactic acid and 3-hydroxy-2-butanone, may all be formed by the action of microorganisms (Thimann, 1963). Suggestions to this effect have been reported for the aliphatic acids (Keverne, 1974). The normal human vagina is populated by a number of different microflora with the predominant genus being *Lactobacillus* (Morris and Morris, 1967). Consequently, a number of the major odorous organics in the vaginal secretions may largely be derived from the metabolism of available nutrients by microflora.

Vaginal acidity is thought to be maintained by lactic acid which in turn is assumed to arise at least in part by the action of the *Lactobacillus* of Döderlien on vaginal glycogen and/or simpler carbohydrates found in the vagina (Bo, 1970; Gross, 1961; Masters and Johnson, 1966; Moghissi, 1972; Moursi et al., 1971; Rakoff, Feo, and Goldstein, 1943; Wylie and Henderson, 1969). Acetic acid may also be formed by the same microbiological pathways as lactic acid (Thimann, 1963). In addition, several of the other genera of microflora reported to be in the vagina are capable of producing lactic, acetic, and the other aliphatic acids (Holdeman and Moore, 1972). Consequently, the 3 subjects who consistently produce fatty acids may have a different bacteriological environment than those subjects which produce only lactic and acetic acid.

Increases in estrogens and combined estrogen/progesterone have been shown to produce increases in carbohydrates in both the vaginal epithelium and secretion (Gross, 1961; Rakoff, Feo, and Goldstein, 1943; Moursi et al., 1971; Bo, 1970; Ayre, 1951; Gregoire, Kandil, and Ledger, 1971). These increases in the amounts of carbohydrates available to microflora could account for the midcycle and luteal phase rises consistently seen for both lactic acid and the C_2–C_5 acids. However, it is difficult to account for the premidcycle rise and late luteal rise (i.e., the latter may be seen in cycles from A-2, B, and I in Figures 3 and 4) for lactic acid, seen in a majority of the cycles, solely on the basis of increased carbohydrates.

The rapid decrease in lactic acid after midcycle could be the result of increased alkaline cervical mucus making its way into the vagina (Rakoff, Feo, and Goldstein, 1943, Moursi et al., 1971; Bo, 1970). Semen may also neutralize the vagina (Masters and Johnson, 1966), and this may account for some of the sharp decreases in lactic acid in cycles where amounts of raw lactic acid were determined. In a fifth documented ovulatory cycle from subject

C, 3 precipitous dips and rises in lactic acid amounts preceded the large midcycle rise on day 0. However, this subject did not report having intercourse during this time.

During the course of this study, 45 incidents of coitus were reported during days when collections were made and in only 4 cases did incidence of coitus correspond to the highest or second highest values of lactic acid for that cycle. This result was anticipated, since previous work (Masters and Johnson, 1966) has shown that vaginal pH does not decrease during sexual foreplay when large amounts of vaginal transudate are produced. This indicates that the transudate may contain little or no free acidic compounds.

The data presented above demonstrate that cyclical changes in the concentration of acidic organic materials of human vaginal secretions do occur. However, the nature and abundance of these materials do differ in individuals and even across cycles in certain individuals. Many of the organics which may contribute to and/or influence vaginal odors throughout the cycle were revealed. In considering human vaginal secretions as a possible carrier for volatiles which may communicate intraspecies information (particularly the optimum time for fertilization), in the absence of appropriate bioassays, all volatiles which contribute to vaginal odor and their concentration changes with respect to the day of ovulation should be monitored. The detection of these cyclical changes using GC and GC–MS indicate that this instrumentation may offer a new approach in determining hormonal changes via analysis of reproductive tract secretions.

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SEX PHEROMONE OF THE STABLE FLY[1]: ISOLATION AND PRELIMINARY IDENTIFICATION OF COMPOUNDS THAT INDUCE MATING STRIKE BEHAVIOR[2]

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Abstract—Cuticular rinses of the female stable fly, *Stomoxys calcitrans* (L.), contained saturated and unsaturated hydrocarbons that incited the male fly to attempt couplation. These compounds present in GLC trappings of the saturated hydrocarbon fraction showing highest biological activity were mono- and dimethyl-substituted hentria- and tritriacontanes. Active trappings from the unsaturated hydrocarbon fraction contained (Z)-9-hentriacontene, (Z)-9-tritriacontene, and methyl-branched hentria- and tritriacontenes.

Key Words—stable fly, *Stomoxys calcitrans* (L.), pheromone, sex pheromone, C_{31} carbon chain, C_{33} carbon chain.

INTRODUCTION

The stable fly, *Stomoxys calcitrans* (L.) is a major pest of livestock and also has been reported to be a serious nuisance to humans in the Gulf Coast States where it is known as the "dog fly" (Wright, 1972). Efforts to bring this pest under control have included such diverse approaches as conventional insecticides, chemosterilants (Harris, 1962), synthetic repellants (Yeoman and Warren, 1970), carbon monoxide and dioxide attractants (Hoy, 1970),

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

and juvenile hormone minicking compounds (Wright, 1970). Because of the continuing high interest in controlling this species, we were prompted to examine the stable fly for the presence of mating stimulants (pheromones)—chemicals that are produced by the female fly and that induce copulatory attempts ("mating strikes") by males of that same species (Rogoff et al., 1964; Cowan and Rogoff, 1968). Pheromones that induce this behavior have been found in the house fly, *Musca domestica* L. (Carlson et al., 1971; Rogoff et al., 1973), the face fly, *Musca autumnalis* De Geer (Chaudhury, Ball, and Jones, 1972; Uebel et al., 1975), and independent of this report in the stable fly (Muhammed, Butler, and Carlson, 1975).

MATERIALS AND METHODS

The bioassay used in these tests was essentially that described for the isolation and identification of the sex pheromone of the face fly (Uebel et al., 1975). Thus, 15 male stable flies were isolated in quart mason jars before they were 24 hours old. Then, in the morning before the confined males were fed, bioassays were conducted by applying the test material to 3- to 5-day-old male stable flies and presenting these treated flies to the males in the quart jars. An activity quotient was derived from the test data by dividing the number of mating strikes made by male flies on treated males by the number of strikes made by male flies on untreated virgin females of the same age.

All flies used in the tests were held at $80 \pm 2°F$ and $50 \pm 5\%$ relative humidity, kept in artificial light from 0800 to 2400, and fed twice daily from a sponge saturated with warm citrated bovine blood.

The extracts used in the assays were obtained by rinsing batches of 1500 4-day-old virgin flies of each sex with petroleum ether. The extracts were freed of solvent and then chromatographed on Florisil, eluting sequentially with hexane, 3% ether in hexane, and 4% acetic acid in ether. Saturated and unsaturated hydrocarbons were obtained from the hexane eluent, separated by column chromatography on silver-nitrate-impregnated Florisil, and chromatographed by gas-liquid chromatography with 5% SE-30 on Gas Chrom Q programmed from 195 to 275°C at a rate of 1°C/min. Then three 0.5-mg portions of the female saturated and unsaturated hydrocarbons were injected into the gas chromatograph, and the material from the different areas of the chromatogram (Figures 1 and 2) were collected and tested. Active saturated components in the trapped GLC areas were subjected to mass spectroscopy. A Finnigan Corp. 1015 quadrupole mass spectrometer interfaced with a glass Gohlke separator to a gas chromatograph equipped with a glass column containing 3% OV-1 on Varaport was employed.

When the unsaturated hydrocarbons that occurred in areas 3 and 4 of

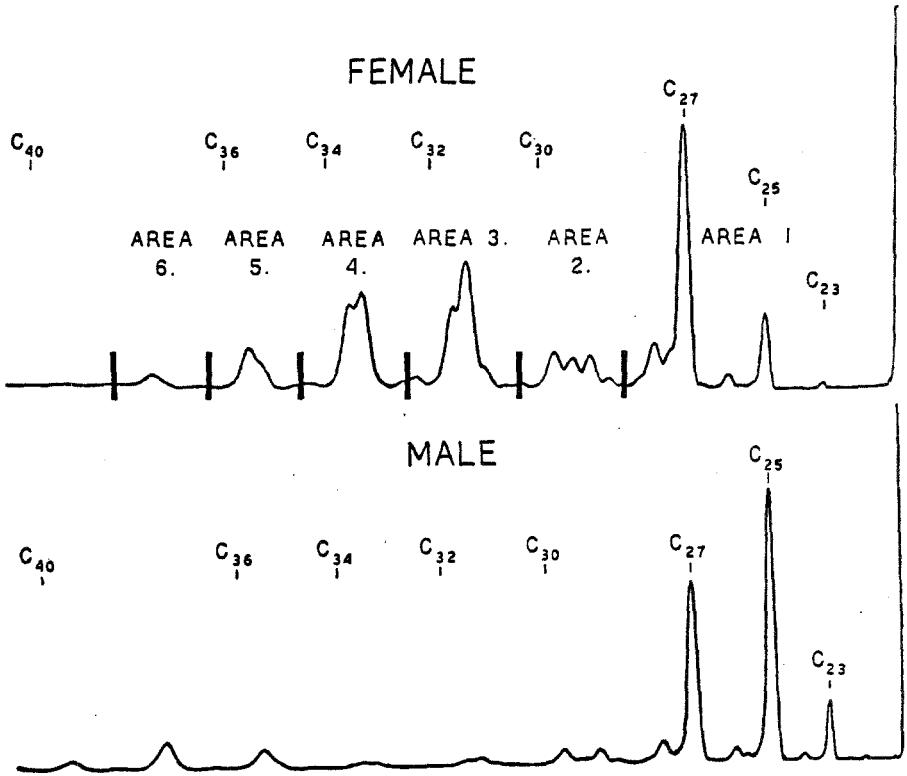

FIG. 1. Gas-liquid chromatograms of the saturated hydrocarbons from the cuticular wash of 4-day-old female (80 μg, 6.6 fly equivalents) and male (70 μg, 16.1 fly equivalents) stable flies. C with subscript indicates the retention time of saturated hydrocarbon standards.

Figure 2 proved to be active, they were investigated. Each component was individually trapped. The position of the double bonds was determined by ozonolysis and gas chromatographic analysis of the resulting aldehydes by the method of Beroza and Bierl (1967). Hydrogenation of the trapped materials allowed a comparison with the saturated hydrocarbons already identified. Configuration of the unsaturated compounds were determined by spotting them on $AgNO_3$-impregnated silica gel thin-layer plates and comparing their movement with those of (Z) and (E) standards.

RESULTS AND DISCUSSION

The cuticular lipids from 4-day-old male and female stable flies contained 16.7 and 14.2 μg of hydrocarbon per insect, respectively. The saturated

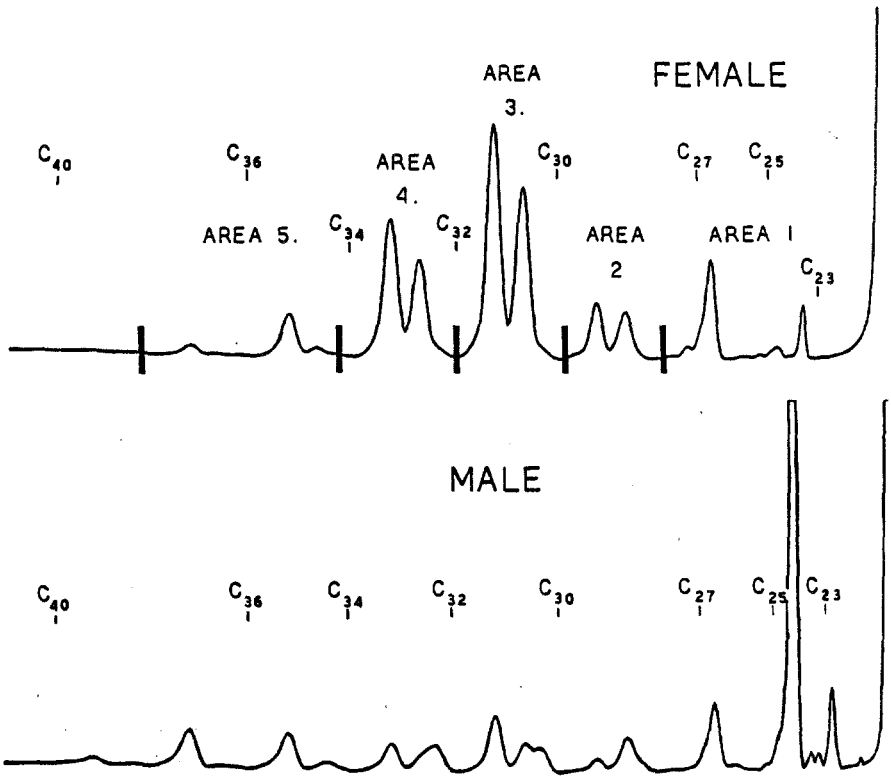

FIG. 2. Gas-liquid chromatograms of the unsaturated hydrocarbons from the cuticular wash of 4-day-old female (50 μg, 23.5 fly equivalents) and male (40 μg, 3.2 fly equivalents) stable flies. C with subscript indicates the retention time of saturated hydrocarbon standards.

hydrocarbon made up ca. 85% of the total hydrocarbon content on the female. This value was corroborated by weighing the saturated and unsaturated materials from the silver-nitrate–Florisil fractionation and by measuring peak areas on the chromatograms. A similar value was obtained for the males when the saturated and unsaturated fractions were weighed. However, when peak areas on the chromatograms were measured, an unsaturated peak that chromatographed between the saturated C_{23} and C_{25} standards made it appear that the proportion of unsaturated material on the male was considerably higher. The identification of this peak is being pursued.

Few mating strikes were made toward untreated male stable flies.

The mating strike activity produced by applying hexane solutions of cuticular lipid and hydrocarbon fractions to the dorsal part of the abdomen and thorax of male flies is shown in Table 1. Plainly, both the saturated and

TABLE 1. MATING STRIKE ACTIVITY PRODUCED BY THE CUTICULAR LIPID AND HYDROCARBON FRACTIONS FROM 4-DAY-OLD MALE AND FEMALE STABLE FLIES

	Activity quotients[a] produced by indicated amounts (μg)			
	50	100	150	200
♂ Cuticular lipid	0.02	0.00	0.00	0.00
♀ Cuticular lipid	0.04	0.19	0.13	0.25
♂ Hydrocarbon	0.02	0.04	0.00	0.00
♀ Hydrocarbon	0.08	0.27	0.33	0.20
♂ Saturated hydrocarbon	0.00	0.09	0.03	0.08
♀ Saturated hydrocarbon	0.09	0.11	0.42	0.68
♂ Unsaturated hydrocarbon	0.05	0.07	0.27	0.10
♀ Unsaturated hydrocarbon	0.35	0.37	0.40	0.30

[a] Each test included five 5-min replicates. Values are average activity quotients for one test.

unsaturated cuticular hydrocarbon fractions obtained from the females were more active than the corresponding fractions from male flies. No activity was found in the two more polar fractions of the column chromatography.

The highest activity was in the female saturated and unsaturated hydrocarbons in area 3 of the GLC trappings. Material collected in area 4 of the saturated and unsaturated hydrocarbons gave about half the activity of area 3. Only slight activity was obtained from the materials collected in the other areas. The relatively small amount of material in the same areas of the male chromatograms (Figures 1 and 2) is consistent with the observation that the male hydrocarbons are inactive.

The active saturated hydrocarbon of the female stable fly consisted primarily of two homologous series. One of these is methyl-branched C_{31}, C_{33}, C_{35}, and C_{37}; the other is doubly methyl-branched C_{31}, C_{33}, C_{35}, and C_{37}. The greatest activity appears to be associated with C_{31} and C_{33} lengths (Figure 1, areas 3 and 4), although definite assignments of activity to specific types of individual structures must await synthesis of these compounds. Mass spectrometry corroborated the GLC analyses and indicated that each member of each series was a mixture of several isomers (Table 2).

Favored fragmentations of branched hydrocarbons occur adjacent to centers of branching. In the case of a methyl-branched chain, an M-15 peak corresponding to the loss of methyl and fragments derived by cleaving the chain on either side of the branch are expected. These latter fragments locate

TABLE 2. COMPONENTS OF THE SATURATED HYDROCARBONS FROM THE FEMALE STABLE FLY

Area (Figure 1)	Structure[a]	*m/e*
1	*n*-$C_{25}H_{52}$	
1	*n*-$C_{27}H_{56}$	
2	*n*-$C_{29}H_{60}$	
2	$C_{29}H_{59}(CH_3)$	[b]
2	$C_{29}H_{58}(CH_3)_2$	[b]
3	11-$C_{31}H_{63}(CH_3)$	169,309
3	13-$C_{31}H_{63}(CH_3)$	197,281
3	15-$C_{31}H_{63}(CH_3)$	225,253
3	11,15-$C_{31}H_{62}(CH_3)_2$	239,323
3	13,17-$C_{31}H_{62}(CH_3)_2$	267,295
4	13-$C_{33}H_{67}(CH_3)$	197,309
4	15-$C_{33}H_{67}(CH_3)$	225,281
4	11,15-$C_{33}H_{66}(CH_3)_2$	239,351
4	13,17-$C_{33}H_{66}(CH_3)_2$	267,323
4	15,19-$C_{33}H_{66}(CH_3)_2$	295
5	13-$C_{35}H_{71}(CH_3)$	197,337
5	15-$C_{35}H_{71}(CH_3)$	225,309
5	17-$C_{35}H_{71}(CH_3)$	253,281
5	11,15-$C_{35}H_{70}(CH_3)_2$	239,379
5	13,17-$C_{35}H_{70}(CH_3)_2$	267,351
5	15,19-$C_{35}H_{70}(CH_3)_2$	295,323
6	13-$C_{37}H_{75}(CH_3)$	197,365
6	15-$C_{37}H_{75}(CH_3)$	225,337
6	11,15-$C_{37}H_{74}(CH_3)_2$	169,239,337,407
6	13,17-$C_{37}H_{74}(CH_3)_2$	197,267,309,379
6	15,19-$C_{37}H_{74}(CH_3)_2$	225,295,281,351

[a] Only the major components are given.
[b] Positions not determined.

the methyl branch (Figure 3). Examples of spectral analyses of similar compounds have been reported (Martin and MacConnell, 1970; Nelson, Sukkestad, and Zaylskie, 1972), and we have tabulated those fragments that aided our assignments (Table 2). The parent ion peaks are, as expected, of extremely low intensity, and chain length was assessed by gas chromatography retentions rather than by relying on mass spectrometry for molecular weights. The material making up the first and second peaks of the unsaturated hydrocarbons of the female in areas 3 and 4 (Figure 2) were separated by GLC trappings and identified as C_{31} and C_{33} alkenes and methylalkenes of the same chain lengths.

Hydrogenation of each component allowed GLC comparison with the

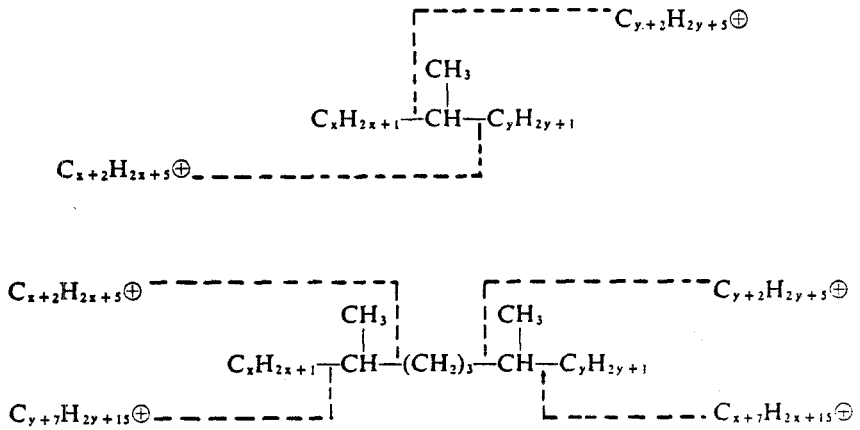

FIG. 3. Fragmentations used to determine position of methyl substitution. In most cases involving the dimethylalkanes, only the C_{x+7} and C_{y+7} fragments were observed.

saturates of the female, and ozonolysis of each indicated the positions of the double bonds. The C_{31} alkene gave rise to nonanal. Docosanal, the other fragment expected from the ozonolysis of a 31-carbon olefin, was hidden by the triphenylphosphine used to cleave the ozonides to aldehydes. The C_{33} alkene gave nonanal and tetracosanal, the latter identified positively with an authentic sample. Thus, with these results and those from the thin-layer studies, the unbranched alkenes were identified as (Z)-9-hentriacontene and (Z)-9-tritriacontene.

The methyl-branched alkenes produced in each case a single identifiable ozonolysis product of such residence time that only a 1-carbon fragment (formaldehyde) could have been lost from each one. The position of methyl branching for these 1-alkenes has not yet been determined. We expect that these compounds will be analogous to the corresponding saturates and occur as mixtures. Moreover, the positions of branching would be determined by hydrogenation of the methyl-branched 1-alkenes, so their positions with respect to the double bond would remain in doubt. Should the activities of our synthetic material not match that of the natural pheromone, a more detailed investigation of the structures of the methyl-branched alkenes would have to be undertaken. The greatest activity thus far has been associated with the saturated hydrocarbons of the female.

CONCLUSIONS

The cuticular lipid of female stable flies contains both saturated and

unsaturated hydrocarbons that induce the male to attempt copulation. These materials appear to be borne solely by the female. The most active components have C_{31} and C_{33} carbon chains. Although we have determined the presence of a large number of individual chemical species contributing to these active components, the task of synthesizing and testing these compounds individually and in several of the many possible combinations still lies ahead.

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STABLE FLY[1] SEX ATTRACTANT AND MATING PHEROMONES FOUND IN FEMALE BODY HYDROCARBONS[2,3]

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Abstract—Investigation into the nature and function of stable fly pheromones indicated that both male sex attractant and mating pheromones are present. Polyolefin fraction of female body hydrocarbons attracted virgin males while the *trans* and *cis* olefins were found to be responsible for mating stimulation. Characterization of the attractive components is under way.

Key Words—stable fly, *Stomoxys calcitrans* (L.), pheromones, sex attractant, mating pheromones, isolation bioassay.

INTRODUCTION

The demonstration of a sex attractant pheromone in the house fly *Musca domestica* by Rogoff et al. (1964) and its isolation, identification, and synthesis by Carlson et al. (1971) suggested that other higher Diptera utilize pheromonal communication. This was later shown by investigations on the

[1] Diptera: Muscidae *Stomoxys calcitrans* (Linnaeus).
[2] Florida Agricultural Experiment Station Journal Series No. 5687.
[3] This paper reflects the results of research only. Mention of a pesticide or a commercial or proprietary product does not constitute a recommendation or an endorsement of this product by the USDA.

face fly, *Musca autumnalis* (Chaudhury, Ball, and Jones, 1972; Uebel et al., 1975*a*) and the tsetse fly, *Glossina morsitans* (Langley, Pimley, and Carlson, 1975). The similarities in morphology and mating behavior to the house fly indicate that similar pheromone response may be present on like body regions of the stable fly, *Stomoxys calcitrans*. Furthermore, the work of Lewis (1961) on stable fly antennal morphology showed sexual dimorphism in the type and number of olfactory sensillae suggesting a strong possibility that olfactory communication exists between the sexes. It is against this background that the present study was undertaken to find the existence and the nature of the sex pheromones involved in stable fly mating.

Initial studies aimed at determining the nature and presence of a sex attractant pheromone revealed that, in addition to a possible attractant pheromone, a mating stimulant was present. That such a mating stimulant is present in stable flies is corroborated by the work of Uebel et al. (1975*b*).

METHODS AND MATERIALS

Stable flies used in the study were originally obtained from the USDA ARS Laboratory, Gainesville, Florida. Adults were maintained under continuous lighting, at a temperature of $27 \pm 4°C$, and relative humidity of $60 \pm 10\%$. Citrated bovine blood was supplied twice a day on blood pads. Extractions were made on 5-day-old adult virgin females (for bioassays of major lipid classes) or mixed sexes (for other bioassays) either by homogenization in a Waring blender or maceration with mortar and pestle using *n*-hexane or petroleum ether at 5 flies/ml. The homogenate was allowed to stand overnight after which the solvent was decanted, filtered, and concentrated to a small volume using a rotary evaporator. The crude extract obtained was fractionated on a 45×2-cm column of silica gel (60–200 mesh, Baker) into various lipid classes using mixtures of hexane and ether, with the ether in increasing concentrations (100:0, 99:1, 90:10, 50:50). 200 ml of each mixture were collected. The hydrocarbon portion of the crude extract was chromatographed on a 45×2-cm column of 20% $AgNO_3$/silica gel (60–200 mesh, Analtech) for fractionation into paraffins and *trans* and *cis* olefins. Fractions of 15 ml were collected using 225 ml of *n*-hexane, 150 ml of hexane ether (99:1) and hexane ether (90:10) eluants. The purity of the neutral lipid components was determined by comparison with authentic standards by thin-layer chromatography (TLC) on silica gel plates and on $AgNO_3$/silica gel TLC plates (250 μm, Analtech). Authentic *cis* and *trans* olefin standards were cochromatographed with natural materials to establish the stereochemistry of the double bonds. A portion of the *cis*, *trans*, and total olefins were each fractionated using a Varian 90-P preparative gas chromato-

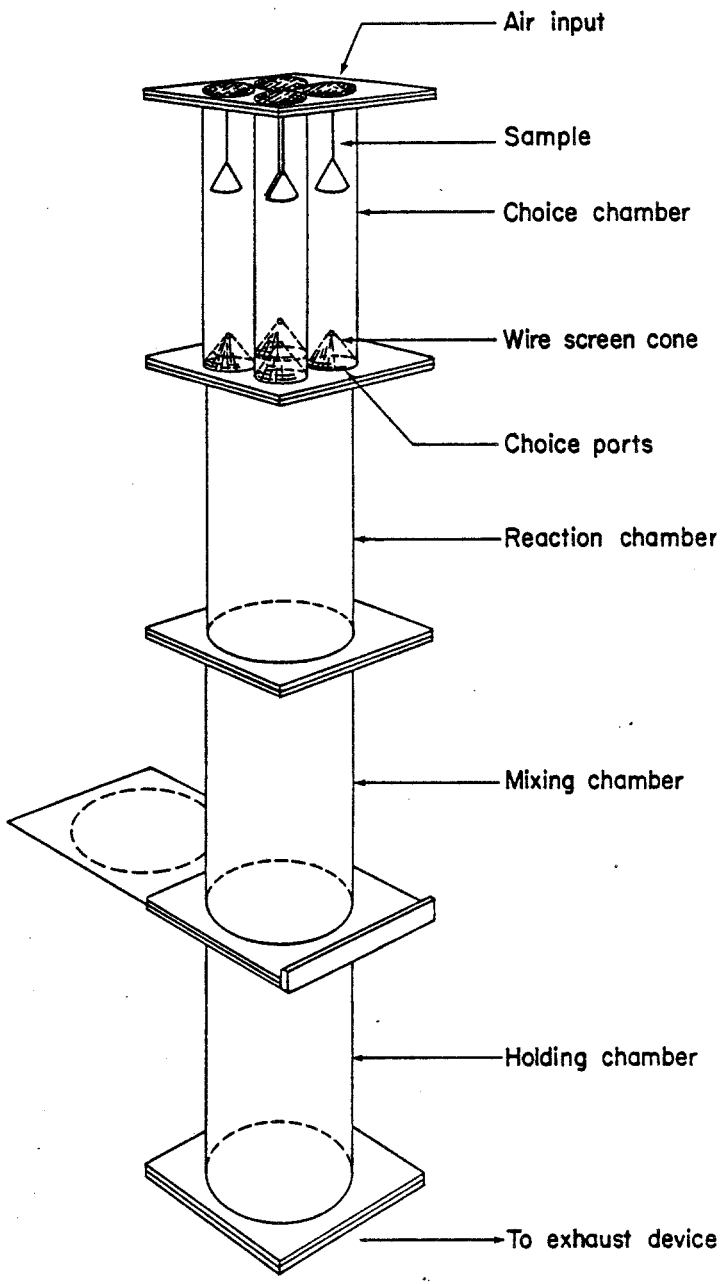

FIG. 1. Diagrammatic representation of the four-port olfaction chamber.

graph equipped with a 1.6 m × 1/4 mm ID stainless steel column packed with 5% SE-30 on Varaport 30, helium carrier gas at 40 ml/min, and thermal conductivity detector. The column oven was temperature programmed at 10°/min from 175 to 310°C for the *cis* and total olefins and 150–300°C for the *trans* olefin. The fractions were collected in air-cooled Pyrex melting point capillaries, after which the compounds were recovered by elution with 500 μl of hexane.

Three types of bioassays were conducted on the activities of the crude extracts and fractions using: (1) an olfactometer, (2) the pseudofly method (Rogoff et al., 1964), and (3) an activity meter. The olfactometer consisted of an oxygen tent modified to supply filtered air at near constant temperature, flow, and relative humidity (26.7±4°C and 50±10%). Air then entered a plexiglass air pressure stabilization chamber before passing into the olfaction chambers (Figure 1). The olfaction chambers consisted of a set of choice chambers (two or four 5-cm-diameter × 23-cm plexiglass cylinders), a mixing

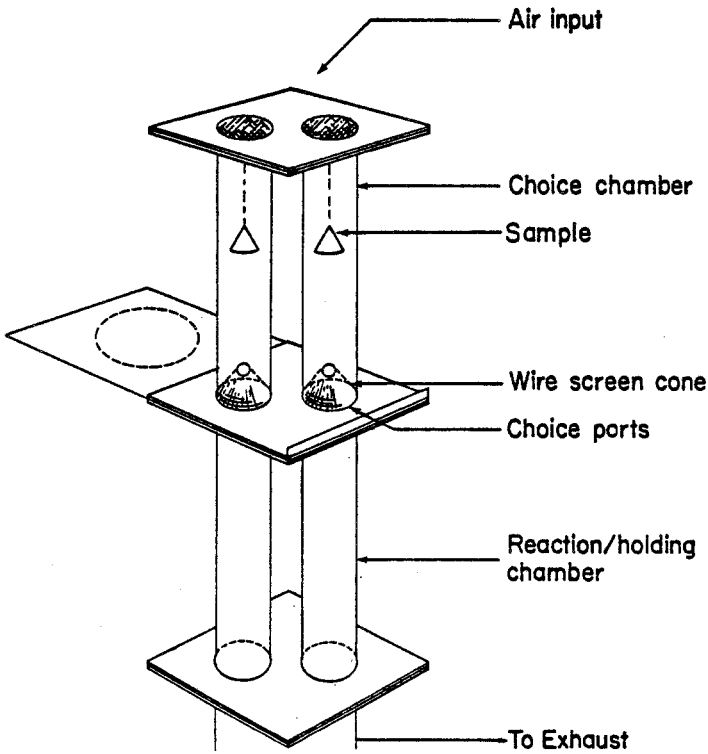

FIG. 2. Diagrammatic representation of a two-port olfaction chamber with reaction/holding chamber attachment.

chamber (10-cm-diameter × 23-cm plexiglass cylinder) and a holding chamber (10-cm-diameter × 23-cm plexiglass cylinder) which connects to an air exhaust device. During competitive bioassays (i.e., concurrent exposure of more than one test fraction), virgin males were introduced into the holding chamber and held for 10 min before exposure to treated filter paper strips (samples) that were held in each of the choice chambers. The flies were then allowed to make a choice by flying into the preferred chamber, where they were trapped behind wirescreen cones held at the top of each choice chamber. Counts were made of both trapped males and males that congregated inside the wirescreen cones. In instances where attraction and mating activity were measured at the same time, the reaction and holding chambers were replaced with a set of 2 or 4 holding/reaction chambers consisting of 5-cm-diameter × 23-cm plexiglass cylinders (Figure 2). Counts for attraction were similarly taken as described above, and the number of jumps by males on other males (mating jumps) were recorded for mating activity.

The pseudofly tests were conducted in plastic petri dishes basically as described by Rogoff et al. (1964), except that counts were made of mating jumps for either 15- or 30-min periods and at various time intervals.

The activity meter consisted of a wirescreen platform attached to a phonograph cartridge. The phonograph cartridge was attached to an incidence recorder through a sensitive relay so that flies landing on the platform or on the cartridge triggered the relay to record the mating activity index. Pseudoflies treated with hexane alone or active fractions dissolved in hexane were suspended on tips of stainless steel wire levers pivoted on the phonograph cartridge. Treated pseudoflies were then covered with 6-oz plastic cups after which 4 virgin males were introduced into the cups. Mating activity as indicated by either mating jumps by males on other males or on the treated pseudofly was recorded.

RESULTS AND DISCUSSION

The results of attraction and mating stimulation by the crude extract and the various fractions obtained from silica-gel column chromatography are summarized in Tables 1–3. Table 1 is a summary of the attraction and mating activity obtained when 3- and 5-day-old males were exposed to the crude extract and other fractions. Significantly, the crude extract and the hexane fractions had a higher level of mating activity than any of the other fractions. This relationship was also observed when 50 FE (fly equivalents) of each test material were exposed to 3–5-day-old virgin males in a 2-port olfactometer (Table 2).

The polyunsaturated olefins (polyolefins) attracted the largest number of

TABLE 1. ATTRACTION AND MATING ACTIVITY DISPLAYED BY 3- AND 5-DAY VIRGIN MALES[a] EXPOSED TO VARIOUS CONCENTRATION RANGES[b] OF EACH TEST MATERIAL[c] IN A 4-PORT OLFACTOMETER[d]

Test material	Component(s)	Attraction[e] and mating activity[f]				
		Reps	A	Δ	MA	Δ
Check	Hexane	46	3.7[i]	0.0	3.9[i]	0.0
Crude lipids		23	5.5[g,i]	1.8	13.6[h]	9.7
Hexane fractions (100:0)	Hydrocarbons	18	4.8[i]	1.1	12.7[h]	8.8
Hex/ether (99:1)	Polyolefins	18	7.6[h]	4.9	3.3[i]	−0.6
Hex/ether (90:10)	Cholesterol esters, triglycerides	6	1.8	−1.9	6.1[i]	2.2
Hex/ether (50:50)	Triglycerides, free fatty acids	6	1.4	−2.3	6.1	2.2

[a] 40 males exposed in holding chamber.
[b] 5–100 fly equivalents.
[c] Obtained from females only.
[d] Competitive tests during which check, crude lipids, and other fractions were in each run.
[e] A indicates mean number of males attracted to or into traps, Δ indicates mean number of mating jumps per test material minus that of check.
[f] MA indicates mating activity based on number of jumps on other males per minute, indicates mean number of mating jumps per test material minus that of check.
[g] Difference between mean of treatment and check significant at 5% (Duncan's multiple range).
[h] Differences between mean of treatment and check significant at 1% (Duncan's multiple range).
[i] Differences between means of treatments not significant at 1% (Duncan's multiple range).

5-day-old males when the test materials were competitively exposed to them (Table 1). A similar performance at a lower significance was seen when the test materials were tested only with check (Table 3). No significant difference was found between the crude, the hydrocarbon, and the hexane/ether (90:10) fractions. Analysis of the Hexane (100) fraction used in this test (Table 3 only) showed that it contained small amounts of polyolefin as shown by spots on silica gel TLC (*Rf* 0.68), and on $AgNO_3$ silica gel TLC (*Rf* 0.1). This means that complete separation was not obtained and activity was probably due to the polyolefin traces.

The low performance of the crude when tested against the check in the 2-port olfactometer can be ascribed to the effect of concurrent stimulation from both the attraction and mating activity components. Observations of both fly movement and mating in the olfactometer during the tests with the crude extract showed that the flies in the reaction chambers tended to mate

TABLE 2. MATING ACTIVITY BY 3–5-DAY-OLD VIRGIN MALES[a] EXPOSED TO 50 FE OF EACH TEST MATERIAL[b] IN A 2-PORT OLFACTOMETER

Test material	Component(s)	Reps	Mating jumps by virgin males on other males/min.
Check	Hexane only	16	14.9[d]
Crude extract	Lipids	11	27.4[c]
Hexane (100:0)	Hydrocarbons	11	36.7[c,e]
Hex/ether (99:1)	Polyolefins	9	20.3[d]
Hex/ether (90:10)	Cholesterol esters, triglycerides	5	18.1[d]
Hex/ether (50:50)	Triglycerides, free fatty acids	8	18.7[d]

[a] 20 males exposed in holding chamber.
[b] Obtained from females.
[c] Differences between means of this treatment and check significant at 1% (Duncan's multiple range).
[d] Differences between means of these treatments not significant at 1% (Duncan's multiple range).
[e] Difference between the means of the treatments significant at 5% (Duncan's multiple range).

TABLE 3. ATTRACTION OF 5-DAY-OLD MALE FLIES[a] TO 10 FE OF THE CRUDE AND VARIOUS FRACTIONS[b] EXPOSED IN A 2-PORT OLFACTOMETER

Test material		Reps	Mean number of virgin males trapped in treated port 15 min after exposure
Check (hexane only)		21	1.1[d]
Crude extract (lipids)		10	1.8[d]
Hexane (100:0) hydrocarbons		8	3.2[d]
Hexane/ether (99:1)	Polyolefins	16	4.7[c]
Hexane/ether (90:10)	Cholesterol esters, triglycerides	12	2.4[d]
Hexane/ether (50:50)	Triglycerides, free fatty acids	12	1.0[d]

[a] 20 males exposed in holding chamber.
[b] Obtained from females.
[c] Differences between the means of this treatment and check significant at 1% (Duncan's multiple range).
[d] Differences between means of these treatments not significant at 1% (Duncan's multiple range).

TABLE 4. MATING ACTIVITY BY MALE STABLE FLIES EXPOSED TO FRACTIONS[a] OBTAINED FROM $AgNO_3$/Si GEL COLUMN

Test material	Response[b] to test materials applied to pseudoflies							
	Reps	0.5 FE[c]	Reps	1 FE	Reps	10 FE	Reps	100 FE
Check (hexane only)	(7)	1.8	(7)	0.4	(7)	0.4	(7)	0.1
Paraffins	(6)	1.2	(6)	4.9	(7)	6.1	(6)	2.3
Trans olefins[d]	(5)	7.4	(5)	15.4	(5)	12.0	(5)	21.2
cis olefins[e]	(6)	3.4	(6)	15.0	(8)	10.8	(5)	17.4

[a] Obtained from mixed sexes.
[b] Response indicated by mean jumps by a male on either pseudofly or other male/hr.
[c] FE = fly equivalent.
[d] Fractions 10–13 of hexane (100:0) elution on $AgNo_c$/silica gel column.
[e] Fractions 14–25 of hexane ether (99:1) elution on $AgNO_3$/silica gel column.

and fall back to the bottom of the holding chambers, leading to low numbers trapped in the choice chambers. In addition, while males attracted by the polyolefin fraction displayed searching movements after reaching the pheromone source, those attracted by the crude displayed mating jumps on other

TABLE 5. MATING ACTIVITY BY MALE STABLE FLIES EXPOSED TO *cis* OLEFINS[a] OBTAINED FROM PREPARATIVE GC

Fraction no.	Kovat's indices for major peaks	Response[b] to 10 FE[c] applied to pseudoflies
Check (hexane only)		6.0
1	Less than 1800	0.0
2	1800–1900	13.0
3	2100, 2110	9.0
4	2440	13.0
5	2480	21.0
6	2680	17.0
7	2890, 2920	8.5
8	3100, 3140	16.5
9	3340, 3370	18.0
10	3700	6.5
11	3900	7.5

[a] Obtained from mixed sexes.
[b] Response indicated by mean jumps by a male on either pseudofly or another male for 6 reps.
[c] FE = fly equivalent.

trapped males. These observations are consistent with the roles of the two pheromones in nature: the attractants serve to lure male insects from a distance, while the mating stimulants elicit mating attempts once the two sexes are in close proximity.

Table 4 shows the comparative mating activity of fractions obtained from $AgNO_3$/silica gel chromatography. The olefin fractions showed higher activity at all concentrations than the paraffin fraction, with *trans* olefins showing overall superiority at most concentrations. Adequate quantitation of the responses was not possible with the pseudofly technique because it was difficult to distinguish between single strikes and episodes during which a single fly maintained a prolonged mating attempt with psuedofly. Prolonged mating of a male with a pseudofly was occasionally observed to last 14 min.

Tests on preparative GC fractions of the *cis* iomers revealed that fractions 5, 6, 8, and 9 (with respective Kovats Indices of 2480, 2680, 3100–3140, and 3340–3370) (Kovats, 1966), were the most effective in elicitation of the mating response (Table 5, Figure 3). The *trans* isomers showed fractions 4

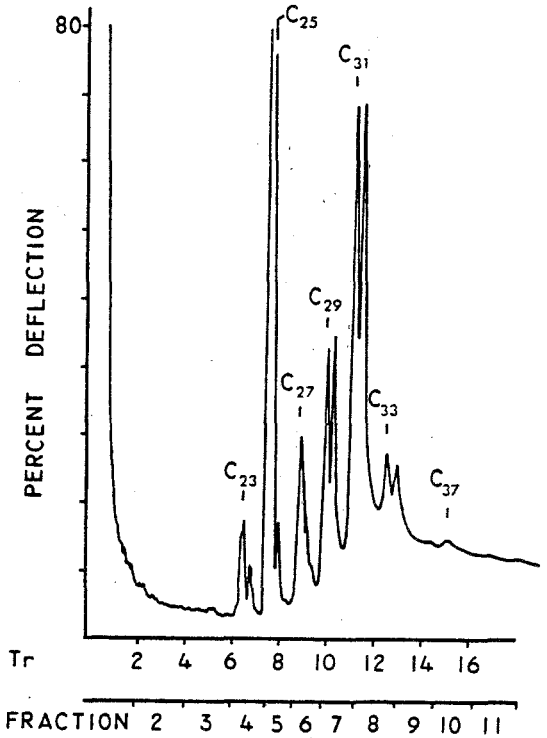

FIG. 3. Analytical GC of *cis* olefins (mixed sexes) as fractionated for bioassay.

TABLE 6. MATING ACTIVITY BY MALE STABLE FLIES EXPOSED TO TRANS MONO OLEFINS[a] OBTAINED FROM PREPARATIVE GC

Fraction no.	Kovat's indices for major peaks	Response[b] to 10 FE[c] applied to pseudoflies
Check (hexane only)		1.6
1	1900	2.0
2	2090, 2105	2.0
3	2300	3.6
4	2430	20.2
5	2500	3.4
6	2685	5.8
7	2900	0.6
8	3020, 3100	6.8
9	3300, 3380	18.2
10	3700	2.6
11	3900	7.8

[a] Obtained from mixed sexes.
[b] Response indicated by mean jumps by a male on either pseudofly or another male/hr for 6 reps.
[c] FE = fly equivalent.

and 9 (KI of 2430 and 3300–3380) to excel all other fractions, although 6, 8, and 11 (KI of 2685, 3020–3100, 3900) displayed moderate activity (Table 6, Figure 4). When the two isomers were combined, activity as measured by activity meter was highest for fraction 7 (KI 3085) (Table 7). Although much less *trans* olefin was present in the total hydrocarbon fractions (3% *trans* olefins as against 15% and 82% for *cis* olefins and paraffins, respectively), mating activity was observed in the order *trans* = *cis* > *paraffins*. This implies that there is more activity associated with the *trans* compounds.

The data so far obtained point to the presence of male sex attractant and mating stimulant pheromones in stable fly female body hydrocarbons. While the mating stimulants primarily reside in the olefins, the attractant pheromones are in the polyolefin fractions. The manner in which they operate is not established; however, our observations during bioassays lead us to advance the following:

1. The processes of mating activity stimulation involve chemical, as well as visual, stimulation. That chemical stimulation acts in the process is verified because most of the mating jumps were directed to treated pseudoflies rather than to untreated pseudoflies or male flies.

2. Attraction, on the other hand, appears to be brought about only by chemical stimulation.

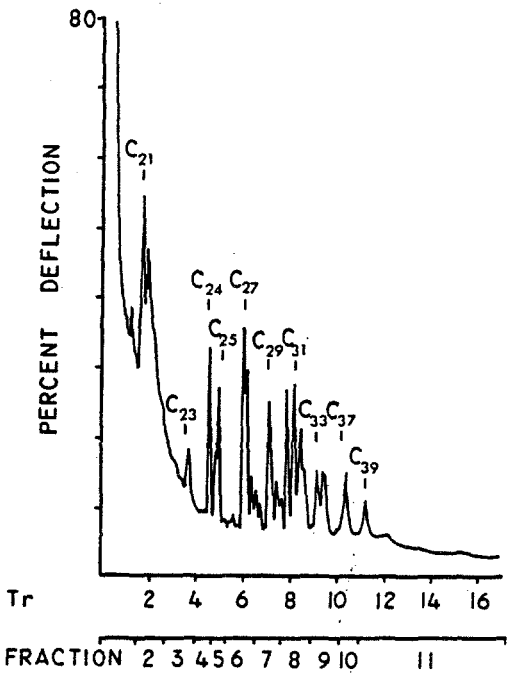

FIG. 4. Analytical GC of *trans* olefins (mixed sexes) as fractionated for bioassay.

TABLE 7. MEAN MATING ACTIVITY OF 5-DAY-OLD MALES[a] EXPOSED TO FEMALE CRUDE AND TOTAL OLEFIN FRACTIONS[b] BY ACTIVITY METER

Preparative GC fractions at 10 FE	Kovat's index	Reps	Mean no. of mating jumps/min[c]	Δ[d]
Crude extract		(4)	22.70	10.97
6	2910	(4)	13.00	1.07
7	3085	(5)	71.75	59.82
8	3320	(5)	15.50	3.57

[a] 4 males used/test.
[b] Obtained from females.
[c] Mean number of mating jumps for untreated = 11.93.
[d] Indicates number of mating jumps by males exposed to treated pseudoflies minus mating jumps after exposure to untreated.

3. When the two stimuli operate concurrently at close range to the stimulation source, the mating activity stimulant overcomes the attractant stimulant. Thus, when the crude extract which contains both the attractant and the mating activity pheromones was exposed to virgin males in the noncompetitive test, the effect of the mating activity stimulant decreased catches of trapped individuals.

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WING SCENTS AND SCENT-RELEASED PHASES IN THE COURTSHIP BEHAVIOR OF *Lycaeides argyrognomon* (LEPIDOPTERA: LYCAENIDAE)

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Abstract—Habitats of *Lycaeides argyrognomon* Bgstr. are described. The most ritualized courtship comprises: male approaching, male fluttering around, female mate refusal posture, female escape flight, sexual chase, female alighting, male fluttering around, female receptive posture, parallel position, male abdomen-bending, male attaching and copulatory position. Field experiments have shown that the refusal posture of mated females is released by odors from the male wings. In unfertilized females this behavior is inhibited by male odor signals. In SEM pictures of the androconial scales of dried specimen, holes and fissures in the upper scale lamella were found. Experiments with models indicate the existence of behavior-releasing odors from the female wings though the females lack scent scales comparable to those of the males. The female scents provoke the whole terminal sequence of the male courtship behavior when combined with a suitable visual object. The volatile material has been analyzed by capillary gas chromatography and mass spectrometry. Wings of males were found to contain nonanal, hexadecyl acetate, and a cyclic sesquiterpene alcohol, tentatively identified as torreyol (δ-cadinol).

Key Words—butterfly, sesquiterpene, scent scales, mating behavior, *Lycaeides argyrognomon*, nonanal, hexadecyl acetate, torreyol, δ-cadinol, pheromone.

INTRODUCTION

According to the anthropomorphic grouping of the butterfly scents used in

several reports (Müller, 1880; Dixey, 1906, 1911) the male wing scents of the blues, which are distinctly perceptible to our nose, must be characterized as pleasant. They are faintly sweet and give associations with vanilla, chocolate, and apple. Butterfly females have usually been considered to lack wing scents. Nevertheless we have isolated volatile compounds also from the wings of females of *L. argyrognomon* Bgstr. and from the wings of some other species of blues. In the present report the occurrence of behavior-releasing odor signals from the wings of both sexes is discussed.

Lycaeides argyrognomon is distributed from France and Switzerland across central Europe and southern Russia to Amurland and perhaps to Japan (Higgins and Riley, 1970), but it is more common in the southeast part of the distribution area. In Sweden, it is both localized and very rare.

Habitats

Lycaeides argyrognomon is in Sweden restricted to a few places in the northeast of the province of Småland and the southeast of the province of Östergötland, where it is bound to the food plant, *Astragalus glycyphyllus*. We have not found *Coronilla varia*, an alternative food plant (cf. Nordström et al., 1941), in any Swedish habitat. The three habitats we have investigated in Sweden are all sunny forest edges facing the southwest, where a road cut has provided an open area to the food plant.

Though *Astragalus glycyphyllus* requires good soil and is demanding about temperature, the food plant is far more widespread than the butterfly. Its northern limit in Sweden is over 600 km further north than the butterfly. The ecological demands of *L. argyrognomon* are insufficiently known. We have found localities with numerous *Astragalus* plants in the neighborhood of the flying places but no *L. argyrognomon* specimens. Michel (1948) writes on this species in Bohemia: "Merkwürdig ist es, dass die Art nur gewisse Flugplätze bevorzugt während sie an anderen gleichartigen fehlt." South of Wiener Neustadt in Austria, where we caught butterflies for the chemical analyses in 1968, *L. argyrognomon* flies in the glades of the pine forests (*Pinus nigra austriaca*). The eggs were to a large extent laid on *A. glycyphyllus*. We have also studied the species on the dry and hot slopes of the Jura valleys north of Regensburg. In this area the prevailing food plant is *Coronilla varia*.

Courtship

The beautifully colored blues ought to have invited numerous entomologists and poets to describe the mating dance. There are, however, only some short communications on separate details in the courtship of a few of these species (e.g., Temple, 1953). The complete course of the courtship behavior

in butterflies is very hard to observe. Even an experienced field observer usually sees butterflies only in whirling fragments of the sexual chase or in copula. It is very often the vigorous refusal behavior of the females that attracts the observer's eye. When we tethered living females to rods in the field, it was obvious that the flying behavior of the males results in an effective searching of the habitat. The females are, as a rule, discovered and fertilized shortly after their emergence. It is of vital importance to the females that they produce progeny before they are exposed to too many dangers. Repeated copulations by the males and proteandry (i.e., the males emerge and are active before the females) therefore have a positive survival value. Most *L. argyrognomon* females probably copulate only once, although butterfly females of other species (e.g., *Pieris brassicae*, Dussaussoy and Delplanque, 1964; *Colias eurytheme*, Stern and Smith, 1960) have been shown capable of copulating more than once. Fertilized females are, however, still attractive to the males, which explains why most courtships are unsuccessful.

The general course of the courtship in *L. argyrognomon* does not seem to be different from that of three other Lycaenid species (*Polyommatus icarus* Rott., *Cupido minimus* L., and *Plebejus argus* L.) which we have been able to study in more detail. The most sustained and ritual courtship is performed when a male detects an unfertilized sitting female at a low level of activity. The following then usually happens:

1. The visually attracted male flies towards the female—*male approaching.*
2. The male flutters close to the female with sweeping wing movements—*fluttering around.*
3. The female vibrates her half open wings and raises the abdomen—*mate-refusal posture.*
4. The female flies away—*escape flight.*
5. The male flies after the female—*sexual chase.*
6. The female alights—*female alighting.*
7. (= 2) Fluttering around. This behavior can be performed by the flying male or when he has alighted close to the female.
8. The female folds her wings and remains quiescent—*receptive posture.*
9. The male walks up beside the female—*parallel position.*
10. The male bends the abdomen, spreads the valves, and exposes the copulatory apparatus—*abdomen bending.*
11. The copulatory apparatus is attached—*attaching.*
12. The butterflies orientate themselves in the typical reversed position—*copulatory position.*

If the female is very receptive, the courtship can be reduced only to fluttering around, receptive posture, male alighting, parallel position, attaching. The main interspecific differences seem to concern the sexual chase

and the spatial performance of the courtship. In *Lycaeides argyrognomon* a form of helicoptering chase was once observed, i.e., the pair was flying about 1½ m above the ground, the male behind the female, but the forward movement was practically absent. The male occasionally struck against the female. Shortly afterwards the female alighted and attaching followed.

Indications of Behavior-Releasing Odors from the Male Wings

The mate-refusal behavior by the female—vibrating the half open wings and raising the abdomen at an ordinary level of activity—seems to be based on male pheromones. In unfertilized females on the other hand, the same behavior is suppressed by male odor signals. Most observed females are mated. Our field experiments show that the mate-refusal behavior can be released by a newly dead male affixed to the tip of a straw of grass. The dummy is presented to sitting but nonfeeding females. Wing vibrating and abdomen raising are also released by a fresh male wing. On the other hand a male or a male wing washed in ether and dried 12 hr at 100°C does not provoke this behavior in the females. Mate-refusal postures of butterflies are reviewed by Scott (1972).

Structure of Androconial Scales

The delicate structure of the scent-disseminating scales of the blues attracted the interest of the early microscopists (Deschamps, 1835). These transparent unpigmented scales turned out to be very difficult to study through the light microscope. We examined the wing scales of *L. argyrognomon* in a Cambridge stereoscan S-4 microscope operated at 3–5 kv. The wing surface was coated with evaporated carbon, silver, and gold. In Figure 1 the numerous scent scales called "battledore scales" are seen projecting between the rows of ordinary scales. Figure 2 shows the stalk attaching the androconial scale to the wing and the perforated upper scale lamella. Sellier (1972) has published some SEM pictures of the scent scales of *Lysandra bellargus* Rott. which show holes and fissures in the upper lamellae. In our dried specimens of *L. argyrognomon* an aberrant type of androconial scale with a practically unpierced upper lamella was found (Figure 3). Further investigation on newly dead butterflies at different ages is needed.

Indications of Behavior-Releasing Odors from Female Wings

In the males the fluttering-around and abdomen-bending behaviors were released in field experiments by a newly dead female and also by a fresh female wing, but not by a female that had been dead for some time or a female

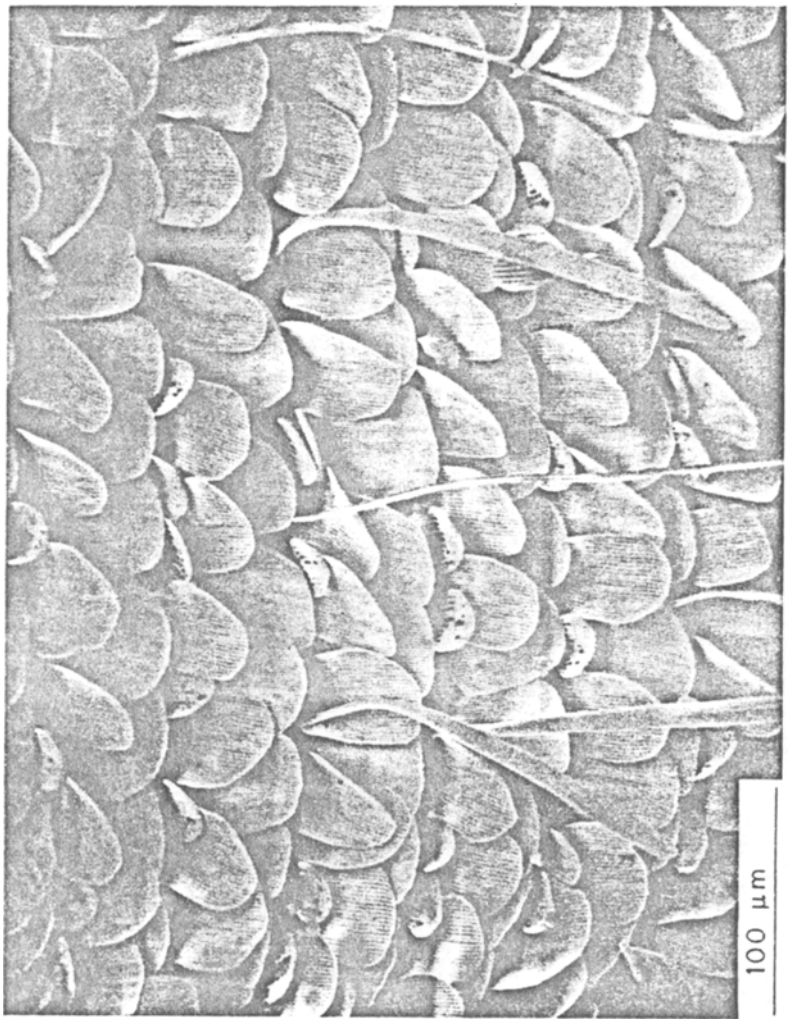

FIG. 1. *L. argyrognomon*; the battledore scales are projecting between the rows of ordinary scales.

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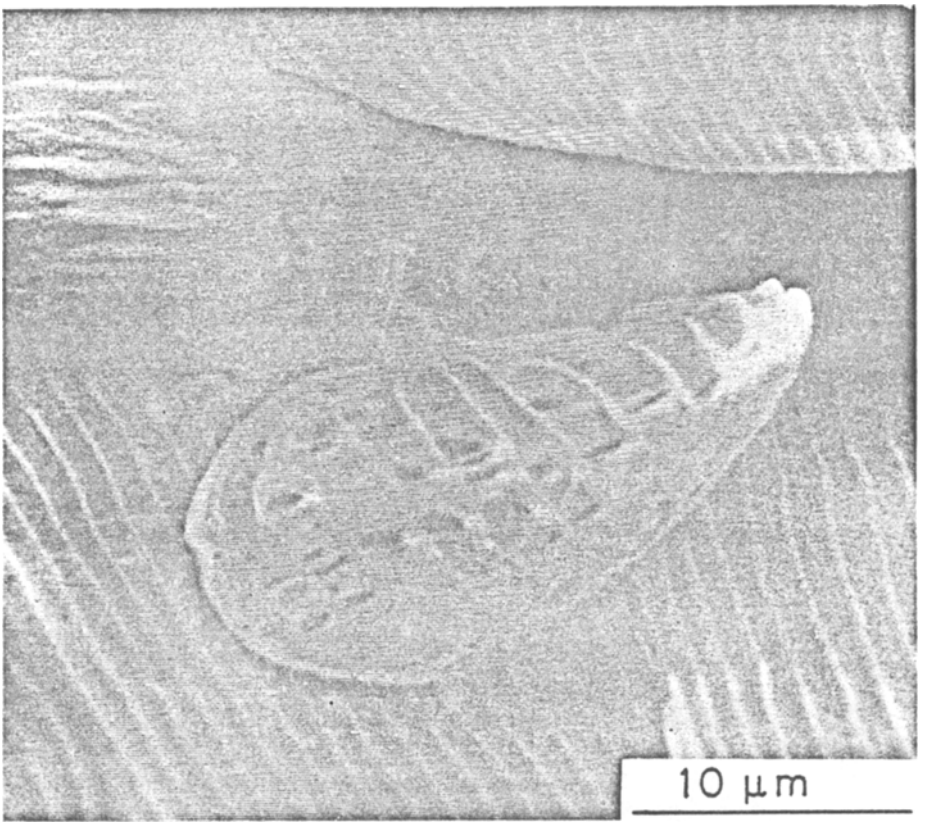

FIG. 2. *L. argyrognomon*; the scent scale is attached to the wing surface with a thin stalk.

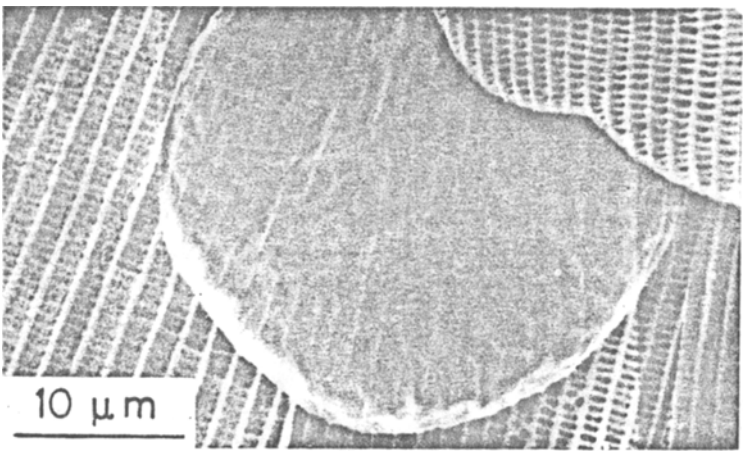

FIG. 3. *L. argyrognomon*; an aberrant type of androconial scale.

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wing washed in ether and dried 12 hr at 100°C. These results conflict with the opinion that the females lack scent scales on the wings. Of course, the females may have glandular cells on the wings, although they do not have the scent-spreading scales. We have not as yet had the opportunity to search for such structures. The explanation could also be that the "wing" scent represents contamination from the abdominal glands.

METHODS AND MATERIALS

Isolation of the volatile compounds has been done mainly through the use of a splitter-free inlet system coupled to a capillary gas chromatograph (Ställberg-Stenhagen, 1972) and through a precolumn tube coupled to the gas chromatograph–mass spectrometer instrument (Bergström, 1974). Wings of butterflies were put directly on the splitter-free inlet system or on the precolumn tube (cf. Bergström and Lundgren, 1973). Extracts of wings in diethyl ether have also been used. The material collected in 1974 was analyzed by a coupled gas chromatograph–mass spectrometer with capillary column and splitter-free inlet system (modified LKB-2091 instrument).

RESULTS

A typical capillary gas chromatogram of the volatile compounds emanating from the wing scales of one male *L. argyrognomon* is shown in Figure 4. The capillary column used is 23 m long, coated with OV-101 as stationary phase. Separation efficiency was 88,000 theoretical plates, measured isothermally relative to methyl pentadecanoate. The temperature of the oven was programmed from 50°C to 208°C, 4°C/min. Three major components (1, 3, and 4) have been identified directly by their mass spectra and their capillary gas chromatographic retention indices as nonanal, hexadecanol, and hexadecyl acetate, respectively.

The mass spectrum of component 2 is given in Figure 5. It indicated that the compound was a cyclic sesquiterpene alcohol, molecular weight 222, with characteristic fragments at $m/e = 204$, corresponding to the ion $[M—H_2O]^+$, $m/e = 161$, corresponding to $[M—H_2O—C_3H_7]^+$, and a series of fragments with $m/e = 105$ as the largest fragment. Catalytic hydrogenation in ethyl acetate with platinum oxide as catalyst produced a compound with the molecular weight 224.

Comparisons between the capillary gas-chromatographic retention indices and mass spectra of component 2 of the secretion and those of some cyclic sesquiterpene alcohols, available as reference compounds, revealed

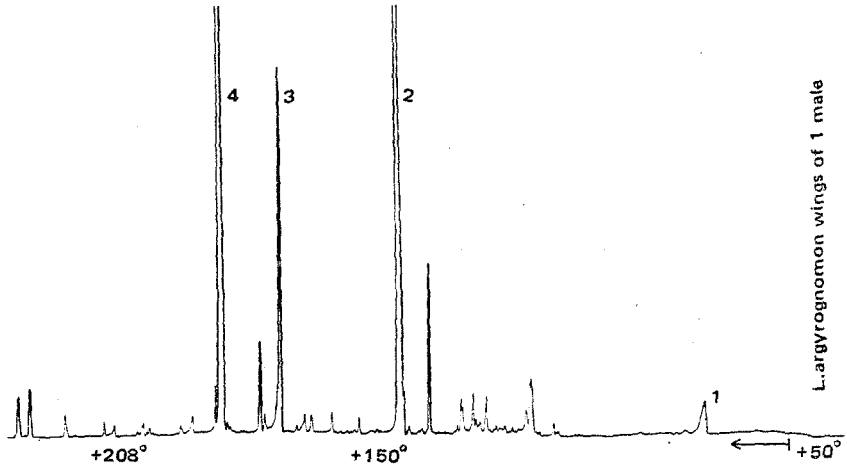


FIG. 4. Capillary gas chromatogram of volatile secretion from wings of one *L. argyrognomon* male.

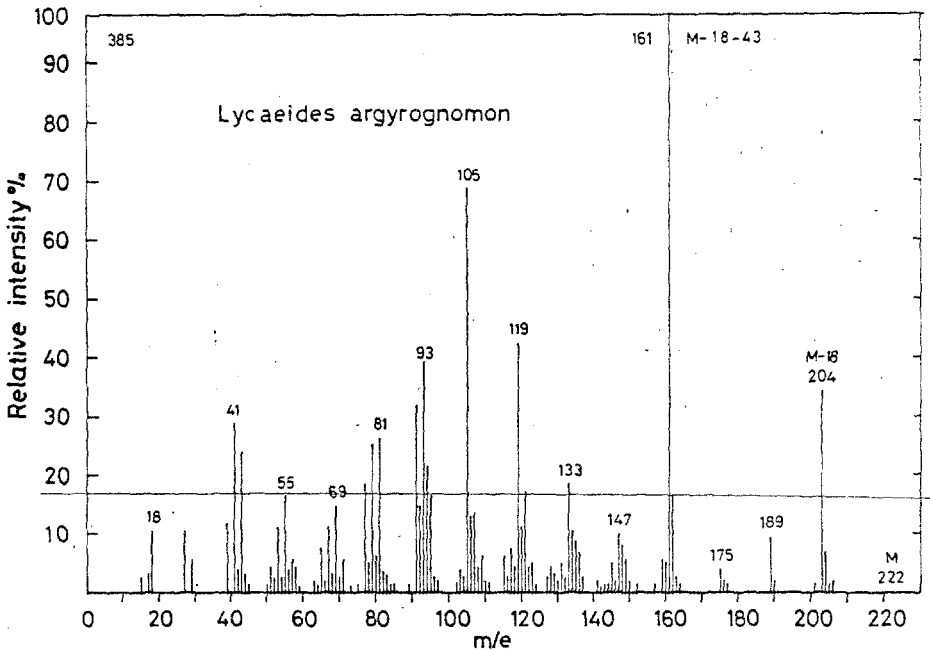
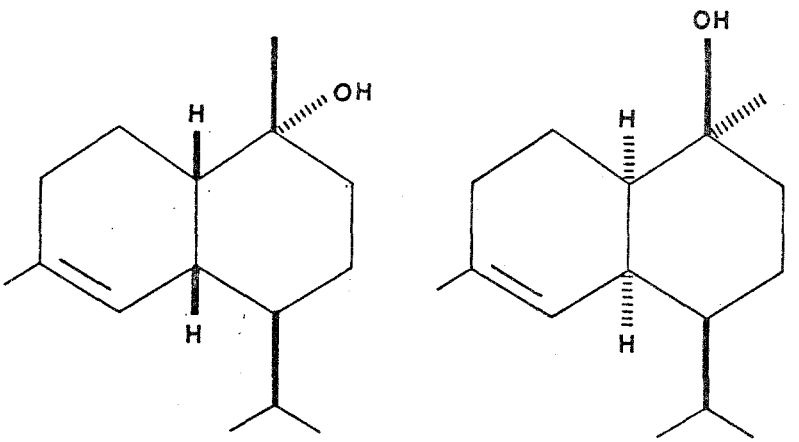


FIG. 5. Mass spectrum of component 2 in the volatile secretion from wings of *L. argyrognomon* males.



A B

FIG. 6. Possible structural formulae of torreyol (= δ-cadinol). A is a muurolol, B is an amorphol.

identical mass spectra and capillary gas-chromatographic retention indices between component 2 and a compound designated as "torreyol". This compound was obtained through the laboratories of Prof. T. Norin and Dr. L. Westfelt in Stockholm. It has been isolated from Swedish sulfate turpentine and from the oil of *Pinus silvestris*. We also obtained a reference sample from Prof. V. Herout, Prague, Czechoslovakia, which was designated as "δ-cadinol." This compound is also indistinguishable from the torreyol and from component 2 of *L. argyrognomon*.

Torreyol, in its dextrorotatory form, was first isolated from the leaves of *Torreya nucifera* Sieb et Zucc. which belongs to Taxaceae (Shinozake, 1922). Several compounds occurring in pines have been shown to be identical with (+)-torreyol. Other compounds, isolated from pines and various plants are identical with (−)-torreyol. Torreyol has frequently been referred to as δ-cadinol. As long as there are doubts about the absolute structures of these compounds we prefer to use the trivial name torreyol, referring to the original isolation. Westfelt (1970) holds that (−)-torreyol [=(−)-δ-cadinol] isolated from *Pinus silvestris* has the structure given in Figure 6A. This is a muurolol type of bicyclic sesquiterpene alcohol. He bases his view on chemical as well as spectroscopic data. Lin et al. (1971) have arrived at the conclusion that (−)-δ-cadinol [=(−)-torreyol] isolated from *Taiwania cryptomerioides* has the structure given in Figure 6B. This, on the other hand, is an amorphol sesquiterpene.

The conclusive identity of component 2 cannot be determined until a

TABLE 1. COLLECTION AND ANALYSIS OF *L. argyrognomon*

Date of collection	Locality	No. of individuals	Sex	Preparation	Date of analysis
7/22–23 1966	Västra Ed	15	m	Ether extract	8/12 1966
7/12 1967	Västra Ed	7	m	Ether extract	7/31 1967
6/17–19 1968	Wiener Neustadt		m	Ether extract	6/23 1968
6/17–19 1968	Wiener Neustadt	7	f	Ether extract	6/24 1968
6/17–19 1968	Reared at ESÖ[a]			Ether extract	7/4 1968
7/9–10 1968	Dalhem		f	Ether extract	7/11 1968
7/9–10 1968	Dalhem	25	m	Ether extract	7/11 1968
7/9–10 1968	Dalhem	25	m	Ether extract	7/13 1968
7/9–10 1968	Dalhem	10	m	Ether extract	7/15 1968
7/10 1968	Dalhem	25	m	Abdomen, tip	7/11 1968
	1–4 days, reared at ESÖ	25	m	Ether extract	7/22 1968
	1 day, reared at ESÖ	7	f	Ether extract	7/22 1968
	1–3 days, reared at ESÖ	25	f	Ether extract	7/22 1968
7/18 1970	Dalhem	10	m	Precolumn	7/19 1970
	Västra Ed	5	f	Precolumn	7/19 1970
7/9 1971	Dalhem	15	m	Precolumn	7/20 1971

7/9 1971	Dalhem	15	m	Precolumn	7/13 1971
7/9 1971	Dalhem	15	m	Precolumn	7/13 1971
6/7 1972	Regensburg	1	m	Precolumn	6/12 1972
6/7 1972	Regensburg	1	m	Precolumn	6/13 1972
7/2 1973	Dalhem	3	m	Precolumn	7/3 1973
7/2 1973	Dalhem	1	m	Precolumn	7/3 1973
7/2 1973	Dalhem	1	m	Precolumn	7/4 1973
7/2 1973	Dalhem	1	m	Precolumn	7/4 1973
7/2 1973	Dalhem	3	m	Precolumn	7/4 1973
7/2 1973	Dalhem	1	m	Precolumn	7/4 1973
7/2 1973	Dalhem	1	m	Precolumn	7/5 1973
7/2 1973	Dalhem	1		Precolumn	7/6 1973
7/2 1973	Dalhem	1	m	Precolumn	7/6 1973
7/2 1973	Dalhem	5	m	Precolumn	7/6 1973
7/29 1973	Västra Ed	5	f	Precolumn	7/30 1973
7/29 1973	Västra Ed	10	f	Abdomen, tip	7/31 1973
7/29 1973	Västra Ed	5	f	Abdomen, tip	7/31 1973
7/20 1974	Dalhem	6	f	Ether extract	7/24 1974
7/20 1974	Dalhem	6	f	Abdomen, tip	7/24 1974

[a] ESÖ = Ecological Station of Uppsala University on Öland.

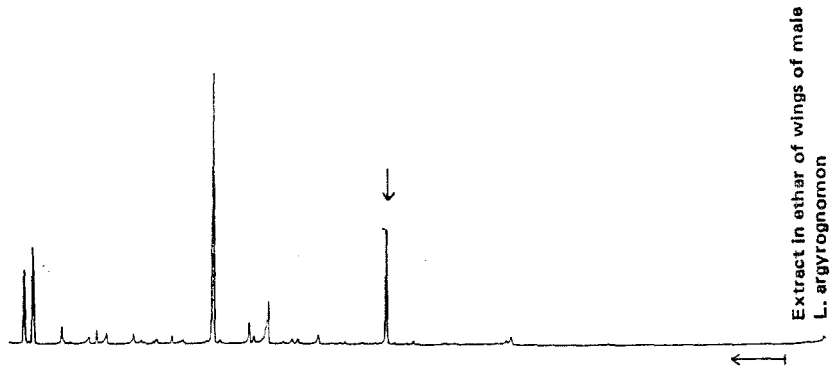


FIG. 7. Capillary gas chromatogram of an extract in diethyl ether of wings from *L. argyrognomon* male.

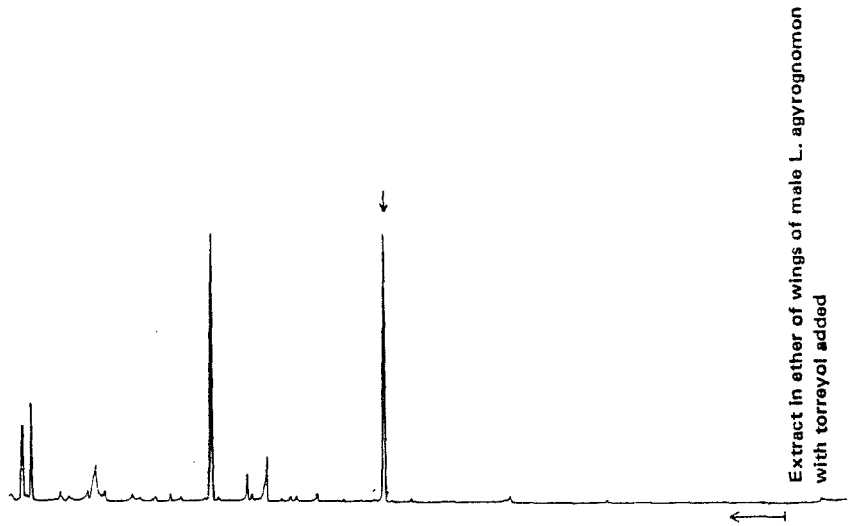


FIG. 8. Capillary gas chromatogram of the same extract as in Figure 7, with torreyol = δ-cadinol added.

larger number of isomeric structures have been isolated and characterized and comparative field and laboratory tests of these compounds have been performed. Addition of torreyol (= δ-cadinol) to an extract from male wings of *L. argyrognomon*, followed by capillary gas chromatography (see Figures 7

and 8), shows that torreyol fully coincides with component 2 of the secretion. We therefore identify this compound tentatively as torreyol (= δ-cadinol). The optical rotation has not been measured. It should be pointed out that other bicyclic sesquiterpene alcohols studied as reference compounds differed from torreyol, both as regards retention index and mass-spectral fragmentation pattern, and therefore they could not be identical with component 2 of the secretion.

The volatile secretion from male wings contains some minor components (see Figure 4), in addition to the major ones. Noteworthy among these are several isomeric sesquiterpene hydrocarbons, probably structurally related to torreyol. They can be seen in the chromatogram (Figure 4) between components 1 and 2.

As shown in Table 1, male wings of *L. argyrognomon* have been collected on several occasions (1966, 1967, 1968, 1970, 1971, 1972, and 1973) from different localities (Västra Ed and Dalhem, Sweden; Wiener Neustadt, Austria, and Regensburg, West Germany). Butterflies were also bred at the laboratory on Öland (1968). The analytical results show no differences in the composition of the secretion from different collections. Analyses of reared males show that the production of wing odors begins during the first day of adult life.

Wings and abdominal tips of females have also been studied on various occasions. So far, no appreciable amounts of volatile compounds have been detected from the abdominal tip. Female wings on the other hand have been found to have trace amounts of a few volatile substances. One of these, found on some occasions, is in all likelihood identical with the torreyol from male wings. It is still an open question if this compound in female wings is produced by the female itself or if it is transferred on contact with a male during the fluttering-around phase of the courtship. The quantitative relation between the sesquiterpenol in the male and in the female (of those in which it has been found) is on the order of 200:1. The amount of torreyol present in a single male is about 1 μg.

Electrophysiological Tests of Butterfly Odors

In this field we are collaborating with Dr. Michael Boppré (Max-Planck-Institut für Verhaltensphysiologie, 8131 Seewiesen). It has been possible to obtain reproducible electroantennograms (EAG) from living animals and from isolated antennae in several species of blues. Females and males react to both female and male odors. The EAGs indicate that the females have pheromones on the wings as well as on the abdomen. Efforts are now being made to make single cell recordings.

DISCUSSION

The aim is to understand the role of the sex pheromones in the intersexual communication in Lepidoptera. Compared to nocturnal species, the chemical communication in butterflies is a close-range communication. In *Lycaeides argyrognomon* the long-range effect fulfilled by the sex attractants in moths is accomplished by the effective search flight of the males. From eye range the males are visually guided to the females. The experiments with natural male odors show that the model must be brought within a distance of a few centimeters from the female in order to provoke behavioral response. The stimulation is most efficient if the odorous object is gently brought in contact with the female antennae. In fact this is what often happens during the intense fluttering-around phase of the courtship. The female odors also are effective only at extremely short range. This is shown by the experiments with models and by the fact that a male fails to locate a female which has crept out of sight a few centimeters away among the ground vegetation.

Conceivable biological functions of the male scents have been surveyed in an earlier communication on the androconial wing secretion in *Pieris napi* L., *P. brassicae* L., and *P. rapae* L. (Bergström and Lundgren, 1973).

In *Lycaeides argyrognomon* the following effects of the male scents are suggested: (1) inhibition of the mate-refusal behavior in unmated females; and (2) releasing of the same refusal behavior in mated and in insufficiently stimulated females. We do not know if the same components are active in both cases.

The mate-refusal behavior, which in similar form occurs in Pierids and also in other butterflies, was originally interpreted as a receptive position of the female as the external genitalia are exposed when the abdomen is raised. Although the mate-refusal function is now well established, it is interesting to speculate on the evolution of the rejection behavior. It may be that it is primarily a scent signaling behavior which secondarily has acquired a copulation evasive effect in mated females. In *L. argyrognomon* the wing vibrating and the abdomen raising occur only in response to the fluttering-around behavior of the males. In *Colias philodice eurytheme* Boisduval, it seems to have a signaling effect according to Stern and Smith (1960). Repellent pheromones may be released in the species which extrude the abdomen glands during the rejection posture (Scott, 1972). Conceivable biological functions of the female scent in *L. argyrognomon* are as: (1) releaser of the fluttering-around behavior; (2) releaser of abdomen bending; and (3) releaser of attaching attempts. We do not know if, as in some species of Lepidoptera, a quantitative increase in concentration of the female sex pheromone is sufficient for the initiation of each successive step in this response sequence or if there are several active components. Behavior-releasing odors are

apparently present on the female wings as well as on the abdomen. As far as this investigation shows, the *L. argyrognomon* scents are aphrodisiacs in the sense that they prepare the opposite sex for copulation after the sexes have been brought together.

Acknowledgments—We are indebted to Prof. Torbjörn Norin and Dr. Lars Westfelt, Stockholm, for their generous gift of several sesquiterpenes and to Prof. V. Herout, Czechoslovakia, for supplying us with a sample of δ-cadinol. We thank Prof. Erik von Sydow, Göteborg, for putting a scanning electron microscope at our disposal. The microphotographs were recorded by Dr. Arne Liljemark and Mrs. Gunnel Schallin, at the Swedish Institute for Food Preservation Research. We acknowledge the skilled assistance of Mrs. Anna-Karin Borg-Karlsson in collecting and rearing. This work has been financially supported by the Swedish Natural Science Research Council and the Ekhaga Foundation.

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STUDIES ON THE ISOLATION AND BIOASSAY OF THE SEX PHEROMONE OF THE DRUGSTORE BEETLE, *Stegobium paniceum* (COLEOPTERA: ANOBIIDAE)[1]

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Abstract—Females of *Stegobium paniceum* (L.) produce a sex pheromone that causes an excitation and attraction of the males. Male response was highest 5–12 days after adult emergence. Pheromone titer of unmated females increased steadily after 1 day, reached a plateau after 5 days, and continued until at least 14 days. Mature males showed a threshold responsiveness of 3×10^{-7} μg pheromone. The pheromone titer per female ranged from 50 to 200 ng. On the basis of a high-resolution mass spectrum the empirical formula of the pure isolated pheromone molecule was determined to be $C_{13}H_{20}O_3$. The pheromone was further defined by its chromatographic behavior and spectroscopic properties.

Key Words—*Stegobium paniceum*, pheromone, sex attractant, drugstore beetle, insect behavior, stored-product insects.

[1] This study was a cooperative project between the Pesticide Research Institute, Kyoto University, Kyoto, Japan; the Stored Product and Household Insect Laboratory, ARS, USDA, and Department of Entomology, University of Wisconsin, Madison. It was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin, NSF Grant GB-36700, and the Agricultural Research Service, USDA. Mention of a proprietary product does not imply endorsement by USDA.

INTRODUCTION

The drugstore beetle, *Stegobium paniceum* (L.), is a cosmopolitan pest of warehouses, stores, houses (Mallis, 1960), and pigeons' nests (Woodroffe, 1953) and has been shown to be capable of completing its development on almost any dry organic substance (Pant and Fraenkel, 1954).

This universality of taste as well as the delitescent behavior and extremely low economic thresholds for stored-product commodities prompted us to examine the possibility of the presence of sex pheromone(s) in this insect which might be ultimately exploited in pest management as sensitive monitoring aids for low-level infestations. Such sex pheromones have recently been found in adult females of the closely related Anobiid *Lasioderma serricorne* (F.), the cigarette beetle (Burkholder, 1970; Coffelt and Burkholder, 1972). Preliminary experiments confirmed the presence of such a sex pheromone and indicated it to be present only in adult females. Barratt (1974) has recently reported the presence of a sex pheromone in females of *S. paniceum* to which only males are attracted; however, no chemical isolation or identification studies were conducted.

This paper is concerned with the extraction, isolation, purification, and bioassay of extracts of this pheromone. The complete characterization of the attractant, which appears to be of complex structure, will be the subject of a further communication. Independent programs were simultaneously established in Japan and the United States but subsequently both were amalgamated at Madison, Wisconsin.

METHODS AND MATERIALS

The insects were reared on either powdered dog food (in Kyoto) or on whole-wheat flour and brewer's yeast (19:1 wt/wt) (Madison) with a 16:8 light–dark photoperiod at $27\pm1°C$ and $60\pm10\%$ relative humidity. Both virgin females and a mixed population of males and females were used for extraction of the sex attractant. Females for extraction and males for bioassay were sexed at the pupal stage. Insect materials were stored for no more than 4 months at $-40°C$ before extraction.

Extraction

For initial bioassays males and females were extracted separately with methylene chloride. 10 females of each age were extracted with methylene chloride to determine amounts of sex attractant produced in relation to age. Batches of insects of mixed sex were extracted with methylene chloride for three independent trials of attractant isolation.

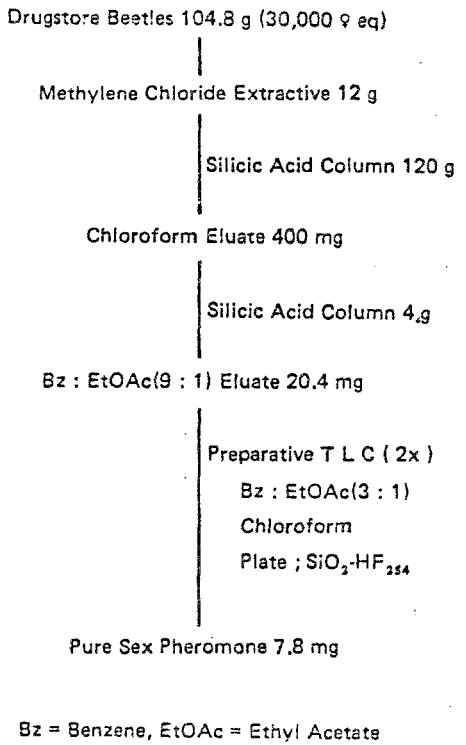

FIG. 1. Isolation procedure of the sex pheromone.

Isolation and Purification

The pure pheromone was isolated in three independent trials. Batch A was obtained from the culture in Kyoto and contained about 4 liters of insect material (856,000 adults of mixed sex). This batch was used to develop a proper purification method. Batches B and C were obtained in Madison, consisting of approximately 1216 ♀ equivalents (4.25 g) and 30,000 ♀ equivalents (104.8 g), respectively. While the efficiency and the yield of each purification trial were recorded, only data for batch C are reported. The overall purification procedure has been summarized in Figure 1.

Extracts were collected by decantation, and the residue was rinsed by vigorous shaking three successive times with 200 ml each of methylene chloride. Solvent was removed by rotary evaporation and 12 g of oily residue was obtained. This residue was then directly transferred to a silicic acid column (Mallinckrodt Chemical Works, analytical reagent 100 mesh, non-activated, 120 g, 3×30 cm), and eluted with benzene (500 ml), and chloroform (2 liter). In all cases, each 50-ml fraction was collected separately. The active substance was found in the third chloroform fraction (1000 ml chloro-

TABLE 1. RETENTION TIME OF *Stegobium paniceum* SEX PHEROMONE UNDER VARIOUS COLUMN CONDITIONS OF GAS CHROMATOGRAPH

Column and condition[a]	nC_{13}—OH	n-C_{12}—OAc	Pheromone
5% Apiezone L (75 cm, 130° C)	24.1	22.7	16.9
5% SE-30 (75 cm, 120° C)	—	8.4	8.7
5% SE-52 (180 cm, 120° C)	9.8	—	9.6
5% OV-17 (75 cm, 150° C)	6.7	6.3	11.4
30% DEGS (75 cm, 150° C)	7.4	3.7	35.3
15% PEG-20M (75 cm, 150° C)	4.4	4.2	16.7

[a] Flow rate = 30 ml helium/min.

form to 1500 ml eluate). The solvent was removed through rotary evaporation, and the residue (400 mg) was further purified on a small silicic acid column (1.2 × 8.8 cm, 4 g) by using 40 ml benzene and 100 ml of benzene–ethyl acetate mixture (9:1). 10-ml fractions were collected and bioassayed. Fractions 4 and 5 of the benzene–ethyl acetate eluate were combined, and the solvent was removed through rotary evaporation, leaving 20.4 mg of clear oil. This oily residue was then purified by thin-layer chromatography (TLC) on activated silica gel HF 254 (benzene–ethyl acetate, 3:1), followed by a second TLC on activated silica gel HF 254 using chloroform for development. The region of the second chromatogram corresponding to the active spot was scraped off and extracted with chloroform and examined on several different gas-chromatographic systems (polar and nonpolar columns). Retention times of the product were compared with those of the standard compounds (*n*-tridecanol and *n*-dodecanol acetate). Each GC system gave a clean symmetrical peak at the area coinciding with the biological activity (Table 1).

Two systems of gas–liquid chromatography (I and II) were used in this study. System I consisted of a Beckman GC-4 with a fraction collector equipped with an 8% SE-52 column on Gaschrom P (all glass 1.8 m × 3 mm ID). System II was that of a Yanaco G-80FTP with various columns (stainless steel 75 cm × 3 mm ID). Both systems were run at a helium flow rate of 30 ml/min.

The pheromone levels of females aged 1–14 days were monitored by injecting crude extract equivalent to one female directly into chromatographic system I. The pure sex attractant, which was obtained by the procedure described above, was used as the reference standard to identify the position and the amount of the attractant present in each extract.

Bioassay

The bioassay procedures of Coffelt and Burkholder (1972) utilized for *L.*

serricorne were employed in monitoring the pheromone activity. In a typical assay a 12.7-mm filter-paper disk impregnated with the test material was placed in a 1.5 dr Teflon-coated vial, and the insect response observed for 1 min. Males 6–10 days old were used to monitor the activity of female extracts. Bioassays were conducted 5–10 hr after the onset of light.

RESULTS

It was found that only males responded to female extract, and that no other materials tested produced detectable attraction of either sex. The threshold amount (i.e., the minimum amount of female pheromone needed per disk to elicit male sexual behavior) was determined as 10^{-2} μg of the crude extract, or in other words, 10^{-5} ♀ equivalents per disk as judged by the minimal positive response: (1) raising of the antennae, (2) extension of the forelegs, and (3) zig-zag locomotive movements with occasional stopping

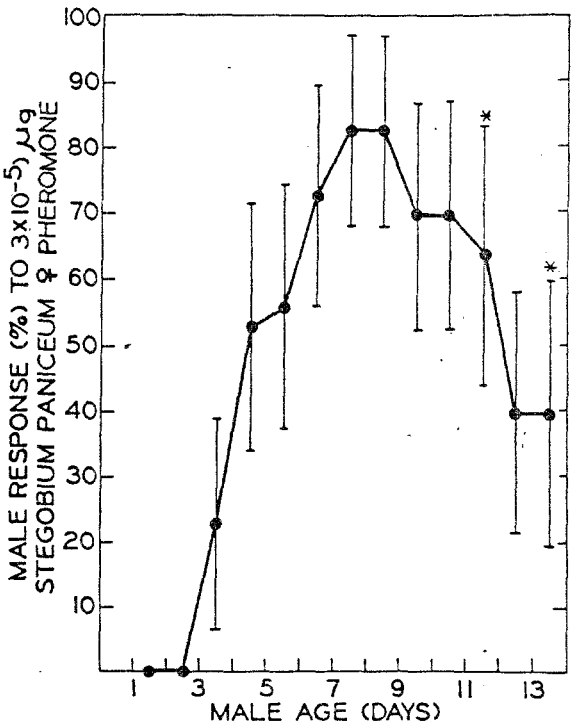

FIG. 2. Response of *Stegobium paniceum* males (aged 1–14 days) to 3×10^{-7} μg of the female pheromone. (*n = 25; no mark, n = 30; 95% confidence interval.)

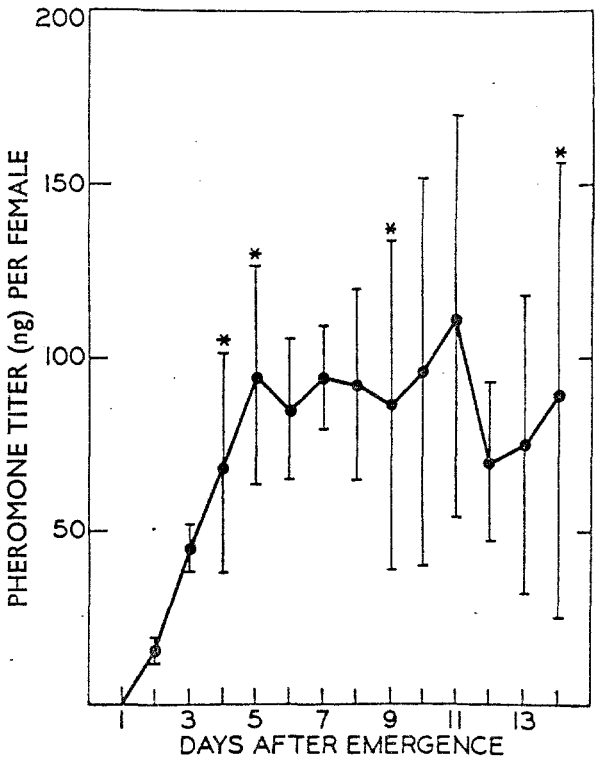

FIG. 3. Changes in the pheromone content of virgin females of different ages. Pheromone levels were determined by using a gas chromatographic system with a hydrogen-flame ionization detector. (*n = 3; no mark, n = 4; 95% confidence interval.)

just below the disk. The results shown in Figure 2 indicate that the degree of responsiveness increases for the first few days after male emergence, then starts declining after 9 days.

The amounts of sex attractant produced by females varied according to the age of the female (Figure 3). The level of pheromone production appears to reach a plateau about the fifth day after emergence.

Results of the thin-layer chromatographic analyses indicated that the activity was closely aligned with a UV-quenching spot (as detected by a mercury lamp shortwave UV detector on a silica gel HF 254) in both cases (Figure 4).

Yields of the sex pheromone and those quantities per female in each batch (A, B, and C) are summarized in Table 2. On the basis of the weight of

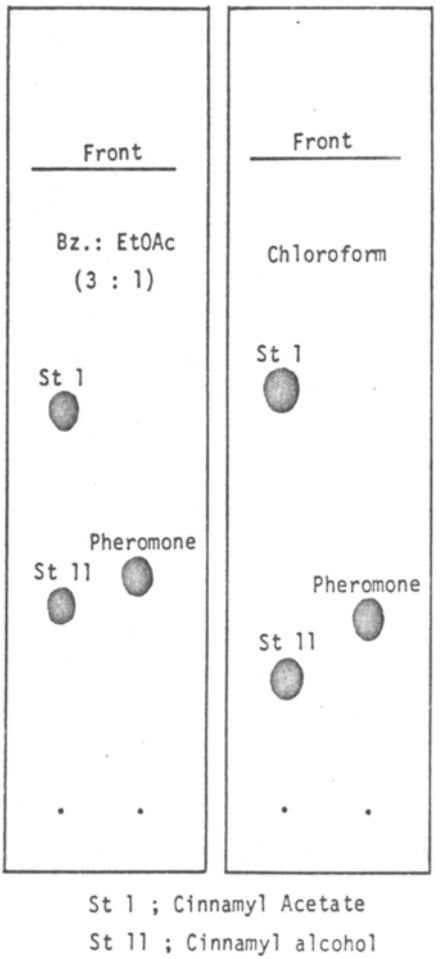

FIG. 4. Thin-layer chromatogram of the sex pheromone. The pheromone on a glass plate coated with Silica gel HF 254 gave a blue spot in yellow background under UV light.

the pure attractant, the threshold amount was determined as 3×10^{-7} μg (Table 3).

Spectroscopic Analyses

Both GC–mass and direct-probe mass spectrometric analyses gave an identical spectrum as shown in Figure 5. On the basis of a high resolution mass spectrum the empirical formula of the molecule was determined to be $C_{13}H_{20}O_3$ (molecular ion observed *m/e* 224.14125). Outstanding peaks were 168 (M—56), 113, 83, and 57 (base peak). The infrared spectrum (Figure 6) indicates the presence of two carbonyl groups without —OH absorption.

TABLE 2. YIELDS OF THE *Stegobium paniceum* SEX PHEROMONE FROM THREE BATCHES

Batch	Number of insects[a]	Origin	Weight of pheromone isolated	
			Total (mg)	Per female (ng)
A	856,000	Kyoto (Japan)	94.8	221
B	2,432	Madison (U.S.A.)	0.2	167
C	60,000	Madison (U.S.A.)	7.8	260

[a] Total of male and female insects in a 1:1 ratio.

TABLE 3. BIOLOGICAL ACTIVITY OF THE ISOLATED *Stegobium paniceum* SEX PHEROMONE AS DETERMINED BY VIAL ASSAY METHOD

Dose (μg/assay)	% Response (20 males)
1×10^{-7}	0
3×10^{-7}	10
1×10^{-6}	30
3×10^{-6}	40
1×10^{-5}	50
3×10^{-5}	70
1×10^{-4}	90
3×10^{-4}	100

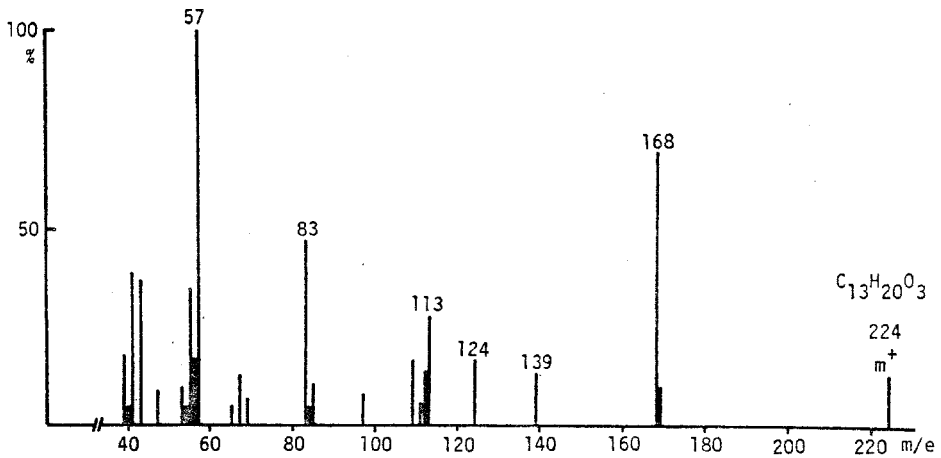

FIG. 5. Mass-spectrogram of the sex pheromone measured by a Hitachi RMS-4 at 70 eV.

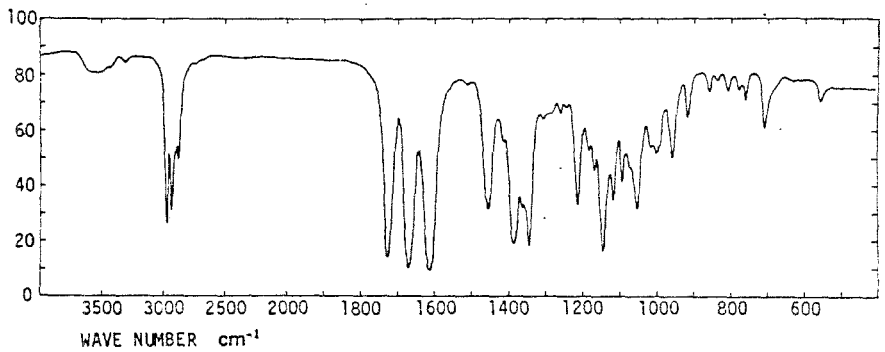

FIG. 6. Infrared spectrum of the sex pheromone measured by a Beckman IR-33 with a KBr pellet. The broad peak at 3500 cm^{-1} also appears in the blank KBr spectrum and therefore represents an artifact and not an $-OH$ signal.

The UV absorption maximum in *n*-hexane was at 266 nm with $E = 9500$ and in 95% ethanol at 272 nm with $E = 8400$. The compound is optically active, showing intense circular dichroic absorption bands at 260 nm (+), 285 nm (−), 345 nm (−), and 360 nm (+), indicating the likelihood of several chiral centers.

DISCUSSION

Owing to the complexity of the molecule, the chemical identity of the pheromone has not been established at this time. On the other hand, the substance is definable by its chromatographic and spectroscopic behavior and properties, as well as by the characteristic sexual responses produced by the males. Such responses are also observed in experiments where highly purified pheromone samples were used. There is no evidence to indicate that any other pheromones are involved in the elicitation of male sexual responses in this species.

One interesting observation is that the females of this species produce comparatively enormous quantities of the sex pheromone; indeed it was possible for us to measure directly the amounts of pheromone present per female using only a few insects per test. As much as 50–200 ng of the pheromone was present in each female. Despite such a high level of production in the female, the male sensitivity was also high. Mature males showed a threshold responsiveness (minimum dose per assay to elicit the response) at 3×10^{-7} μg/disk. In comparison, males of *Attagenus megatoma* (F.) showed threshold responsiveness at 3×10^{-4} μg/disk to their pheromone, megatomoic acid, under identical test conditions (Burkholder, 1970). Similarly the males of

Anthrenus flavipes LeConte show threshold responsiveness at 1.4×10^{-2} μg/disk (Fukui, et al., 1974). Furthermore, the females of the drugstore beetle continue the level of pheromone production for at least 14 days.

It is not clear why females of this species produce such high amounts of pheromone. It is likely, however, that such a phenomenon is closely related to their mode of life and sexual behavior, such as the distance of travelling required for the males to approach the female. Further related research appears worthwhile.

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SPECIFICITY OF THE PHEROMONE SYSTEM OF *Adoxophyes orana* AND *Clepsis spectrana*

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Abstract—The pheromone system of the summer fruit tortrix moth *Adoxophyes orana* (Fischer von Röslerstamm) consists of a mixture of *cis*-9- and *cis*-11-tetradecen-1-ol acetate (*cis*-9- and *cis*-11-TDA, respectively) in a ratio of 9:1. Substitution of one or both of these compounds by related unsaturated acetates reduced the attractancy. Only *cis*-11-TDA could be replaced by *cis*-11-tridecen-1-ol acetate (*cis*-11-TriDA), although the 9:1 mixture of *cis*-9-TDA and *cis*-11-TriDA was less attractive in the field than the pheromone system itself. The major component of the pheromone system of the leaf roller *Clepsis spectrana* (Treitschke) (*cis*-9-TDA: *cis*-11-TDA = 1:9) could also be replaced by *cis*-11-TriDA, again with some reduction in attractancy.

Key Words—insect sex attractants, pheromones, *cis*-9-tetradecen-1-ol acetate, *cis*-11-tetradecen-1-ol acetate, *cis*-11-tridecen-1-ol acetate, *Adoxophyes orana*, *Clepsis spectrana*, specificity.

INTRODUCTION

Males of the summer fruit tortrix moth *Adoxophyes orana* (Fischer von Röslerstamm) (Lepidoptera: Tortricidae) are attracted by a 9:1 mixture of two positional isomers, *cis*-9-tetradecen-1-ol acetate (*cis*-9-TDA) and *cis*-11-tetradecen-1-ol acetate (*cis*-11-TDA). These compounds form the natural pheromone system of this species (Ritter, 1971; Tamaki et al., 1971; Meijer et al., 1972). The attractancy of this mixture can be influenced negatively by many compounds, for instance by small amounts of *trans*-9- or *trans*-11-tetradecen-1-ol acetate (Minks and Voerman, 1973), some alkoxyalkan-1-ol acetates (Voerman and Minks, 1973), or some 9- or 11-alkyn-1-ol acetates

(Voerman et al., 1974). Replacing *cis*-9-TDA or *cis*-11-TDA or both by an acetate with a shorter or longer carbon chain with the double bond in position 9 or 11, resulted in mixtures that did not attract male insects in the field (Voerman et al., 1974).

Males of the leaf roller *Clepsis spectrana* (Treitschke) (Lepidoptera: Tortricidae) are also attracted by *cis*-9-TDA and *cis*-11-TDA, but the female of this species produces these two compounds in a 1:9 ratio (Minks et al., 1973, 1974).

Traps baited with a more attractive mixture than the identified pheromone system of *Adoxophyes orana* should improve their competitiveness over natural females. In the search for such a mixture we studied the specificity of the components in laboratory and field tests in more detail than we did in 1973.

We again replaced *cis*-9-TDA or *cis*-11-TDA or both by a homologous compound, now also including *cis*-11-tridecen-1-ol acetate (*cis*-11-TriDA) which was not available for our 1973 program. It was this compound that appeared interesting. Hence we studied replacement of *cis*-9-TDA or *cis*-11-TDA by *cis*-9- or *cis*-11-tridecen-1-ol acetate in the pheromone system of *Clepsis spectrana* too (field experiments only). These investigations are the subject of this paper.

METHODS AND MATERIALS

The monounsaturated acetates used in this study were prepared by known methods (Voerman et al., 1974; Schwarz and Waters, 1972), and they were ultimately purified by liquid chromatography on a silver-loaded resin (Houx et al., 1974). Purity of the compounds was checked by gas–liquid chromatography (2.1% PDEAS on Varaport-30 100/120 in a glass column 5 m × 2.5 mm ID) and estimated to be over 98%.

Electroantennogram (EAG) recordings were obtained from antennae of male *A. orana* moths by methods similar to those described by Minks et al. (1974).

Laboratory bioassays were done by observing the reactions of groups of 10 male *A. orana* moths, kept in tubes of transparent material (22 cm high, 10 cm ID), upper end closed with cloth, lower end placed on a filter paper in a petri dish. A small quantity of air was blown into the tube out of a test pipette with a small balloon. In the pipette was 50–75 μg test mixture on a strip of filter paper. After 5 sec the number of moths that showed the typical sexual excitation (wing fluttering with high frequency, agitated walking) was recorded. Tests took place in dim light (15 lux) right after the dark period.

TABLE 1. RELATIVE RESPONSES OF ANTENNAE OF MALE *A. orana* MOTHS TO ACETATES OF MONOUNSATURATED LONG-CHAIN ALCOHOLS

No.	Name	Abbreviation	Relative EAG ±S.D.[a]
1	9-decen-1-ol acetate	9-DA	—
2	*cis*-9-undecen-1-ol acetate	*cis*-9-UDA	46± 9
3	*cis*-9-dodecen-1-ol acetate	*cis*-9-DDA	76±12
4	*cis*-9-tridecen-1-ol acetate	*cis*-9-TriDA	76±12
5	*cis*-9-tetradecen-1-ol acetate	*cis*-9-TDA	100
6	*cis*-9-pentadecen-1-ol acetate	*cis*-9-PDA	57±18
7	*cis*-9-hexadecen-1-ol acetate	*cis*-9-HDA	23± 7
8	11-dodecen-1-ol acetate	11-DDA	—
9	*cis*-11-tridecen-1-ol acetate	*cis*-11-TriDA	76±16
10	*cis*-11-tetradecen-1-ol acetate	*cis*-11-TDA	58±11
11	*cis*-11-pentadecen-1-ol acetate	*cis*-11-PDA	40±15
12	*cis*-11-hexadecen-1-ol acetate	*cis*-11-HDA	29± 8

[a] Mean of 5–12 replicates; response of *cis*-9-TDA set equal to 100.

For field evaluation the same traps, adhesive material, and polyethylene caps were used as described by Voerman et al. (1974). Each cap contained 400 μg of a mixture of two compounds in a 1:9 ratio. In this way 360 μg *cis*-9-TDA was combined with 40 μg of *cis*-11-TDA homologs and 360 μg *cis*-9-TDA homologs with 40 μg *cis*-11-TDA. Binary combinations of 3 and 8, 4 and 9, 6 and 11, and 7 and 12 (360 μg of the first + 40 μg of the second) were also included (Table 1). These tests were performed in three orchards, two series per orchard.

The field trials took place during the first and second flight period of *A. orana* in 1974 in orchards in the central and southern part of the Netherlands. Controls with caps containing standard pheromone mixtures were suspended at regular intervals between the other traps.

From the field trials it was soon apparent that *cis*-11-TriDA was an interesting compound. Therefore it was tested more thoroughly in an apple orchard near Wageningen at two concentration levels with seven replications for *A. orana*. In the same orchard mixtures of *cis*-9-TDA and *cis*-11-TriDA and *cis*-9-TriDA and *cis*-11-TDA (1:9) were evaluated for attractancy for *Clepsis spectrana* with four replications. Thus there were seven blocks of 5 traps and four blocks of 3 traps. The distance between the blocks was 25 m, the interval between traps within the same block was 10 m. The number of moths captured in the traps was counted every 7 or 10 days. At the same time the traps were cleaned if necessary and rerandomized within the same block.

RESULTS AND DISCUSSION

Table 1 shows the relative response of male antennae of *A. orana* to the *cis*-9- and *cis*-11-alken-1-ol acetates (*cis*-9-TDA = 100). Each value is the mean of at least five observations. Both *cis*-9-TriDA and *cis*-11-TriDA show a response higher than *cis*-11-TDA, one of the two components of the natural pheromone system. Also the response of *cis*-9-DDA is of the same magnitude.

In the bioassay the combination of *cis*-9-TDA/*cis*-11-TriDA is as good as the natural pheromone system itself (at least with this dosage), whereas the other combinations have much lower scores (Table 2).

In the field trials only the *cis*-9-TDA/*cis*-11-TriDA (9:1) combination was attractive to male *A. orana* moths and therefore was tested at two concentration levels in one orchard, where traps with 1 living female were also hung. At the 100-μg level this combination is as good as the natural pheromone system or 1 living female. At the 500-μg level the number of captures was less than that with *cis*-9-TDA/*cis*-11-TDA, but was still significantly better than that with 1 living female (Table 3).

In the pheromone system of *Clepsis spectrana*, *cis*-11-TDA (here the major component!) can also be replaced by *cis*-11-TriDA as is shown in

TABLE 2. RELATIVE RESPONSES OF MALE *Adoxophyes* MOTHS IN LABORATORY BIOASSAY TO DIFFERENT BINARY COMBINATIONS OF *cis*-9-TDA OR *cis*-11-TDA AND ANALOGS OF *cis*-11-TDA AND *cis*-9-TDA, RESPECTIVELY

Test mixture (ratio 9:1, 50–75 μg/pipette)	Relative response[a]
cis-9-TDA/11-DDA	2
cis-9-TDA/*cis*-11-TriDA	98
cis-9-TDA/*cis*-11-TDA	100
cis-9-TDA/*cis*-11-PDA	0
cis-9-TDA/*cis*-11-HDA	0
9-DA/*cis*-11-TDA	0
cis-9-UDA/*cis*-11-TDA	9
cis-9-DDA/*cis*-11-TDA	24
cis-9-TriDA/*cis*-11-TDA	43
cis-9-PDA/*cis*-11-TDA	0
cis-9-HDA/*cis*-11-TDA	0

[a] Mean of at least 5 replicates; response to pheromone mixture set equal to 100.

TABLE 3. TOTAL CATCHES OF MALE *Adoxophyes* MOTHS WITH 7 TRAPS BAITED WITH 1 LIVING FEMALE OR WITH MIXTURES OF *cis*-9-TDA AND *cis*-11-TRIDA OR *cis*-11-TDA

Bait	Number of moths captured	
	1st flight period	2nd flight period
1 Living female	2	259
90 μg *cis*-9-TDA + 10 μg *cis*-11-TriDA	32	292
450 μg *cis*-9-TDA + 50 μg *cis*-11-TriDA	52	713
90 μg *cis*-9-TDA + 10 μg *cis*-11-TDA	62	264
450 μg *cis*-9-TDA + 50 μg *cis*-11-TDA	171	1308

TABLE 4. TOTAL CATCHES OF MALE *Clepsis spectrana* MOTHS WITH 4 TRAPS BAITED WITH 3 DIFFERENT MIXTURES

Mixture	Number of moths captured
1. 50 μg *cis*-9-TDA + 450 μg *cis*-11-TDA	87
2. 50 μg *cis*-9-TDA + 450 μg *cis*-11-TriDA	23
3. 50 μg *cis*-9-TriDA + 450 μg *cis*-11-TDA	0

Table 4 although captures decreased. Replacement of the minor component *cis*-9-TDA by *cis*-9-TriDA resulted in a totally unattractive mixture.

The figures in Tables 1, 2, and 3 prove once again that neither EAG data nor laboratory bioassay are conclusive about attractancy in the field. Especially the EAG results of this series of compounds show clearly that a compound with a less striking response (as *cis*-11-TDA) can still be an important (part of a) sex pheromone (system). Evidence for the presence of a compound in an extract of females can only be obtained by a combination of gas chromatography and electroantennography.

The specificity of *cis*-9-TDA and *cis*-11-TDA in the pheromone system of *A. orana* and *C. spectrana* is high. Replacement of *cis*-11-TDA by *cis*-11-TriDA (one carbon atom shorter than *cis*-11-TDA) results in a less attractive mixture. However, *cis*-9-TDA cannot be replaced by any of the investigated homologs.

Similar specificity was found for *cis*-7-dodecen-1-ol acetate, the sex pheromone of the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera:

Noctuidae) (Toba et al., 1970; Gaston et al., 1972). Also the attractancy of disparlure, *cis*-7,8-epoxy-2-methyloctadecane, the sex attractant of the gypsy moth *Porthetria dispar* (L.) (Lepidoptera: Lymantriidae) could not be improved by small changes in the molecule (Sarmiento et al., 1972). The geometrical isomers of *trans*-8,*trans*-10-dodecadienol, the sex attractant of the codling moth *Laspeyresia pomonella* (L.) (Lepidoptera: Tortricidae) are hardly attractive (Roelofs et al., 1972). The geometrical isomers of *trans*-10, *cis*-12-hexadecadien-1-ol, the sex attractant of the silkworm moth *Bombyx mori* (L.) have a much lower activity than the pheromone itself (Truscheit and Eiter, 1962).

Roelofs and Comeau (1970) have reported that *cis*-11-TriDA is an attractant for the male redbanded leafroller, *Argyrotaenia velutinana* (Walker), but this could not be confirmed by Klun and Robinson (1972). Most probably this was caused by a difference in purity of their test compounds (Klun et al., 1973). However, combinations of *cis*-11-TriDA and *trans*-11-TriDA or *cis*-11-TriDA and 11-tridecyn-1-ol acetate proved to be attractive to the male European corn borer moth *Ostrinia nubilalis* (Hübner) (Klun and Robinson, 1972).

Thus the sensitivity of the male antennae of Lepidoptera for chemical signals in the field is very selective. From these results it should be concluded that a compound with strong attractancy, found by (field) screening, is very likely to possess a chemical structure equal or at least similar to the natural pheromone of the attracted moths. Whether this is true or not must ultimately be proven by chemical and physical methods.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS: III. STIMULATION OF *Trichogramma achaeae*,[1] *T. pretiosum*,[1] AND *Microplitis croceipes*[2] WITH HOST-SEEKING STIMULI AT TIME OF RELEASE TO IMPROVE THEIR EFFICIENCY[3,4]

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Abstract—Frass from larvae of the corn earworm, *Heliothis zea* (Boddie) and scales from *H. zea* moths (that are known to contain the host-seeking stimulus, tricosane) stimulate and orient host-seeking activity in female *Microplitis croceipes* (Cresson), a larval parasite of *H. zea*, and *Trichogramma* spp., egg parasites of *H. zea*. When larval frass, moth scales, and tricosane were used as sign stimuli (releasers) for *M. croceipes*, *T. pretiosum* (Riley), and *T. achaeae* Nagaraji and Nagarkatti, respectively, at time of their release from laboratory containers, parasite performance improved, resulting in significantly increased rates of parasitization over that of unstimulated parasites. Stimulation of *M. croceipes* with larval frass had an overriding effect on this parasite's innate tendency to disperse upon release, thereby increasing the numbers remaining and prolonging their retention in the target area. Supplying the appropriate host-seeking stimuli to these 3 hymenopterous parasites of *H. zea* at time of their release to improve their efficiency greatly increases the probability of their effective utilization in pest management systems.

Key Words—kairomones, *Trichogramma achaeae*, *Trichogramma pretiosum*, *Microplitis croceipes*, *Heliothis* spp., host-seeking stimuli, insect behavior, releasers, pest management, biological control.

[1] Hymenoptera: Trichogrammatidae.
[2] Hymenoptera: Braconidae.
[3] In cooperation with the University of Georgia College of Agricultural Experiment Stations, Coastal Plain Station, Tifton, Georgia 31794.
[4] Mention of a proprietary product does not constitute endorsement by the USDA.

INTRODUCTION

The success of an insect-management program dependent on the field release of laboratory-reared entomophagous insects is governed by a multitude of biotic and abiotic factors, any one of which may be determinant. Of necessity, the primary initial concern is the successful establishment and retention of the parasite in the target area. In nature, chemical guidance systems—kairomones (Brown et al., 1970, Whittaker and Feeny, 1971)—emanating from the host and/or its byproducts aid the parasite in effectively orienting to its host once within the host habitat. Recent studies by Lewis et al. (1975*a*,*b*) have shown that the efficiency of released *Trichogramma* spp. was significantly improved by the application of the host-seeking stimulus, tricosane, in target areas. However, the probability of field-released parasites encountering these host-seeking stimuli, either natural or introduced, is left to chance. These parasites may just as readily follow their primary innate response, upon release, to escape and disperse before settling into a normal behavioral pattern. Oriented movement may then again predominate at indeterminant distances from the release site.

During 1973 and 1974, kairomones were evaluated as sign stimuli (releasers)[5] at time of parasite release to determine whether the egg parasites, *T. achaeae* and *T. pretiosum* (Riley), and the larval parasite, *Microplitis croceipes* (Cresson), could be stabilized and oriented in a host-seeking pattern, thus increasing their frequency of retention in the target area and thereby improving their overall efficiency.

METHODS AND MATERIALS

Microplitis croceipes

Greenhouse Tests. In tests conducted on 0.75-m-high cement tables within a 7.6 × 7.6-m greenhouse section, the bottom halves of 10-cm petri dishes were placed atop 3 overturned 15.2-cm-high plastic planter pots arranged in a triangular design with ca. 0.5 m separation between pots. Each petri dish contained one 3rd- or 4th-instar larvae of the corn earworm, *Heliothis zea* (Boddie) obtained from the Tifton, Georgia, laboratory colony. The larva was mounted on a single crowder pea leaf with a minuten, nadeln pin sticking from a 3 × 6 × 10-mm cork beneath the leaf. The pin passed through the larva in one of the last 3 abdominal segments entering from the ventral surface, thus retaining the larva on the leaf. Also, newly deposited frass from *H. zea* larvae was spotted on the leaf to aid the searching parasite in locating the

[5] Sign stimuli (releasers) are stimuli that evoke a particular instinctive behavior or so-called fixed-action pattern, a sequence of coordinated motor actions that appear without the animal having to learn it by the usual learning process (Hess, 1965).

host, since frass elicits a host-seeking response by *M. croceipes* (Lewis and Jones, 1971). Laboratory-reared *M. croceipes* were collected in 2-dr vials from emergence cages (Lewis and Burton, 1970) when 3–6 days old. Prior to their release, fresh frass from larvae that has fed the previous 24 hr on crowder pea foliage was smeared over the entire lip of the release vial to ensure contact by the parasite upon emerging. One such vial containing a single parasite was then placed upright, at ground level, equidistant between the 3 pots. The parasites were allowed 30 min to locate larvae, after which larvae were collected and dissected to determine percentage parasitization (Jones and Lewis, 1971). The test was replicated 5 times on each of 7 days. Temperatures within the greenhouse ranged from 25.0°C to 28.9°C during the studies.

In other tests, *M. croceipes* were exposed as above to 13-methylhentriacontane (1 mg/ml hexane), an identified host-seeking stimulus of this parasite (Jones et al., 1971).

Field Tests. The stability of stimulated and unstimulated *M. croceipes* was observed under field conditions by releasing them onto a potted crowder pea plant ca. 25 cm high. Again frass from *H. zea* larvae that had developed on crowder pea foliage was smeared on the lip of the 2-dr release vial. Unstimulated parasites were released from untreated vials. Each parasite was permitted to crawl from the vial to the potted crowder pea plant. Stability of the parasites was then measured by their retention and antennal examination of the pea plant vs. their dispersion without initiating search.

Trichogramma achaeae

Laboratory Tests. Effects of stimulating the egg parasite, *T. achaeae*, with the host-seeking stimulus, tricosane, before their release was measured in 14-cm petri dishes. Laboratory and field bioassays have shown tricosane (a biochemical isolated from moth scales of *H. zea*) to elicit orientation and stimulate parasitism by *T. evanescens* (Jones et al., 1973). Eggs of *H. zea*, 1–2 days old, collected from the Tifton laboratory culture were arranged in 2 intersecting rows of 4 eggs/dish. Then 2 female *Trichogramma* spp., cultured as described by Lewis and Redlinger (1969), with the substitution of *H. zea* eggs for those of *Cadra cautella*, were collected from emergence tubes into a 2-dr shell vial and transferred to a release vial. The inner surface of the release vial had been treated with ca. 1/8 ml tricosane (1 mg/100 ml hexane) and allowed to dry. After the parasites had been confined for 5 min on the treated surface, they were released into petri dishes containing the eggs. Unstimulated parasites were released from untreated vials. Parasites were permitted 40 min to 1.25 hr to parasitize eggs. Eggs were then removed and dissected (Lewis and Redlinger, 1969) to determine percentage parasitization. Paired comparison (30 replications) were evaluated on 6 days.

Field Tests. The effect of stimulation with tricosane (1 mg/100 ml hexane) at time of release was evaluated on *T. achaeae* in field plots of early to late green tassel stage White Cross Bantam sweet corn 18 rows wide by 30.4 m long. Nine sites, 1.8 m long (3 lines equidistant from one another at 3 locations) were marked with plastic nursery tags in each plot. Then at each site, 20 *H. zea* eggs were attached to the upper leaf surfaces at the top one third of the corn plants (ca. 1.5 m high) with a saliva-moistened camel's hair brush. Circular sections of ca. 100 eggs of *H. zea* containing developing *T. achaeae* (av. 2/egg) were individually clipped from oviposition cards, placed into 8-dr plastic cups and held until emergence at 26.7 ± 1°C. (A raisin was placed in each cup to provide food and moisture before release.) At 6 sites (2 lines of 3 cups each, equidistant between the center and sides of the plot) cups were opened and placed within 48-dr screw-cap jars which had been sprayed to runoff (ca. 5 ml) with tricosane and allowed to dry. Parasites were retained ca. 5 min, then the jars were opened, allowing the parasites free movement. Unstimulated parasites were released from untreated jars. Eggs were collected within 4–5 hr after parasite release, returned to the laboratory, and dissected to determine percentage parasitization. Paired comparisons of stimulated vs. unstimulated parasites were made on each of 7 days.

In addition, an influx of *Heliothis* spp. moths into cotton near Tifton, Georgia, during mid-August, 1974, permitted us to measure the response of *T. pretiosum* to naturally oviposited eggs, after the parasites had been stimulated with moth scales of *H. zea*. Paired comparisons of stimulated vs. unstimulated *T. pretiosum* were made in 21 plots 8 rows wide by 6.1 m long as follows: 25 eggs of *H. zea* parasitized by *T. pretiosum* were placed in 16-dr plastic cups 1 day before the scheduled emergence of the parasites. A paper cap (1 mm thick) containing a 6-mm hole to permit parasite emergence was brushed with moth scales of *H. zea* on the inner surface to ensure contact by the parasite as it emerged. Unstimulated parasites were handled identically except for the absence of moth scales. 16 such 16-dr cups containing ca. 800 total parasites were placed in each plot (2 on each of 8 rows) at the base of the cotton plant. Examination of a random sample of the cups 24 hr later revealed that 95+% of the emerging parasites had left. Efforts were made the following day to collect 15 eggs/plot from the plant terminals. Eggs were taken into the laboratory and dissected to determine percentage parasitization. Separation of means in all studies was done with the Student's *t* test.

RESULTS AND DISCUSSION

Microplitis croceipes

Greenhouse Tests. Because of their innate tendency to disperse upon release, *M. croceipes* are difficult to use in experimental studies. Being

positively phototactic, they usually move upward and away from the release site. Unstimulated parasites in the greenhouse study responded accordingly, by moving upon release to the ceiling of the greenhouse where they remained without initiating search. Parasites stimulated via antennal contact with the larval frass as they exited from the vial established a host-seeking pattern and generally had little difficulty locating the dishes containing larvae despite the dissimilarity between the test area and their natural habitat. These observations therefore led us to believe that location of larvae by the parasites established within the host habitat is dependent primarily on chemical stimuli, and little, if at all, on visual perception of habitat conformation. Rates of parasitization by female *M. croceipes* stimulated at the time of release were significantly higher, 1% level of probability, than that of unstimulated parasites (Table 1). However, on each test day a higher incidence of attempted parasitization was observed than the dissections revealed and the tabular data indicate. This discrepancy, we feel, was primarily the result of misses by the parasite and the loss of larval hemolymph (and probably parasite eggs) when larvae were removed from the pins.

Attempts to stabilize *M. croceipes* with 13-methylhentriacontane were unsuccessful. Parasites were stimulated by antennal contact with this biochemical (Lewis and Jones, 1971), but they remained in the area only a short time, and no oriented flight in search of additional sources of the stimulus was observed. There appear to be components in addition to the 13-methylhentriacontane, some perhaps plant derived, which together produce the oriented searching pattern observed by stimulation with larval frass.

Field Tests. Sixteen stimulated *M. croceipes* remained and searched on potted crowder pea plants; only 1 dispersed upon release. Of the unstimulated parasites, 21 dispersed while one remained and searched. As in the greenhouse test, the stimulated parasites had no tendency to disperse; instead they performed extensive antennal examinations of the stem and leaf surfaces of

TABLE 1. COMPARATIVE PARASITIZATION OF LARVAE OF *H. zea* BY FRASS-STIMULATED AND UNSTIMULATED *M. croceipes* RELEASED IN THE GREENHOUSE

	% Larvae parasitized on indicated days[a]							
	1	2	3	4	5	6	7	Mean[b]
Stimulated	33.3	33.3	13.3	40.0	13.3	33.3	26.6	27.6
Unstimulated	0	0	0	0	0	0	0	0

[a] 15 total larvae available for parasitization on each day.
[b] Means significantly different at the 0.01 level of probability.

the plants. Only after parasites searched for several minutes without receiving additional reinforcing stimuli did they disperse.

Trichogramma achaeae

Laboratory Tests. Parasitization of *H. zea* eggs by stimulated *T. achaeae* was significantly higher, 1% level of probability, than by unstimulated parasites when evaluated in petri dishes (Table 2). It is now apparent that stimulation of *T. achaeae* with tricosane before release activates the host-seeking behavior, thus increasing the frequency and/or intensity of search for an undetermined period. We suspect, however, that oviposition may in itself act as an independent reinforcing stimulus for host seeking.

Field Tests. Observations of *T. achaeae* upon release indicated that they apparently do not have an initial tendency to unoriented dispersal but rather move short distances on the plant in search of host eggs. Nevertheless, *T. achaeae* that were stimulated with tricosane upon release produced signifi-

TABLE 2. COMPARATIVE PARASITIZATION OF EGGS OF *H. zea* BY TRICOSANE-STIMULATED AND UNSTIMULATED *T. achaeae* RELEASED IN PETRI DISHES

	% Eggs parasitized on indicated days						
	1	2	3	4	5	6[a]	Mean[b]
Stimulated	29.8	32.4	28.4	14.6	19.7	32.2	26.2
Unstimulated	14.4	15.0	18.0	18.8	5.8	14.4	14.4

[a] 20 paired comparisons on day 6.
[b] Means are significantly different at the 0.01 level of probability.

TABLE 3. COMPARATIVE PARASITIZATION OF EGGS OF *H. zea* BY TRICOSANE-STIMULATED AND UNSTIMULATED *T. achaeae* RELEASED IN FIELD PLOTS OF TASSEL-STAGE SWEET CORN

	% Eggs parasitized on indicated days							
	1	2	3	4	5	6	7	Mean[a]
Stimulated	53.8	37.6	60.3	10.0	4.3	3.6	22.1	27.4
Unstimulated	48.5	21.4	42.3	7.4	2.4	4.7	14.8	20.2

[a] Means are significantly different at the 0.01 level of probability.

cantly higher rates of parasitization in the field than did unstimulated parasites (Table 3). Instances of apparent lack of response are shown in Table 2 for day 4 and in Table 3 for day 6. However, an individual statistical analysis of data for both days indicated that the reversals were not significant. Age of *T. achaeae*, which was not standardized during the study, may eventually prove to be crucial to a favorable response by stimulated *Trichogramma*.

Data are not yet available to determine how much of the higher parasitization by stimulated *Trichogramma* is attributable to retention of larger numbers of parasites in the target area for longer periods of time and how much is caused by a more oriented and intensified search by the parasites. Our petri-dish studies and those of Lewis et al. (1975*b*) demonstrate that at least part of the increased parasitization is the result of a more efficient search.

Lewis et al. (1975*a*) demonstrated the ability of field-applied tricosane to increase rates of parasitization by *Trichogramma* spp. The data in the present text showed that tricosane stimulation of *T. achaeae* at release significantly improves their efficiency. We suspect that the effect of stimulating parasites before release is additive to the effect of field-applied kairomones, and produces additional retention of *Trichogramma* in the target area.

T. pretiosum stimulated with moth scales of *H. zea* at time of release likewise responded positively, producing higher rates of parasitization (30.5%) on naturally oviposited *Heliothis* spp. eggs than did parasites that were not stimulated (21.0%). Separation of means with the Student's *t* test indicated significance at the 0.02 level of probability.

CONCLUSIONS

An oriented host-seeking behavior is evoked in *M. croceipes*, *T. achaeae*, and *T. pretiosum* when they are stimulated at the time of release from containers by exposure to their respective host-seeking stimuli. Frass of *H. zea*, a stimulus for *M. croceipes*, prolongs the retention of the parasite in the target area, thereby improving their overall efficiency. *T. achaeae* stimulated with tricosane at time of release produced significantly higher rates of parasitization than unstimulated parasites in both petri-dish bioassays and in field plots of whorl-stage sweet corn. *T. pretiosum* stimulated with moth scales of *H. zea* at release produced significantly higher rates of parasitization on naturally occurring eggs of *Heliothis* spp. in cotton than did unstimulated parasites.

The demonstrated principle of utilizing kairomones as releasers to activate host-seeking behavior before parasite release has far-reaching implications for future parasite release programs.

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EVOLUTIONARY CONSEQUENCES OF EATING: *Trichosurus vulpecula* (MARSUPIALIA) AND THE GENUS *Eucalyptus*

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Abstract—While being somewhat specialized on a diet of mature *Eucalyptus* leaves (66% of feeding time), wild *Trichosurus vulpecula* consume an average of three different foods per night. Usually, these foods are two different species of *Eucalyptus* leaves, and "ground feeding." Laboratory feeding experiments indicate that this ingestion of a variety of foods is due to severe limitations on the quantity of *Eucalyptus* leaves a possum is capable of consuming. It is argued that the limitation is due to plant toxins (volatile oils, phenols) present in *Eucalyptus* leaves, rather than to "normal" nutritional factors. We hypothesize that *Eucalyptus* toxins indirectly regulate possum populations at levels that afford the *Eucalyptus* trees some degree of protection from possum predation. In addition, we suggest that the "New Zealand phenomenon" can be explained by a lack, and acquisition, of plant chemical defenses against herbivores introduced from chemically more complex environments.

Key Words—*Trichosurus vulpecula*, *Eucalyptus*, herbivore, plant chemical defenses, feeding behavior, detoxification, population regulation.

INTRODUCTION

There is a growing realization that animals do not perceive the world as green, but as a kaleidoscope of potentially deleterious chemical compounds. Freeland and Janzen (1974) attempted to demonstrate the importance of this

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difference in perception and formulated a series of hypotheses as to how a generalist herbivore can be expected to behave in order to overcome plant chemical defenses. In this paper we use an arboreal leaf-eating marsupial, *Trichosurus vulpecula*, in order to examine the hypotheses presented by Freeland and Janzen (1974). These hypotheses are (1) that a generalist herbivore has to ingest several different plant foods in order to meet its energy requirements, (2) that large amounts of a single plant food are not eaten when a generalist herbivore first encounters it, the animal initially taking small samples in preference to eating a large meal, (3) that the amount of a single plant eaten can be gradually increased as the animal gains "experience" with it, probably because the animal induces enzymes to detoxify the food, and (4) that foods lacking toxic secondary compounds are recognized quickly, larger amounts of them being eaten than can be eaten of single toxic foods, or even a restricted range of toxic foods. In addition, we hypothesize a mechanism whereby the feeding behaviors enforced by the evolution of plant chemical defenses can lead to the maintenance of predator populations at levels where the major prey item is not exterminated.

Natural History

The brush-tailed possum (*Trichosurus vulpecula*) is a solitary, nocturnal, arboreal marsupial weighing 1.5–2.5 kg (Dunnet, 1956). It is found over much of the forested areas of Australia, its natural habitat being grassy and shrubby *Eucalyptus* forests (Troughton, 1951). Populations reach densities of 2.2 possums per hectare and exhibit little or no fluctuations in size within a given year or from one year to the next (Winter, personal observation). There are usually two breeding seasons per year although a female will often not breed during the second, minor breeding season (Dunnet, 1964; Smith *et al.*, 1969). A single young is produced. When the young is about 9 months of age the mother drives it away (Winter, personal observation). Most young do not manage to establish a home range. This age class is subject to considerable mortality (Dunnet, 1964). If a young animal does establish a home range, it may remain there for up to 6 or more years (MacLean, 1967). *T. vulpecula* is a leaf eater (Gilmore 1967; Mason 1958), and in Australia the majority of its diet is mature *Eucalyptus* foliage.

Mature *Eucalyptus* leaves contain large concentrations of volatile oils and phenols (Baker and Smith, 1920; Penfold and Willis, 1961). *T. vulpecula* lacks the type of complex stomach (Tyndale-Biscoe, 1973) that would be expected to maintain a bacterial flora capable of degrading plant secondary compounds prior to their reaching the absorptive surfaces of the small intestine. Brush-tailed possums must rely on other means to protect themselves from the toxic effects of *Eucalyptus* secondary compounds. Hinks (1956) and

Hinks and Bolliger (1957*a*,*b*) found that *T. vulpecula* fed *Eucalyptus* leaves produced large concentrations of glucuronides in the urine. When fed non-toxic, artificial diets (domesticated vegetables) possums produced only small amounts of glucuronides in the urine. Animal microsomal enzymes degrade toxic plant secondary compounds via a two-phase reaction (Williams, 1959). The second of these phases involves the conjugation of the modified toxin with glucose or some other molecule such as an amino acid or sulfate group. These glucuronides are excreted in the urine. From this it would appear that microsomal enzymes of *T. vulpecula* protect it from the potentially toxic effects of its food.

Habitat

The study area was near Brisbane, Queensland (latitude; 27°33′ S; longitude, 152°56′ E), in a slightly disturbed open forest. The forest originally had a shrubby understory. Selective tree felling and the grazing of cattle have converted it into an open grassy forest. Most of the trees in the forest are in the genus *Eucalyptus* (*E. tesselaris*, *E. territicornis*, *E. crebra*, *E. hemiphloia*, and *E. maculatus*). Other common species include *Angophora subvelutina* and *Tristania suaveoleus* (Myrtaceae), *Acacia alocarpa* (Leguminosae), and several species of rain-forest tree (including *Ficus* sp.) along a nearby river. The species and location of all trees were mapped with a plane table and each tree was individually marked.

METHODS AND MATERIALS

Feeding Behavior in the Wild

During a study of *T. vulpecula* social organization (Winter, in preparation), observations on 1 of 6 possums (3 males and 3 females) were made 1 night a week for the 12 months December, 1965, to December, 1966. During any one night a single possum was followed with the aid of a spotlight and binoculars. The possums had been individually marked, and the location of each individual's den tree was known. A night's observation began by waiting for the possum to emerge from its den. The possum was followed all night until it retired in the early morning. During observation the location of a possum (on the ground, in a particular tree, etc.) and what its behavior was (feeding on a particular species of plant, traveling, grooming, resting, etc.) were recorded at least every 5–6 min. Records were taken more frequently when behavioral changes occurred. For the purpose of analysis an hour was divided into tenths. Any behavior recorded in a particular tenth was taken to have been in progress for the whole of that tenth.

Individual possums differed in their response to the light. Extremely shy possums could be followed only at a distance of 50–60 m, and the light frequently had to be dimmed with orange cellophane paper, a diaphragm, or the light was not shone directly on the possum. Most individuals rapidly became accustomed to the light and could be followed at distances much shorter than 50–60 m. Nights when a possum retreated to its den in response to the light, spent long periods of time (e.g., 1 hr) staring back at the light, or was mislaid for more than an hour, were eliminated from the sample. This left a total of 36 nights of observation: 7 nights with Gus, 5 with Alec, 5 with Jack, 6 with Gert, 6 with Jill, and 7 with Alice.

Cage Feeding Experiments

Feeding experiments were designed to determine: (1) how the amount of *Eucalyptus* leaves eaten differed between when an individual was fed a single species of *Eucalyptus*, and when it was provided with three *Eucalyptus* species; and (2) how much artificial food (low toxic plant secondary compound content) would be consumed by possums after having been fed nothing but *Eucalyptus* for varying periods of time, and how this compared to the amount of *Eucalyptus* that would be consumed after similar periods of time feeding on nothing but *Eucalyptus*.

The species of *Eucalyptus* leaves chosen for the experiments were those known to be "preferred" by free-living *T. vulpecula*. Where possible, leaves were removed from individual trees known to be fed on by possums. To ensure that all individuals would eat while in captivity, possums were kept on an artificial diet for 5 days prior to beginning the experiments.

Experiment 1. Two possums were fed *E. crebra* leaves (and nothing else) for a period of 5 days. Leaves were replaced daily and the net weight of the leaves placed in the cages was recorded. When the remaining leaves were removed 24 hr later, they were oven-dried at 60–70°C. In no case did a possum eat all the leaves provided. Control bunches of leaves were oven-dried each day. This provided a conversion factor for determining the dry weight of leaves eaten by individual possums per 24 hr. After 5 days on the *E. crebra* diet, the possums were given access to three species of *Eucalyptus*: *E. crebra*, *E. territicornis*, and *E. hemiphloia*. Determinations of the dry weight consumption per day were made for each leaf species. This was continued for 5 days. Two similar experiments were carried out, differing only in the species of *Eucalyptus* fed to possums during the first 5 days. Two possums received *E. hemiphloia* and two others received *E. territicornis*.

Experiment 2. Individual possums were fed *Eucalyptus* leaves for varying periods of time, and then placed for 5 days on an artificial unnatural diet of white bread, pumpkin, and apples. These foods are not usually regarded as

containing large concentrations of toxic plant secondary compounds. One possum was kept on *Eucalyptus* leaves for 8 days, one for 5 days, two for 4 days, and three for 2 days. Records were taken of the amounts of unnatural foods consumed.

RESULTS

Field Observations

Observations throughout the study revealed that possums eat a wide variety of food types (Table 1). About half of these different food types were consumed on only one or two occasions. The bulk of the diet was made up of mature *Eucalyptus* leaves supplemented with seasonally available foods such as flowers and fruit of the mistletoe *Amyema miquelii*. This predominance of *Eucalyptus* in the diet is reflected in the results of the 36 nights of following individual possums. Feeding on mature *Eucalyptus* leaves made up 66% of the total observed feeding time. Of this, more than three quarters is accounted for by feeding on *E. territicornis* and *E. hemiphloia* (Table 2).

The next most important type of feeding behavior is ground feeding. This took place on 35 of the 36 nights and accounted for 23% of the total feeding time. Ground feeding involves the consumption of a variety of foods, few of which could be accurately identified. Not all possums had access to cattle-feeding troughs. Because of this inequality, and as feeding from troughs accounted for only 3% of the total feeding time, it is included as ground feeding.

Of the remaining 11% of the feeding time, 5% was spent feeding on the leaves, flowers, etc., of four non-*Eucalyptus* tree species, and 6% was spent eating flowers and fruit of mistletoe (Table 2).

Over the 36 nights of observation an average of three different foods were consumed per night. Two types of food were eaten on 7 nights, three foods on 18 nights, four foods on 7 nights, and five foods on 4 nights. As ground food was consumed on 35 of the 36 nights, this estimate of number of foods per night is conservative since ground food is many different things.

At least one species of *Eucalyptus* was eaten each night. If three foods were eaten, the food additional to one *Eucalyptus* and ground food was usually a second species of *Eucalyptus*. Only once were all three foods *Eucalyptus*. If four foods were taken, they were ground food, two species of *Eucalyptus*, and either an additional species of *Eucalyptus* or one of the non-*Eucalyptus* foods listed in Table 2. If five foods were eaten, the fifth food was usually non-*Eucalyptus*. When two foods were eaten, one was *Eucalyptus*, and the other ground food.

We have been forced to use the amount of time spent feeding on particular foods as an estimate of feeding. An amount of time spent eating leaves is

TABLE 1. TYPES OF FOODS OBSERVED TO BE EATEN BY *Trichosurus vulpecula* DURING THE COURSE OF FIELD WORK

Food items	Frequency of eating[a]
Eucalyptus territicornis (mature leaves, buds, young capsules)	Frequent, nonseasonal
E. hemiphloia (mature leaves, blossoms, buds, young capsules)	Frequent, nonseasonal
E. crebra	
Mature leaves	Frequent, nonseasonal
Young leaves	Rare
E. tesselaris (leaves)	Occasional
E. maculata (leaves)	Occasional
Amyema miquelii (a mistletoe) (flowers, buds)	Frequent, seasonal
Euroschinus falcatus (berries)	Seasonal
Angophora subvelutina (mainly young leaves, blossoms)	Frequent, seasonal
Tristania suaveoleus (leaves)	Occasional
Ficus sp. (leaves)	Rare
Ipomea cairica (a vine) (leaves)	Two observations
Mallotus philippinensis (leaves or fruit)	One observation
Acacia alocacarpa (leaves)	Occasional
Lantana sp. (flowers)	One observation
Hawk moth larva	One observation
Cicada exuvium	One observation
Insect gall on branch of *E. maculata*	One observation
Agaric fungus (ground)	One observation
Low vegetation (ground)	
Grasses (assorted species)	Probably
Coranopus didymus (leaves)	Probably
Solanum nigrum (leaves)	Probably
Aphananthe philippinensis	Probably
Passiflora suberosa	May have killed male 6228
Available through man's activities (ground)	
Cattle feed (lucerne and millet mixture)	Frequent
Bean sprouts	One observation
Sprouting grain in cow pat	One observation
Dried peas	One observation

[a] Ingestion of each food type is indicated in a qualitative way.

TABLE 2. AMOUNT OF TIME SPENT FEEDING ON PARTICULAR FOODS DURING 36 NIGHTS OF OBSERVATION OF INDIVIDUAL POSSUMS

Food item	No. of nights eaten (36 max.)	Average time per night	Total time spent eating	Percent of total feeding
Eucalyptus territicornis	31	1.28	39.6	
E. hemiphloia	12	1.54	18.5	
E. crebra	16	0.58	9.2	66%
E. tesselaris	3	0.57	1.7	
E. maculata	1	0.50	0.5	
Angophora subvelutina	4	0.58	2.3	
Tristania suaveoleus	3	0.63	1.9	
Ficus sp.	2	0.30	0.6	5%
Euroschinus falcatus	1	0.90	0.9	
Amyema miquelii	8	0.81	6.5	6%
Ground feeding	35	0.70	24.5	23%

not comparable to a similar amount of time spent on ground feeding. For a possum, feeding on *Eucalyptus* leaves is simply a matter of ingesting leaves. There is little or no search time involved. Ground feeding involves a larger search component. Possums feeding on the ground continually moved from place to place, ingesting small amounts of food at a time. For example, possums were observed to feed on grass. However, they selected young growth rather than dry, rank grass. Tender young grass was scarce during most of the

TABLE 3. PROPORTION OF FOOD EATEN OVER 10-DAY EXPERIMENTAL PERIOD THAT WAS EATEN DURING THE FIRST AND SECOND SET OF FIVE DAYS

Food for first 5 days	Possum	Percentage of total food eaten over the full 10 days: First 5 days (one *Eucalyptus* sp.)	Second 5 days (three *Eucalyptus* spp.)
Eucalyptus crebra	1	31.65	68.35
	2	28.65	71.35
E. territicornis	3	31.53	68.46
	4	7.48	92.52
E. hemiphloia	5	25.98	74.01
	6	38.31	61.70

year, and possums had to search for it. In terms of quantity of food ingested per unit time, leaf feeding was far more profitable.

Feeding Experiments

Experiment 1. The amount of food ingested when possums were fed one species of *Eucalyptus* for 5 days was consistently smaller than the amount ingested when the same possums were provided with three species of *Eucalyptus* for 5 days (Table 3). During the first 5 days the amount ingested per day

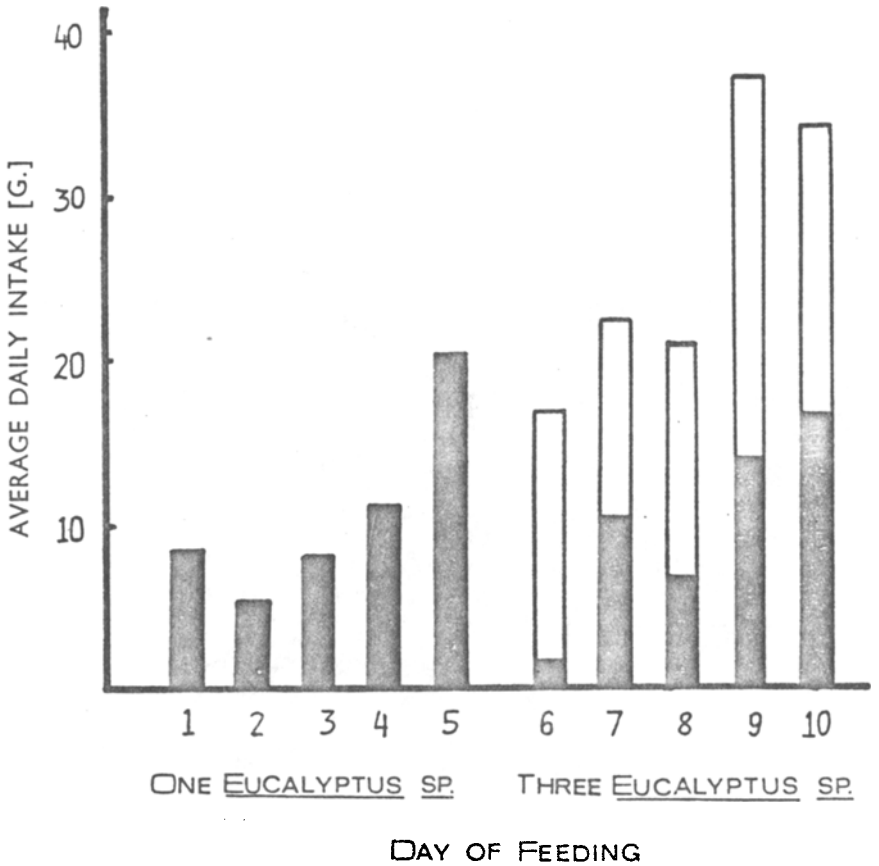

FIG. 1. Average dry weight of leaves eaten per day of the experiment. Consumption of the species provided during the first 5 days of the experiment is indicated by black shading; consumption of the two additional species (provided during the second 5 days) is indicated by the unshaded area.

increased such that the average amount consumed on the fifth day was approximately twice the average consumed on the first day (Figure 1). When possums were given three species of *Eucalyptus*, there was a similar pattern of ingestion. The average amount consumed on day 10 was approximately twice that eaten on day 6 (Figure 1).

Increasing ingestion during the second 5 days was not due to continued increases in consumption of the *Eucalyptus* species eaten during the first 5 days. Rather, it was associated with a form of sampling behavior. Small quantities of two species were eaten on the sixth night, and there was a sampling of a different combination of two species on the seventh night. Not until the last two nights of the experiment did most individuals eat all three species over a 24-hr period. Possums ate more food when feeding on all three species of *Eucalyptus* than they did on any other night. The increase in ingestion during the second 5 days was due to an increase in the number of species eaten in a day, and a gradual increase in the amount of each species eaten.

The average of the maximum amounts each possum ate in any one day when provided with one *Eucalyptus* species is approximately half that of a similar average from when possums were fed three species (Table 4). These average maximum dry weight consumptions per day were converted into kilocalories using a value of 5.4 kcal/g dry weight of leaves. This value is taken from Marples' (1973) data on the caloric content of food in the stomachs of *Schoinobates volans* (the stomachs contained nothing other than *Eucalyptus* leaves, flower buds, and a little bark). Assuming a 60% digestive extraction of the food's energy content, a diet of one *Eucalyptus* species does not provide enough energy to maintain the standard metabolic rate of a 2.19-kg possum (the average weight of the possums used in the experiments). A diet of three *Eucalyptus* species provides for the standard metabolic weight

TABLE 4. ESTIMATION OF CALORIC VALUES OF THE AMOUNT OF FOOD INGESTED AND ASSIMILATED BY 2.19 KG POSSUM FED DIETS OF ONE AND THREE SPECIES OF *Eucalyptus*

	Average maximum dry weight eaten in 1 day	Caloric value (5.4 kcal/g)	Caloric value of assimilated food (60%)	Energy to maintain SMR for 1 day
Possums fed one *Eucalyptus* sp.	21.96 g	118.6 kcal	71.16 kcal	122.29 kcal
Possums fed three *Eucalyptus* spp.	43.63 g	235.6 kcal	141.37 kcal	

(Table 4). A 60% digestive assimilation is simply a rather generous estimation of what could be expected for a nonruminating mammal of this size (Golley, 1967). Standard metabolic rate is taken from Dawson and Hulbert's (1970) data on *T. vulpecula*. If the daily energy requirement is increased by 20% to allow for specific dynamic action, movement, etc. (increased to 146.75 kcal/day), a diet of three *Eucalyptus* species may be just sufficient. A 20% increase in energy requirement is probably too small, the 60% energy assimilation far too generous, and to this must be added the cost of metabolizing the leaf toxins and providing glucose molecules to form glucuronides with the metabolized toxins. If a possum can balance its energy budget on a diet of nothing other than three species of *Eucalyptus*, it is only just doing so.

Experiment 2. When possums were fed nothing other than one species of *Eucalyptus* for periods of 2, 4, and 5 days, and then switched to a diet of apples, white bread, and pumpkin (less toxic artificial diet) for 5 days, the average consumption was 1 apple, 2½ slices of bread, and 170 g wet weight of pumpkin per day. This is equivalent to 74.3 g dry weight of food per day. A similar rate of consumption of artificial food was recorded for a possum following a diet of one *Eucalyptus* for 5 days, and then three species of *Eucalyptus* for 3 days (8 days on a *Eucalyptus* diet).

Consumption of 74.8 g dry weight of nontoxic food per day is much greater than the average maximum consumption of possums given three species of *Eucalyptus* (Table 4). The total caloric content of the daily nontoxic food intake was 302.27 kcal. Caloric values for the artificial foods are taken from Altman and Dittmer (1968). A 60% digestive assimilation would provide a possum with 181.36 kcal/day when feeding on the artificial foods. This is greater than the energy assimilated from three species of *Eucalyptus* leaves (Table 4). It is also greater than the 146.37 kcal hypothesized as the daily energy requirement.

DISCUSSION

During the first 5 days of the feeding experiments individual possums did not ingest enough *Eucalyptus* leaves to meet normal body maintenance requirements. When provided with three *Eucalyptus* species, the possums may have ingested enough to meet these requirements. This limitation in the amount of food eaten was not observed when possums were given artificial foods. These observations can be interpreted as being the result of two mutually exclusive physiological phenomena. *Eucalyptus* leaves of all three species could be nutritionally lacking or imbalanced in some way, so limiting the quantity of leaves ingested. On the other hand, the quantity of leaves ingested could be limited by the rate at which possums are capable of detoxify-

ing *Eucalyptus* secondary compounds. The less restricted consumption of artificial foods could be the result of these foods being less nutritionally lacking, or their being less toxic.

Nutritional factors do not seem to be related to the limited ingestion of *Eucalyptus* leaves. Species of *Eucalyptus* leaves differ little in their gross nutritional content, having 3–7% fat, 6–9% protein, 37–42% carbohydrate, and 6–9% fiber (Pratt, 1937). If nutritional imbalance is the basis of the observed limitation, it must be the result of the leaves lacking minor nutrients. For more leaves to be eaten when three species are provided than when a single species is given, the lack of some minor nutrient in each species must be complemented by the presence of minor nutrients in the other species. While such a phenomenon need not be impossible to imagine, it does not account for the increase in ingestion over the first 5 days of the experiments. When mammals are fed nutritionally lacking or imbalanced diets they will initially eat the food, but soon exhibit feeding depression or a complete refusal to eat the food provided (Rozin, 1967). Feeding depression is also observed in animals fed foods containing digestive inhibitors (Glick and Joslyn, 1970). The observed increase is the reverse of this process: rather than a growing dislike, it is an increase in appreciation for the food. This, together with there being little difference between the gross nutritional content of *Eucalyptus* leaves and that of the artificial food, militates against nutritional factors being the basis of the limited *Eucalyptus* ingestion. In the proportions eaten, the artificial foods had an overall content of 0.9% fat, 2.2% protein, 17.03% carbohydrate, and 0.85% fiber (Altman and Dittmer, 1968). If anything, *Eucalyptus* leaves are the slightly better food.

The presence of plant secondary compounds can explain the possums' limited ingestion of *Eucalyptus* leaves. *Eucalyptus* oils and phenols are known to be toxic to mammals (MacPherson, 1925; Read et al., 1970). As mentioned earlier, possums fed *Eucalyptus* leaves produce much greater quantities of urinary glucuronides than do possums fed less toxic domesticated vegetables (Hinks, 1956; Hinks and Bolliger 1957*a*,*b*). Glucuronides are the metabolic products of microsomal detoxification. It appears that possums absorb and have to detoxify *Eucalyptus* secondary compounds.

Detoxifying mechanisms have to be induced (Conney and Burns, 1972; Freeland and Janzen, 1974). The occurrence of a toxic effect from a foreign compound is dependent on the rate of detoxification, and the quantity of compound ingested. Toxic effects can be eliminated if the compound is ingested at a rate such that the detoxifying enzymes are not overloaded. The quantity of a toxic food that an animal can safely ingest can be expected to increase as enzymes are induced for detoxification. Obviously there is some limit to the quantity of a food that can be safely ingested and detoxified. This process can explain the increase in ingestion of a single *Eucalyptus* species

during the first 5 days of the experiment. It can also explain the limited ingestion of this species during both 5-day periods.

The increase in ingestion during the second 5 days can be explained by induction of additional enzymes to process different toxins present in the two new *Eucalyptus* species. This second phase of increasing ingestion was characterized by two phenomena. First, there was sampling behavior leading to the ingestion of all three species by the ninth and tenth days. Second, on day 6 there was a drastic reduction in ingestion of the *Eucalyptus* species eaten during the first 5 days. Possums either abandoned the species they had been eating and sampled the two new species, or continued to eat some of the old while sampling one of the new. When feeding on a food that is not supplying minimum energy requirements it is obviously advantageous to eat, or attempt to eat, additional foods when they are available. However, when foods are added to an already toxic diet, there is potential for additive, synergistic, or inhibitory interactions to occur among the toxins (Freeland and Janzen, 1974). The potential danger of an additive or synergistic interaction can be minimized by treating both new and old foods in the same way: in combination they can be equally dangerous. Once the potential for detrimental interaction has been assessed by sampling behavior, consumption of large amounts of the old food (for which enzymes have already been induced) and some of the new food can take place. This pattern was observed during the second half of the experiment (Figure 1). The active sampling and later consumption of all three species, the continued limited ingestion of single species, and the continued restriction in total energy intake make it unlikely that continued increase in ingestion could have taken place had only a single species been provided during the second 5 days.

This explanation assumes that different *Eucalyptus* species contain different types of oils and phenols. Although each of the three species used contains oils that are present in one or the other species, the combination of oils in each is different, and two of the species contain at least one type of oil not found in either of the other two species. The quantitative composition of the oil content also differs between the species (Penfold and Willis, 1961; Baker and Smith, 1920). Nothing is known of the phenolic composition of the three species. The observed patterns of *Eucalyptus* ingestion are compatible with an interpretation based on the toxic secondary compound content of *Eucalyptus* leaves. This interpretation also explains the ingestion of large quantities of artificial (low toxicity) foods associated with an apparent absence of a period of enzyme induction.

Consequences of the Experimental Results

Marsupials and *Eucalyptus* can be assumed to have been evolving

together since Australia abandoned Gondwanaland some 45–56 million years ago (Raven and Axelrod, 1972; Tyndale-Biscoe, 1973). Leaf-eating marsupials, in this case *Trichosurus vulpecula*, can be expected to have had an impact on the evolution of *Eucalyptus* antipredator characteristics. *Eucalyptus* chemical defenses are effective in limiting the quantity of *Eucalyptus* leaves individual *T. vulpecula* can eat over a given time period. Individual possums can, however, eat enough *Eucalyptus* to account for the majority of their energy requirements. In the wild, 66% of their feeding time is feeding on species of *Eucalyptus*. For *Eucalyptus* chemical defenses to be effective against possums, they must in some way be directly or indirectly responsible for limiting the number of possums feeding on individual trees. In other words, the chemical defenses must in some way limit the size of the possum population.

The inability of possums to gain all their energy requirements from a single species of *Eucalyptus* is evidenced by free-living possums feeding on an average of three different foods in any one night. Possums fed on *Eucalyptus* every night, but they also went in search of non-*Eucalyptus* foods 35 out of 36 nights. This resulted in 34% of the feeding time being spent eating difficult-to-get foods. They are difficult to get for the following reasons. Most of the 34% was taken up eating a variety of foods found on the ground and involved a large search effort. These ground foods are widely scattered through an animal's home range, and occur in small parcels. In terms of quantity of food ingested per unit time, feeding on *Eucalyptus* leaves was far more profitable than ground feeding. The remaining non-*Eucalyptus* foods can be characterized as being either seasonal in occurrence (e.g., mistletoe flowers and fruits) or as being in some way "unpalatable" (probably due to toxic secondary compounds) as evidenced by the very small amount of time spent feeding on them (e.g., *Ficus* sp. leaves, see Table 2). These non-*Eucalyptus* foods are necessary for a possum to be able to balance its energy budget, and these foods are either temporary, in short supply, or somewhat inedible. This necessity to consume other foods is probably reflected in the carelessness of male 6228. He was observed to have paralyzed hind limbs as he left a *Passiflora suberosa* vine (an introduced plant). He was never seen again. *Passiflora* is extremely toxic, and paralysis is a classic symptom of *Passiflora* poisoning.

We hypothesize that the density of *T. vulpecula* populations is indirectly limited by the occurrence of toxic secondary compounds in its major food item. Although the possums can eat enough *Eucalyptus* to make up the majority of their energy requirement, they have to consume non-*Eucalyptus* food in order to subsist. These other foods are in short supply, and it is this that limits the size of the possum population, rather than the abundance of the major food item.

Also, in this indirect way, *Eucalyptus* species have obtained protection

from the depredations of possums. In the next section we examine evidence that supports the proposal.

The New Zealand Phenomenon

The flora of New Zealand evolved in the absence of mammals, or of any ecological equivalent to an arboreal leaf-eating mammal. From 1840 to the early 1920s *Trichosurus vulpecula* were introduced to New Zealand in order to foster a previously nonexistent fur trade (Pracy, 1962). When introduced to particular forests, the possum population rose rapidly, frequently reaching levels of 29–49 individuals per hectare (Kean and Pracy, 1953; Batcheler *et al.*, 1967). This rapid increase in population density was not due to any change in litter size, number of breeding seasons, age of sexual maturity, or longevity. There appeared to be an increase in juvenile survivorship (Tyndale-Biscoe, 1955, 1960, 1973). In Australia the only potentially important possum predators are domestic dogs. These are also present in New Zealand. Predator release does not seem to be the basis of the New Zealand possum population explosion.

Associated with these rapid increases in possum density was destruction of many individuals of particular tree species. These species were highly "palatable" to possums (Kean and Pracy, 1953; Zotov, 1949). Following this denudation of the forests, the possum populations declined to approximately 14 individuals per hectare (Batcheler et al., 1967). Tree species that in many cases had been almost eliminated gradually recovered as new individuals grew (Kean and Pracy, 1953; Gilmore, 1967). These new individuals were less "palatable" to possums than individuals of the species had been, and certain other tree species increased in importance as possum food (Kean and Pracy, 1953; Gilmore, 1967). Trees were no longer killed by possums, and the possum population did not exhibit any further rapid population increase (Kean and Pracy, 1953).

The rapid increase in density of newly introduced populations can be explained as being due to the presence of an abundance of relatively nontoxic foods. These did not require an individual possum to search for additional foods in order to make up its energy budget. Individuals could, and did, concentrate their feeding activities to one or two trees, ultimately killing them (Kean and Pracy, 1953). Selection due to possum feeding eliminated "palatable" trees, and selected for trees of low "palatability." Loss of "palatability" is associated with the presence of plant defenses, usually toxic secondary compounds (Freeland and Janzen, 1974). The feeding patterns of New Zealand possums can now be expected to be similar to those of Australian possums. Indeed, possums living in areas of New Zealand that have had possums for a long time consume an average of three different types of foods a

night (Gilmore, 1967). The situation closely resembles that in Australia. Trees have evolved defenses against possums, possum populations are stable, and although the quantity of potential food need not have changed, food trees are no longer denuded.

The proposed mechanism for evolutionary regulation of predator carrying capacity depends on prey chemical defenses forcing predators to search for and consume other foods. By forcing one of its predators to feed on foods other than it, the prey is automatically providing the selective force necessary to help bring about resistance to the predator in these other prey populations. Assuming that the predator's ability to overcome prey defenses does not change, increased resistance to predation among these additional prey times must lead to an even greater decrease in the size of the predator population. A limit to the resistance of prey populations to predation is imposed by some level of cost–benefit to individual prey (e.g., Janzen, 1973). This can be expected to depend on the plant part that the predator eats, the plant life form, successional stage, etc. (Janzen, 1969; Cates and Orians, 1975). In turn these limits will ultimately stabilize the predator density at some particular level. The apparent inability of *T. vulpecula* to specialize on a *Eucalyptus*-only diet is probably an evolutionary compromise related to the costs and benefits of locating and consuming non-*Eucalyptus* foods, as opposed to energetic and time costs, involved in processing a more toxic all-*Eucalyptus* diet.

SUMMARY

Field observations and laboratory experiments on the feeding behavior of brush-tailed possums (*Trichosurus vulpecula*) support hypotheses on the feeding behavior of generalist herbivores (Freeland and Janzen, 1974). These were:

1. A generalist herbivore has to ingest several different plant foods in order to meet its energy requirements.
2. Large amounts of a single plant food are not eaten when a generalist herbivore first encounters it, the animal initially taking small samples in preference to eating a large meal.
3. The amount of a single plant eaten can be gradually increased as the animal gains "experience" with it, this probably is a result of the animal inducing enzymes to detoxify the food.
4. Nontoxic foods are recognized quickly, larger amounts of them being eaten than can be eaten of single toxic foods, or even a restricted range of foods.

We hypothesize that *T. vulpecula* populations are indirectly regulated by chemical defenses evolved by their major plant food: *Eucalyptus* spp. The evolution of these defenses is at least in part due to selection induced via possum feeding. Actual limitation of the possum population, and so protection for the *Eucalyptus* trees, is achieved via the chemical defenses forcing individual possums to consume food other than *Eucalyptus*. It is the availability of these other foods that limits the density of *T. vulpecula* populations. The hypothesis is compatible with the New Zealand phenomenon. On being introduced to New Zealand, possum populations reached extremely high densities, followed by severe reduction in density and the occurrence of stable populations. This was associated with possum-induced selection for less palatable (probably more toxic) food tree species. Populations of other generalist herbivores can be expected to be limited by self-induced evolutionary changes in the chemical defenses of their food plants.

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OVIPOSITION STIMULANTS OF THE MOTH *Ectomyelois ceratoniae*: THE EFFECT OF SHORT-CHAIN ALCOHOLS

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Abstract—Females of the moth *Ectomyelois ceratoniae* prefer to oviposit on carobs, *Ceratonia siliqua* L., infested with the fungus *Phomopsis* sp. (Gothilf, 1964). An extract of the steam distillate of fungus-infested carobs was more effective in stimulating female moths to oviposit than was an extract of uninfested carobs. The extract of the fungus-infested carobs was found to be composed mainly of simple alcohols: ethanol (60%), *n*-propanol[3] (15%), 2-propanol (2.5%), isobutanol (15%), *n*-butanol (2.5%), and isopentanol (5%). In addition, the extract contained a small proportion (1–2%) of unidentified compounds. All of the alcohols, with the exception of isopentanol, stimulated oviposition by female moths. However, the alcohols, when tested separately or as a mixture, were not as stimulating as the total extract. The unidentified compounds were less stimulating than the alcohols.

Key Words—*Ectomyelois ceratoniae*, oviposition stimulant, carob, *Ceratonia siliqua*, alcohols, oviposition behavior, *Phomopsis*.

INTRODUCTION

Studies of host selection in phytophagous insects have primarily been concerned with larval reactions. Although the orientation of females to the host and their ovipositional responses after they arrive at the host are often the first stages of host selection, these aspects have received less attention. Consequently, only in few instances have chemical oviposition stimuli of host origin been identified (Gupta and Thorsteinson, 1960; David and Gardiner,

[3] See page 463 for editorial note.

1962; Matsumoto and Thorsteinson, 1968; Städler, 1972; 1974; Traynier, 1965; Wearing and Hutchins, 1973).

Ectomyelois ceratoniae (Zeller) (Lepidoptera: Phycitidae) is a phytophagous insect indigenous to the Mediterranean region (Heinrich, 1956). The larvae feed on the fruits of various trees, including carob (*Ceratonia siliqua* L.), almonds, and citrus. Gothilf (1964) has shown that on carob trees the females prefer to lay eggs on fruits infested with the hyphae of the fungus *Phomopsis* sp. and that they differentiate between fungus-infested and non-infested fruit on the basis of odorous chemical stimuli. The present work was aimed at identification and assessment of ovipositional stimulant activity of the volatile chemicals contained in the infested carob fruit.

METHODS AND MATERIALS

The Insect

Rearing procedures were described previously (Gothilf, 1968). Larvae were fed an artificial diet composed of a mixture of soybean flour, powdered sugar, and water. Following pupation, the rearing trays were placed in a 1-m^3 emergence cage, from which adults were taken for bioassay.

The Fungus

Phomopsis sp. was isolated from carob fruits and was routinely grown in sterilized 500-ml Erlenmeyer flasks containing 220 g substrate composed of 40% ground carob pods and 60% water. The flasks were kept at room temperature. When the fungus had spread over the substrate, the medium was used for chemical isolation of oviposition stimulants or for reinoculation of new substrates. Procedures commonly employed in bacteriological work were used for rearing and handling the fungus.

Isolation and Identification Procedure

The fungus-infested carob medium was placed in 900-g lots in round-bottomed flasks (3 liters) which were connected to a vacuum pump via two traps. The first trap was immersed in a salt-ice bath and the second in a liquid air bath. The flask containing the medium was heated in a hot water bath. When no more liquid distilled over, the distillates from both traps were combined and extracted with ether (3 × 500 ml) in a separatory funnel. The ether extract was dried over Na_2SO_4 and filtered. The ether was then removed by vacuum at room temperature. The oily residue (extract A) was analyzed by GLC using a Varian model 90-P aerograph, with a thermal conductivity

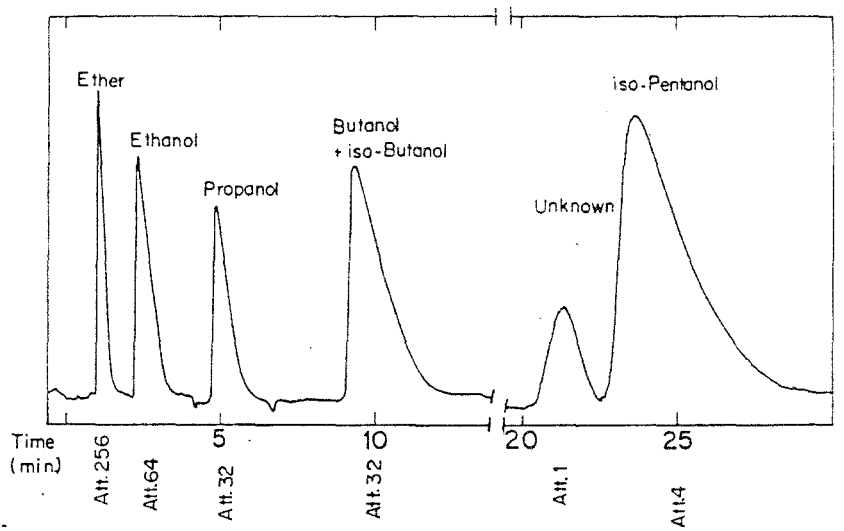

FIG. 1. GLC analysis of extract A showing the retention times of the different components (Att. = attenuation).

detector. The column was a 5-ft × 1/8-in. OD tube packed with Porapak Q. The helium flow was 30 ml/min, and the column temperature was 170°C. The injector and detector temperatures were 210°C and 220°C, respectively. A typical chart is shown in Figure 1. For preparative purposes the same type of column was used, however with a 1/4-in. OD tube. The substances producing the different peaks were collected and identified by NMR, mass spectral analysis, and comparison with authentic samples. The relative concentration (in percent) of each of the components of the volatile fraction was then determined. Similar extraction procedures were performed using fungus-free carob fruit to give extract B. The fungus-free medium was prepared and sterilized as described for the fungus-infested medium but was not inoculated with the fungus.

Distillation of extract A under reduced pressure gave a residue which was dissolved in methanol and extracted with *n*-hexane. The hexane was then evaporated, and the residue was left under vacuum in order to remove all traces of alcohols and solvents as checked by GLC. This fraction was designated "wax A." "Wax B" was obtained by dissolving extract B in chloroform, followed by extraction with 10% aqueous Na_2CO_3 solution (3 × 25 ml) in order to remove all of the isobutyric acid known to be present. The extract was washed with distilled water (3 × 50 ml), dried over Na_2SO_4, and filtered. The chloroform was then removed in vacuum until no solvent or isobutyric

acid could be detected by GLC. It should be noted that no isobutyric acid could be detected in extract A.

Bioassay Procedure

Bioassays were performed in a separated room which was held at 25–26°C and 70% relative humidity. Windows on one wall provided natural illumination.

About 100 females and 100 males of *E. ceratoniae*, 1–3 days old, were transferred from the emergence cage to the bioassay room and placed in a test cage (50 × 60 × 47 cm high) made of plastic screen mounted on a wooden frame. The cage was exposed to even and indirect light from the window. The chemicals to be tested were placed in undiluted form into open vials (12 × 35 mm, with opening diameter 5 mm) in equal volumes ($\leq$0.4 ml/vial) in each experiment. All chemicals completely covered the bottoms of the vials; thus each had the same surface area exposed to the air. Each vial was placed in a plastic cup (42 mm diameter × 52 mm high). The cup was covered with a perforated lid having 12 equally spaced, 1.2-mm-diameter holes. The holes provided a physically suitable substrate for egg laying, while preventing direct contact with the odorous chemicals. The cups were placed on the floor of the test cage. The tests were of the three-choice preference type: three cups were placed 1.5 cm apart, with two of them containing test compounds and the third, containing an empty vial, serving as a control. Four replications of each test were conducted simultaneously, with each cluster of three cups located about 20 cm from the other cluster. The females inserted their ovipositors into the holes and attached their eggs to the inner side of the lid. Occasionally, a few eggs were laid on the outer rim of the lid, and these were not counted. No eggs were laid on the floor of the cage around the cups, but sometimes eggs were found on the screened wall of the cage.

The duration of each experiment was one night, encompassing the activity period of the moths. The same moths were used for 1–3 experiments. On the morning following each experiment, the cups were removed from the cage and the number of eggs laid per lid was counted. Results were subjected to statistical analysis. First an analysis of variance (F test) was performed, and if significant differences were indicated, a t test was used to compare individual pairs of means.

RESULTS AND DISCUSSION

It had been shown previously that gravid females of the carob moth prefer to oviposit on carob pods infested by the fungus *Phomopsis* sp., rather

than on uninfested pods (Gothilf, 1964). Similar results were obtained when an ether extract of the steam distillate of infested carob pods (extract A) was compared with an extract of uninfested pods (extract B) (Table 1, experiment 17). Extract A was found to be composed mainly of simple alcohols, when analyzed by GLC. The alcohols were identified and their respective percentage (by weight) was: ethanol 60%; 2-propanol, 2.5%; *n*-propanol, 15%; isobutanol, 15%; *n*-butanol, 2.5%; and isopentanol, 5%. With the exception of isopentanol, all these alcohols were stimulatory to the females[4] (Table 1, experiments 1–9). The stimulatory effect of the alcohols as a mixture appears to be no greater than the summed activity of the individual components. No synergism was noted when the mixture was compared to a single alcohol having the same surface area (experiment 10). Changing the proportion of the various alcohols did not seem to affect reaction of females to the mixture (experiment 11).

Since extract A is made up predominantly of short-chain alcohols, it could be assumed that the activity of the extract is due to the alcohols. However, some of our results were not in accord with this assumption. More eggs were laid on extract A than on the alcohol mixture or on *n*-propanol alone (Table 1, experiments 12, 13, and 14; difference not significant in experiment 14). Thus, the total extract appears to be more stimulating than the alcohols, and additional components in the total extract must contribute to its observed effect. We found that extract A, in addition to short-chain alcohols, contains a small amount (about 2% by weight) of other compounds, which we designated "wax A." Given a choice between extract A and wax A the moths preferred to oviposit on the former (experiment 15); similarly, *n*-propanol was more stimulatory than wax A (experiment 16). The identity of the compounds which comprise wax A and their effect on oviposition has yet to be investigated.

The ether extract obtained from carob fruits not infested with the fungus—extract B—was less stimulatory than either extract A, *n*-propanol, or wax A (experiments 17, 18, and 19). Nevertheless, extract B had some stimulatory effect; more eggs were laid on cups containing this extract than on control cups. The identity of the stimulatory factors in extract B are as yet unknown. GLC analysis showed that it contains isobutyric acid, and no short-chain alcohols were detected. Isobutyric acid was not stimulatory to female moths (experiment 20). In addition to isobutyric acid, extract B contains other compounds which we designate "wax B." Wax B was less stimu-

[4] We use the term "stimulant" for an airborne chemical whose overall effect on the insect results in a significantly higher number of eggs laid on the oviposition cups containing that chemical, as compared to the control cups. This is a generalized designation. It is possible that besides eliciting egg laying, the chemicals might act as attractants or arrestants. However, the bioassay employed by us could not distinguish between these possibilities.

TABLE 1. OVIPOSITION RESPONSE OF *Ectomyelois ceratoniae* TO CHEMICALS ISOLATED FROM CAROB FRUIT

Experiment No.	Treatment of oviposition cups			% Eggs laid on oviposition cups[a]		
	A	B	C	A	B	C
1	Isobutanol	Isopentanol	none	48 a	27 b	25 b
2	Ethanol	Isopentanol	none	53 a	12 b	35 c
3	*n*-Propanol	Ethanol	none	55 a	37 b	8 c
4	2-Propanol	Ethanol	none	50 a	37 b	13 c
5	2-Propanol	*n*-Propanol	none	46 a	41 a	13 b
6	Isobutanol	*n*-Butanol	none	48 a	46 a	6 b
7	Ethanol	Isobutanol	none	48 a	46 a	6 b
8	2-Propanol	Isobutanol	none	53 a	40 a	7 b
9	*n*-Propanol	*n*-Butanol	none	53 a	41 a	6 b
10	*n*-Propanol	Alcohol mix[b]	none	48 a	47 a	5 b
11	*n*-Propanol	Alcohol mix[c]	none	47 a	48 a	5 b
12	Extract A[d]	Alcohol mix[b]	none	58 a	33 b	9 c
13	Extract A	Alcohol mix[b]	*n*-Propanol	44 a	27 b	29 b
14	Extract A	*n*-Propanol	none	51 a	39 a	10 b
15	Extract A	Wax A[d]	none	64 a	31 b	5 c
16	*n*-Propanol	Wax A	none	51 a	36 b	13 c
17	Extract A	Extract B[d]	none	87 a	8 b	5 b
18	*n*-Propanol	Extract B	none	57 a	35 b	8 c
19	Wax A	Extract B	none	57 a	35 b	8 c
20	Isobutanol	Isobutyric acid	none	80 a	9 b	11 b
21	Extract B	Wax B[d]	none	57 a	26 b	17 c
22	Wax A	Wax B	none	81 a	12 b	7 b

[a] About 100 females per experiment (one night) with an average egg laying of 1600 (range 632–2707) per experiment. Calculated F ratio ($P \leq 0.05$) showed significant differences among means of the three treatments in all experiments. A t test was calculated for each pair of means in each experiment. Means followed by different letters are significantly different ($P \leq 0.05$).

[b] Mixture of short-chain alcohols found in extract A, in natural proportion.

[c] As (*b*) but isopentanol was omitted and alcohols were mixed in equal proportions.

[d] See Methods and Materials.

latory than the total extract (experiment 21); it was also less stimulatory than wax A (experiment 22).

In nature, the female moths lay eggs on uninfested carob fruits, although at a lower rate than on fungus-infested fruits (Gothilf, 1964). It appears that the fungus drastically changes the chemical composition of the fruit and that several of the compounds characteristic of infested fruit serve as better oviposition stimulants than the compounds found in uninfested fruit. The

TABLE 2. OVIPOSITION RESPONSE OF *Ectomyelois ceratoniae* TO WATER

Chemicals applied	% Eggs laid on oviposition cups[a]
n-Propanol	41 a
n-Propanol + water	41 a
Water	12 b
None	6 c

[a] Out of a total of 1460 laid eggs. Means followed by different letters are significantly different (t test, $P \leq 0.05$, between individual pairs of means).

teleology of this preference is clear; larval growth is faster and survival is greater on infested fruit (Gothilf, 1964).

Insects often utilize humidity gradients for orientation to food sources (Thorsteinson, 1960). The gradients are also probably used by many insects for orientation to oviposition sites, as was demonstrated in *Trichoplusia ni.* (Hübner) (Shorey, 1964). The effect of water on ovipositional response was tested during the present research in a four-choice experiment. The results are given in Table 2. The experiment was conducted in a similar manner to those described above. However, 10 ml of water were added to some of the oviposition cups shown in Table 2. Water alone stimulates oviposition but to a much lesser extent than *n*-propanol. Also, water does not enhance the response to *n*-propanol. This implies that in the presence of adequate chemical stimulants the water content of the host is not important for inducing egg laying by females of the carob moth.

EDITORIAL NOTE ADDED IN PROOF

The editors did not wish to delay publication by changing the improper nomenclature of several of the alcohols at the page proof stage:

n-propanol should be 1-propanol
isobutanol should be 2-methyl-1-propanol
n-butanol should be 1-butanol
isopentanol should be 3-methyl-1-butanol

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A SEX PHEROMONE IN THE POTATO TUBERWORM MOTH, *Phthorimaea operculella* (ZELLER)[1]: BIOLOGICAL ASSAY AND PRELIMINARY CHEMICAL INVESTIGATION[2]

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Abstract—A sex pheromone was obtained from extracts of adult virgin female abdomens of the potato tuberworm, *Phthorimaea operculella*. A biological assay method was devised to test males for optimal responsiveness to the pheromone under varying conditions. Chemical analyses of pheromone extracts were initiated using gas chromatograph–mass spectrometry.

Key Words—*Phthorimaea operculella*, potato tuberworm, pheromone, bioassay.

INTRODUCTION

The potato tuberworm, *Phthorimaea operculella* (Zeller) (PTW), is a major potato pest in this country (Hofmaster, 1949). Its damage to potato crops derives from defoliation of plants and damage to tubers in the field and in storage. A female sex attractant has been reported in the potato tuberworm adult (Adeesan et al., 1969). We report here in detail the development of an optimum biological assay method and preliminary chemical studies of the pheromone. The PTW pheromone system is being investigated as a potential

[1] Lepidoptera: Gelechiidae
[2] Mention of a proprietary product or service does not constitute an endorsement by the USDA or The Pennsylvania State University.

field control alternative to pesticides, either by communication disruption (Shorey et al., 1972) or male trapping (Sharma et al., 1971).

METHODS AND MATERIALS

Insect Rearing

A culture for mass rearing of the PTW on natural substrate was established similar to that described by Finney et al. (1944; 1947). Adults were housed in battery jars in which the females laid eggs on potatoes or muslin cloths. The eggs were transferred into cardboard cartons and left for 7 days, during which time the larvae hatched. The potatoes were then transferred to a pupation unit consisting of 6 wooden racks with screen bottoms. As the larvae matured the racks were rotated every 2 days. Larvae which burrowed down into sand at the bottom of the unit were isolated as pupae and placed in the egg-laying jars to maintain the culture or were individually housed in plastic jelly cups to provide virgin adults for testing. Males were separated for biological assays, while females were used for extraction of pheromone. Culture moths are defined as those not individually isolated.

Extraction of Pheromone

Adult virgin females 1–7 days old were anesthetized with CO_2, and the terminal 3 abdominal segments excised into spectrograde CH_2Cl_2. The abdominal material was extracted for 24 hr, filtered, dried over Na_2SO_4, and adjusted to the desired concentration, which we represent in female equivalents (FE, the number of female abdomens extracted per given volume of solution). Extracts were stored at 0°C.

Laboratory Bioassay

The characteristic sexual response of PTW males to females or extract was viewed as being primarily composed of two behavioral components which generally occur together. The first component, sexual excitation, was identified by rapid wing flapping, directed movements in search of the pheromone source, and repeated attempts to copulate with the sample applicator or with other males. The second component, attraction, was quantitated in the bioassay in terms of locomotion to the pheromone source.

The system of choice was a manifold bioassay apparatus (Sower et al., 1973) consisting (for the Pennsylvania group) of a manifold (5 cm diameter × 70 cm long) connected to 10 glass tubes (3.5 × 45 cm). The tubes, containing 10 males each, had 0.5-cm-diameter holes at the upwind end for administering samples and were covered at the distal end with wire mesh. Air flowed through

the system from a compressed-air tank at approximately 100 cc/min/tube, and was exhausted into a fume hood. A 14-hr-light–10-hr-dark cycle was established, and during the latter period the manifold system was uniformly illuminated from below by two dimmed 15-W bulbs (<0.3 lux for initial studies). Test males in the tubes were acclimated to the air flow and background illumination for 15 min before bioassaying. Samples were applied to a wisp of cotton in a glass melting-point capillary through a cork in the small hole at the upwind end of the tube. Males were not tested again for at least 24 hr after bioassaying. Laboratory cultures and all samples were kept in a separate building to avoid contamination.

The Florida bioassays were conducted in a similar manner and with the same equipment (Sower et al., 1973). Moths were kept on a 14-hr-light–10-hr-dark cycle at 27°C and 60% relative humidity. However, males were not reused and samples were applied in ether to the surface of stainless-steel applicators. Investigations were carried out independently and both groups replicated biological experiments, with the exception of the light level series, which was conducted in Florida.

It was observed that a reliable way of quantitating attraction was to define it as movement into an area within 4 cm of the sample. Males in this area were counted at time 0 before administering sample (B), and counted again at 30 sec, the approximate time for maximum response (R). The percent net attraction response was calculated (Bartell and Shorey, 1969) as

$$\frac{R-B}{10-B}(100)$$

This gave a true percentage of males outside the area being attracted into it at 30 sec.

Blank samples of CH_2Cl_2 elicited an apparent net response of approximately 5% in this bioassay system, while a positive response to crude pheromone was an order of magnitude higher, However, this value varied with conditions such as the age of the males, the concentration of the sample, the time of the light cycle, and the background light level. Therefore, before serious chemical fractionation and identification could be pursued, those variables which could complicate the examination of fractions for activity were defined and a consistently reliable biological assay for the male response to pheromone was developed.

RESULTS AND DISCUSSION

Biological Assay of Pheromone

In preliminary bioassays it was confirmed that males respond only to

extracts of female abdomens and not to extracts of newly emerged or older males, while female adults showed no response to PTW extracts of either sex. Both virgin and "culture" males showed similar responses to female extracts, and extracts of both virgin and "culture" females elicited equivalent responses. No difference was detected in bioassay response to extracts of females between 2 and 7 nights after emergence, although response to newly emerged female extracts was lower. One tenth of an FE given at 5 hr into the dark period produced a reliable response. The parameters were used as a starting point for detailed bioassays.

Figure 1 shows the results of five replications (with error bars representing ± 1 SD) of bioassay studies on age variation of adult males. The data indicate that newly emerged males ($< 1/2$ day old) show absolutely no excitation or attraction to the pheromone. By the 2nd night after emergence, males attain a maximal response level to the pheromone which is maintained

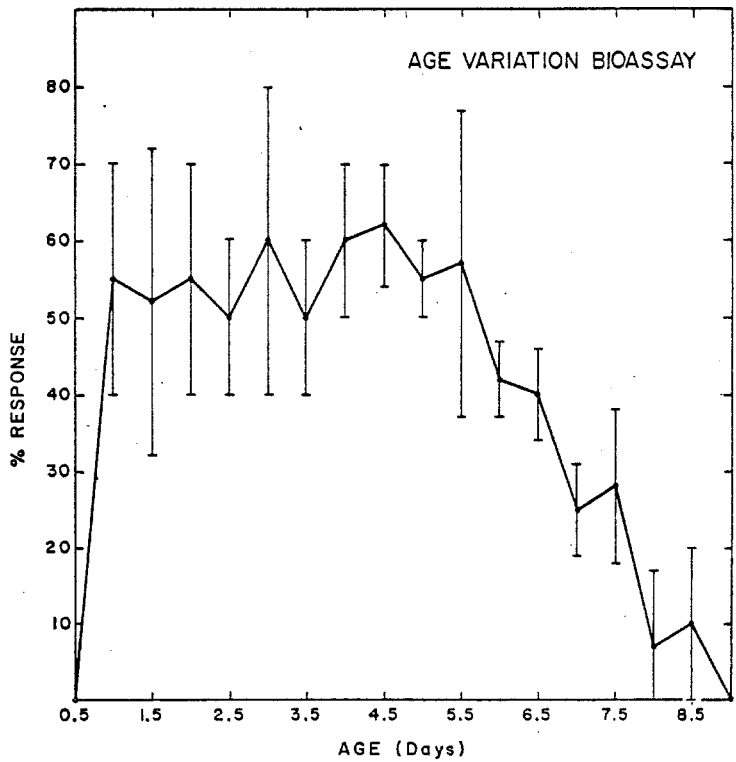

FIG. 1. Attraction of PTW males of varying ages to 10^{-1} FE of sex pheromone at 5 hr into the dark period. Five replicates were conducted; error bars denote standard deviation.

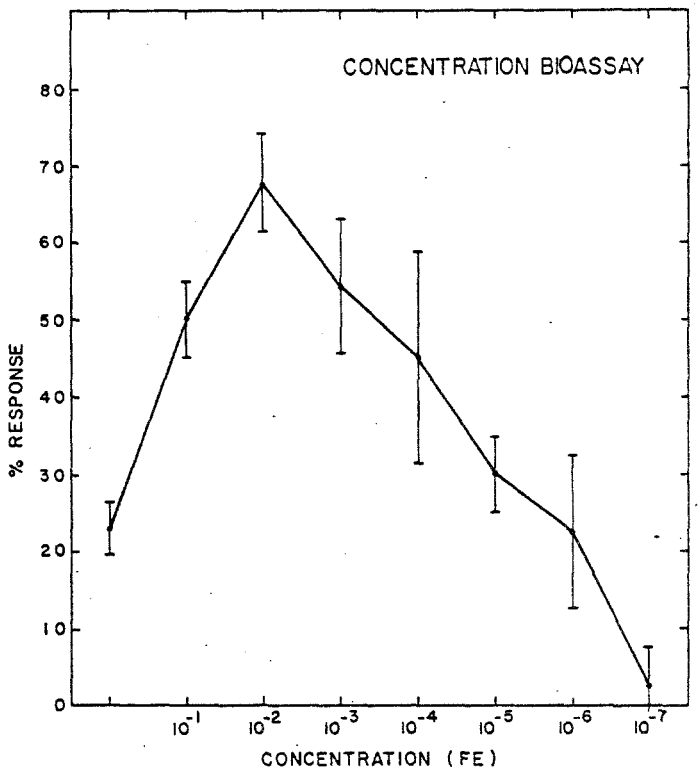

FIG. 2. Attraction of 1–5-day-old PTW males to varying concentrations of sex pheromone at 5 hr into the dark period. Error bars represent SD for five replications.

for about 5 nights with only statistical variation. After this time responsiveness decreases. Hence, 1–5-day-old males were used for bioassays.

The effect of extract concentration on male response was examined over a considerable range. Figure 2 shows the results of five replications of concentration experiments which indicate a very interesting effect. The maximal attraction (69%) occurs at 10^{-2} FE and decreases for either higher or lower concentrations. At the lower concentrations, attraction decreases rather uniformly, but is still detectable down to 10^{-6} FE (23%). Correspondingly, the qualitatively observed excitation (i.e., wing flapping, searching, and copulatory attempts) also decreases. However, at higher concentrations of pheromone, the percent net attraction drops off rather sharply, e.g., to 23% at 1 FE, but excitation does not decrease correspondingly. At these concentrations one observes less wing flapping and searching, but many copulatory

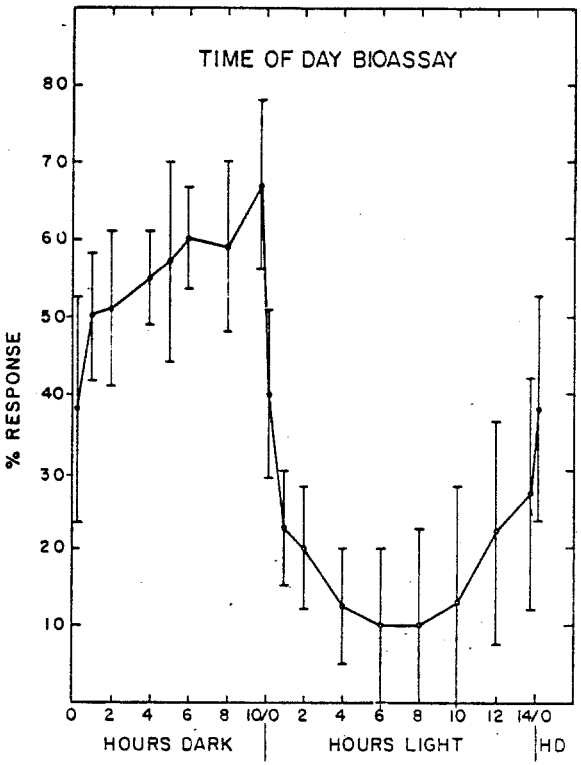

FIG. 3. Attraction of 1–5-day-old PTW males to 10^{-2} FE at different times during the light cycle. Error bars represent SD for 10 replications.

attempts. The males seem unable to locate the point source of the pheromone over a distance at this concentration. This observation may be very important to the use of pheromone for communication disruption.

Photoperiod studies were conducted using 10^{-2} FE samples. A definite preference for sexual activity was found in the dark cycle. Figure 3 summarizes 10 replications which show that soon after the onset of dark cycle, the response to pheromone is high. The first data point, within 15 min, shows a mean 38% response which goes up to 50% at 1 hr. The response level seems to increase gradually to a maximum of 67% at the end of this 10-hr cycle. The onset of the light period rapidly decreases the percent response, almost to the level of solvent blanks.

Studies on the influence of light level on male response were conducted during the period 4–6 hr after the onset of dark cycle with males 2 nights after

TABLE 1. INFLUENCE OF LIGHT LEVEL ON RESPONSE OF 2-NIGHT-OLD MALES TO 10^{-4} FE AT 4–6 HOURS DARK (10 REPLICATIONS)

Light level (lux)	% Mean response	± 1 SD
0.01	53.8	10.5
0.3	45.2	8.9
3.0	41.6	12.6
30.0	20.4	18.3

emergence. Males were preconditioned at the given light level (Spectra Photometer® Model FC-200, Photo Research Division, Kollmerger Corporation) for at least 1 hr prior to bioassay. Table 1 shows the results of 10 replications testing the effect of light intensity on the attraction of males to 10^{-4} FE. Very low levels of background lighting (e.g., 0.01 lux) maximize the response (53.8%), but only at very high light levels (e.g., 30.0 lux) is the response significantly decreased (20.4%).

In summary, from our behavioral studies, male responses to pheromone seemed to be maximized and consistent when using 1–5-day-old adult males which were bioassayed with 10^{-2} FE between about the 4th and 8th hr of a 10-hr dark cycle at low light levels (<0.3 lux).

Preliminary Chemical Investigation of Pheromone

The sex pheromones of 2 related species of the family Gelechiidae have been found to be doubly unsaturated C_{16} acetates. The sex pheromone of the Angoumois grain moth (*Sitotroga cerealella*) was reported to be Z,E-7,11-hexadecadienyl acetate (HDDA) (Vick et al., 1974). The sex pheromone of the pink bollworm (*Pectinophora gossypiella*) is an isomeric mixture of Z,Z- and Z,E-7,11-hexadecadienyl acetate (Hummel et al., 1973). Both of these moths also respond to the parapheromone attractant "hexalure," which is Z-7-hexadecenyl acetate (Green et al., 1969; Sower et al., 1973) (Table 2). Therefore, preliminarily, in our work toward identifying the PTW sex pheromone, it was assumed that similar C_{16} acetates might be involved. Thin-layer chromatography of crude female extract and bioassays of five fractions indicated that the active pheromone migrated with about the same R_f as standard 7,11-HDDA. However, gas chromatograph–mass spectrometric (Finnigan 3200 GC-MS system with model 6000 digital computer) analysis of the active TLC fraction failed to show any GC peak which contained the characteristic mass spectrum of standard 7,11-HDDA. Moreover, mass

TABLE 2. SEXUAL RESPONSE OF MALE ADULTS OF THE FAMILY GELECHIIDAE TO SYNTHETIC PHEROMONES

	Sexual response			
		Hexadecadienyl acetate		
Lepidoptera: Gelechiidae	Hexalure	Z,Z-7,11	Z,E-7,11	Combined[a]
Pink bollworm [*Pectinophora gossypiella* (Saunders)]	Yes[b]	No[c]	No[c]	Yes[c]
Angoumois grain moth [*Sitotroga cerealella* (Olivier)]	Yes[d]	No[e]	Yes[e]	—
Potato tuberworm moth [*Phthorimaea operculella* (Zeller)]	No	No	No	No

[a] 50:50 mixture of Z,Z and Z,E isomers.
[b] Green et al., 1969.
[c] Hummel et al., 1973.
[d] Sower et al., 1973.
[e] Vick et al., 1974.

fragmatograms (Hendry et al., 1975*a*,*b*; Hendry and Hindenlang, 1975) of characteristic ions in the mass spectrum of 7,11-HDDA were not diagnosed in PTW-active TLC fraction.

Even more dramatic were the results of bioassays using these standard C_{16} compounds on males under our previously determined maximal response conditions. Testing the Z,Z- and Z,E isomers and a 50:50 isomeric mixture of the 7,11-HDDAs over a range of 0.01–10 ng gave absolutely no response either in terms of excitation or attraction (Table 2). In addition, bioassaying 10-ng binary mixtures of all four 7,11-HDDA isomers elicited no response. Similarly testing 0.1–100-ng samples of hexalure showed no observable response by the males (Table 2). However, massive doses of hexalure in the 1-μg region did elicit a slight, but consistent, excitation response without attraction.

With these results, we are returning to a classical approach of thorough fractionation of crude female extracts, following the active material with our laboratory bioassay. Chemical identification of the isolated pheromone will be attempted chiefly by gas chromatograph–mass spectrometric techniques.

Acknowledgments—We would like to thank Dr. Hans Hummel and Dr. Helen Su for the standard 7,11-HDDAs and Dr. Martin Jacobson for the hexalure sample used in these studies. We also thank Dr. Patrick Greany, Marilyn Hindenlang, Jim Kostelc, Dave Vanderbilt, and Grace Van Epps for their assistance in various aspects of this project.

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ETHOLOGICAL FUNCTION OF COMPONENTS OF A SEX ATTRACTANT SYSTEM FOR ORIENTAL FRUIT MOTH MALES, *Grapholitha molesta* (LEPIDOPTERA: TORTRICIDAE)

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Abstract—Field studies of male Oriental fruit moth, *Grapholitha molesta* (Busck), behavior indicated that a mixture of *cis*-8-dodecenyl acetate (c8–12:Ac) and ca. 7% *trans*-8-dodecenyl acetate (t8–12:Ac) was requisite for upwind anemotaxis. The simultaneous emission of dodecyl alcohol (12:OH) and the attractant blend of c8–12:Ac containing ca. 7% t8–12:Ac elicited a behavioral repertoire including close-range orientation, landing near the chemical source, wing fanning, and extrusion of the males' abdominal hairpencils in precopulatory display. The effect of 12:OH in increasing trap catches was, therefore, not due to its effect on upwind anemotaxis, but rather to its importance in eliciting landing and other close-range precopulatory behavior. Interestingly, *cis*-8-dodecen-1-ol in extremely low ratios to the attractants (1:333) appeared to duplicate the activity of 12:OH used in ratios of 3:1 to the attractants. Laboratory observations of mating sequences revealed that male hairpencil eversion always preceded copulation. The evidence supports a male response sequence based on specific component combinations and concentrations eliciting successive behavioral steps rather than a response hierarchy dependent on increases in concentration of a single chemical or blend. Additionally, the closeness of the males' approach to c8–12:Ac containing ca. 7% t8–12:Ac was optimal at a discrete emission rate, and male responses were diminished within 30–60 sec after the males' arrival at the attractant source.

Key Words—Tortricidae, *Grapholitha molesta*, Oriental fruit moth, sex pheromone, attractant, synergist, hairpencil, aphrodisiac, *cis*-8-dodecenyl acetate, *trans*-8-dodecenyl acetate, dodecyl alcohol, *cis*-8-dodecen-1-ol.

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INTRODUCTION

Although the majority of Lepidoptera appear to employ female sex pheromone blends (Roelofs and Cardé, 1974*b*), behavioral investigations have been limited to the description of blend attractiveness in nature and excitation in the laboratory. The exact communicative role of the individual pheromone components has remained undefined. The sex pheromone of the Oriental fruit moth, *Grapholitha molesta* (Busck) (Tortricidae: Oleuthrutinae), was identified as *cis*-8-dodecenyl acetate (Roelofs et al., 1969). Recently the requirement for attractancy of ca. 7% *trans*-8-dodecenyl acetate was demonstrated (Beroza et al., 1973*a*,*b*; Roelofs and Cardé, 1974*a*) although this component has not yet been characterized from the female. Male trap catches also were increased approximately twofold by the addition of two intrinsically nonattractive chemicals: dodecyl alcohol (Roelofs et al., 1973; Roelofs and Cardé, 1974*a*) and 8-propoxyoctan-1-ol (Roelofs et al., 1973) in ratios of 4:1 or 3:1 and 10:1, respectively, to 200 μg of attractant pheromone. Neither dodecyl alcohol or 8-propoxyoctan-1-ol are known to be part of the natural pheromone bouquet of *G. molesta*. The present paper details the communicative role of *cis*- and *trans*-8-dodecenyl acetates and two chemicals which increase trap catches. Some preliminary aspects of this study were reported elsewhere (Cardé et al., 1975*a*).

METHODS AND MATERIALS

Mixtures of *cis*-8-dodecenyl (c8–12:Ac) and *trans*-8-dodecenyl acetates (t8–12:Ac) and of *cis*-8-dodecen-1-ol (c8–12:OH) and *trans*-8-dodecen-1-ol (t8–12:OH) were analyzed quantitatively with a 3% phenyldiethanolamine succinate (HI-EFF 10BP, Applied Science Laboratories, State College, Pennsylvania) on 100–120 mesh Chromosorb-W-AW-DMCS gas chromatographic column. Pure c8–12:Ac was obtained by TLC; pure c8–12:Ac was saponified to the alcohol. All test chemicals were placed on 5 × 7-mm rubber septa (Arthur H. Thomas Co., Philadelphia, Pennsylvania) dispensed in 10-μl Skellysolve B solvent. White sticky traps employed were either Sectar® I with a restricted entrance (Roelofs et al., 1973) or Pherotrap® IC (both supplied by Zoecon Corp., Palo Alto, California).

All experiments were conducted in non-insecticide-sprayed apple orchards in Geneva, New York. Relative attractiveness of various chemical blends in sticky traps was evaluated in a randomized complete-block design with 10 replicates positioned 1 trap/tree at a height of 1.5 m on a 10-m tree spacing. Traps were cleaned of males and rerandomized at least every second day. Data were transformed to $\sqrt{x+0.5}$ and submitted to an analysis of

variance, with differences among means being determined according to Duncan's new multiple-range test.

Wild male behavior in the vicinity of a pheromone-baited sticky trap was quantified by 1 or 2 observers situated 2 or 3 m from the attractant source who described individual male behavior onto a tape recorder. Male responses over a 4-min interval near various attractant sources were recorded by allowing individual wild males to enter clear plastic cages $20 \times 14 \times 10$ cm, open to air currents at each of the 4 sides with 2.5×5-cm wire screens. The attractant dispenser was positioned on the center of the floor of these cages. Male behavioral patterns were described for 4 min on tape. These behavioral observations were conducted in May, 1974, between 14:00 and 19:00 (Eastern Standard Time), in accordance with the males' diel response interval as shown by an automated trap (Comeau, 1971) indicating time of attraction to synthetic pheromone. Data from the tape recordings were analyzed subsequently with the aid of a stop watch.

Additional studies of male behavior close to an attractant dispenser were conducted using a flat, circular, table-top arena of galvanized sheet metal with a 60-cm radius. The chemical dispenser was located on the table center. Concentric circles marked on the table surface delineated 10-cm intervals from the center. These arenas were set out at a height of ca. 0.7 m approximately 2–3 m from apple trees.

Recordings of visual observations and subsequent analyses of male behavior were undertaken as described previously over observational periods of 10–15 min. Two arenas were employed simultaneously by single observers. To minimize the possibilities of contamination the lowest dispenser dosages of the components were presented first: subsequent treatments utilized increased charge levels. The arenas were moved to new trees 24 m or more crosswind prior to the start of the next observational period. Both experiments were conducted on several days in September, 1974, between 17:00 and 19:30 (EST) with all treatments being accorded equal observational time on each day. Dispenser septa were stored between tests in individual stoppered vials, and the arenas were rinsed with acetone prior to each test series.

Our brief reports of laboratory mating sequences were undertaken with laboratory reared moths in about the 12th hr of photophase under a 16:8 light/dark regime at 22°C.

RESULTS

Attractancy of Males to c8–12:Ac and to c8–12:Ac with ca. 7% t8–12:Ac

The attractancy (trap catch) of a multicompound blend such as 180 μg c8–12:Ac + 20 μg t8–12:Ac or these compounds plus either 600 μg 12:OH

or 0.6 μg c8–12:OH, could result from individual components acting in a medley in which each component affects all phases of behavior or by some combinations of components eliciting positive anemotaxis and others mediating close-range searching behavior. We investigated the comparative attractiveness of 200 μg of c8–12:Ac (containing <0.5% t8–12:Ac, undetectable by GLC) and 200 μg c8–12:Ac with 6.8% t8–12:Ac. In several tests, traps baited with the pure c8–12:Ac caught no males, whereas traps baited with the appropriate *cis–trans* mixture captured several hundred males (>200) in 10 hr of observations. By situating 2 observers downwind of the pheromone source, it was possible to follow individual males flying upwind to the traps baited with c8–12:Ac containing 6.8% t8–12:Ac. Males routinely were followed 10 m or more to the trap. No instances of *G. molesta* male orientation or positive upwind anemotaxis toward the pure c8–12:Ac were noted, although equal observational time was accorded both dispensers. A related apple-feeding oleuthrutine, *G. prunivora* (Walsh), would be lured to pure c8–12:Ac (Roelofs and Cardé, 1974*a*), and in flight could be confused with *G. molesta*. Although the temporal distributions of these 2 species overlap broadly, adult *G. prunivora* were not present during the period of these observations, as determined by separate monitoring traps. These findings indicate that male attraction or upwind anemotaxis under natural conditions is elicited by the combination c8–12:Ac and t8–12:Ac and that c8–12:Ac alone is intrinsically unattractive to *G. molesta*.

Effect of c8–12:OH on Male Trap Catches

The roughly twofold increase in male trap catch effected by the addition of dodecyl alcohol (12:OH) (Roelofs et al., 1973; Roelofs and Cardé, 1974*a*) and 8-propoxyoctan-1-ol (PrO-8:OH) (Roelofs et al., 1973) to c8–12:Ac (3–7% t8–12:Ac) suggested the potential of c8–12:OH and 12:OH for affecting male behavior, since these 4 compounds likely could interact with the same hydroxyl and terminal C_{12} active sites on a male antennal pheromone acceptor (Roelofs et al., 1973). Preliminary studies in Australia (G. Rothschild, private communication) indicated that low percentages of c8–12:OH did increase *G. molesta* attractancy.

Numerous field trials were conducted in 1973 and 1974 using 200 μg of c8–12:Ac with 4–8% t8–12:Ac (attractant) and various added quantities of c8–12:OH. One trial (Table 1, Test 1) showed that c8–12:OH reduced male trap catch at attractant/secondary compound ratios of 1:3 to 33:1, with the use of 7.8% t8–12:OH in the c8–12:OH giving a lower catch than the use of 3.5% t8–12:Ac. Tests (Table 1, Tests 2 and 3) of c8–12:OH at ratios greater than 33:1 showed some increases in trap catches, with a dispenser load of ca. 333:1 giving roughly a twofold increase in male catch.

TABLE 1. COMPARISONS OF ATTRACTIVENESS OF c8–12:Ac WITH 6.6% t8–12:Ac (ATTR.) WITH VARIOUS ADDED AMOUNTS OF c8–12:OH USING SECTAR I TRAPS

Treatment	Males/trap ($\bar{X}$)[a]
Test 1 (August 13–20, 1973)	
200 μg Attr.	40.6 a
200 μg Attr. + 6 μg c8–12:OH (3.5% t8–12:OH)	43.0 a
200 μg Attr. + 60 μg c8–12:OH (3.5% t8–12:OH)	17.4 c
200 μg Attr. + 600 μg c8–12:OH (3.5% t8–12:OH)	8.2 d
200 μg Attr. + 6 μg c8–12:OH (7.8% t8–12:OH)	29.2 b
200 μg Attr. + 60 μg c8–12:OH (7.8% t8–12:OH)	18.2 c
200 μg Attr. + 600 μg c8–12:OH (7.8% t8–12:OH)	4.6 d
Unbaited	0.0 e
Test 2 (August 27–31, 1973)	
200 μg Attr.	20.6 cd
200 μg Attr. + 0.6 μg c8–12:OH (< 0.1% t8–12:OH)	33.3 a
200 μg Attr. + 2 μg c8–12:OH (< 0.1% t8–12:OH)	23.8 bc
200 μg Attr. + 6 μg c8–12:OH (< 0.1% t8–12:OH)	16.3 d
200 μg Attr. + 20 μg c8–12:OH (< 0.1% t8–12:OH)	9.9 e
Unbaited	0.0 f
Test 3 (May 17–22, 1974)	
200 μg Attr.	8.0 b
200 μg Attr. + 0.06 μg c8–12:OH (6.6% t8–12:OH)	11.2 ab
200 μg Attr. + 0.2 μg c8–12:OH (6.6% t8–12:OH)	11.3 ab
200 μg Attr. + 0.6 μg c8–12:OH (6.6% t8–12:OH)	17.4 a
200 μg Attr. + 2 μg c8–12:OH (6.6% t8–12:OH)	14.5 ab
Unbaited	0.0 c

[a] Within each test, treatment means followed by the same letter are not significantly different at the 5% level.

Together these data show that at certain low ratios relative to the attractant, c8–12:OH will elicit an increase in male trap catch, similar to the effects of 12:OH and PrO-8:OH at much higher ratios.

The Role of 12:OH in Close-Range Searching Behavior

The increase in male trap catch effected by the addition of discrete quantities of PrO–8:OH, 12:OH, and c8–12:OH to c8–12:Ac (6.8% t8–12:Ac) could be related to an increase in the frequency of elicitation of male anemotaxis downwind of the attractant source or in a modification of

close-range searching behavior. Comparative observations of male behavior within 1 m of Pherotraps IC baited with either 200 μg c8–12:Ac (6.8% t8–12:Ac) or 200 μg c8–12:Ac (6.8% t8–12:Ac) and 600 μg 12:OH revealed distinctly disparate patterns of close-range searching behavior. Individual males were followed continuously from the time upwind orientations brought them within approximately 3 m of the trap until they were ensnared on the trap sticky surface or until they ceased apparent searching behavior by leaving the trap vicinity. The direction of departure was almost always directly downwind. Males terminating close-range searching behavior were observed until they flew at least 5 m from the trap, although searching behavior was scored as terminated once males flew more than 3 m from the trap. None of our observations indicated that males which had terminated searching behavior later reoriented to the attractant source. Nonetheless, the possibility of multiple observations of a single moth was minimized by repositioning traps 20 m crosswind within the orchard at 15 min intervals. No more than 13 males were observed prior to trap repositioning.

Male flight within 1 m of a trap was characterized by a decreased precision of orientation and an undulating flight, during which the distance of the males to the attractant source remained relatively constant, while males generally flew up, down, and from side to side in "casting" movements of roughly 5–20 cm and 1–2 sec in duration. Such close-range searching behavior (before capture or flight away from the trap) occurred for a mean of 19.8 sec (range of 5–69 sec, $n = 39$) with a trap baited with 200 μg c8–12:Ac (6.8% t8–12:Ac), whereas the mean time of searching flight with a trap baited with c8–12:Ac (6.8% t8–12:Ac) plus 600 μg 12:OH was 12.5 sec (range 3–50, $n = 40$). These means are significantly different at $P < 0.05$ according to the t test.

The frequency of males landing on the trap's lower sticky surface (and thereby approaching within at least 10 cm of the attractant) differed markedly with the addition of 12:OH to the attractant dispenser. The attractant alone produced a trap capture rate of 56.4%, where the confidence interval (c.i.) at $t = 0.95$ extends from 40.8 to 72.0%, whereas the attractant plus 12:OH resulted in 92.5% of the males being captured, where the c.i. at $t = 0.95$ extends from 80.1 to 98.4%. The communicative function of 12:OH added to the c8–12:Ac (6.8% t8–12:Ac) attractant did not seem to be effecting an increase in the number of males attracted to within 3 m of the attractant source. On the contrary, in this behavioral context, 12:OH appears either to modify the males' close-range searching behavior, or to cause the males to land, or both. Since the ensnarement of males on the sticky trap surface terminates the behavioral response, analyses were conducted utilizing assays which allowed continued observations of male responses.

Behavioral Effects of 4 Minutes' Exposure to Multicomponent Systems

Males were allowed to orient toward and enter a baited 20 × 14 × 10-cm clear plastic cage hand held at a height of 1.5 m. Immediately after a male had flown into the assay device, the top of the box was replaced and recording of behavior commenced. Four distinct categories of behavior were evident: (1) flying and hovering; (2) wing fanning on the cage surface (concurrent with rapid walking or while stationary); (3) rapid walking; and (4) quiescence.

Analyses of these behavioral modalities for the 4-min observational period revealed distinct changes in their relative frequencies dependent on the nature of the stimulus and the time elapsed (Figure 1). During the first

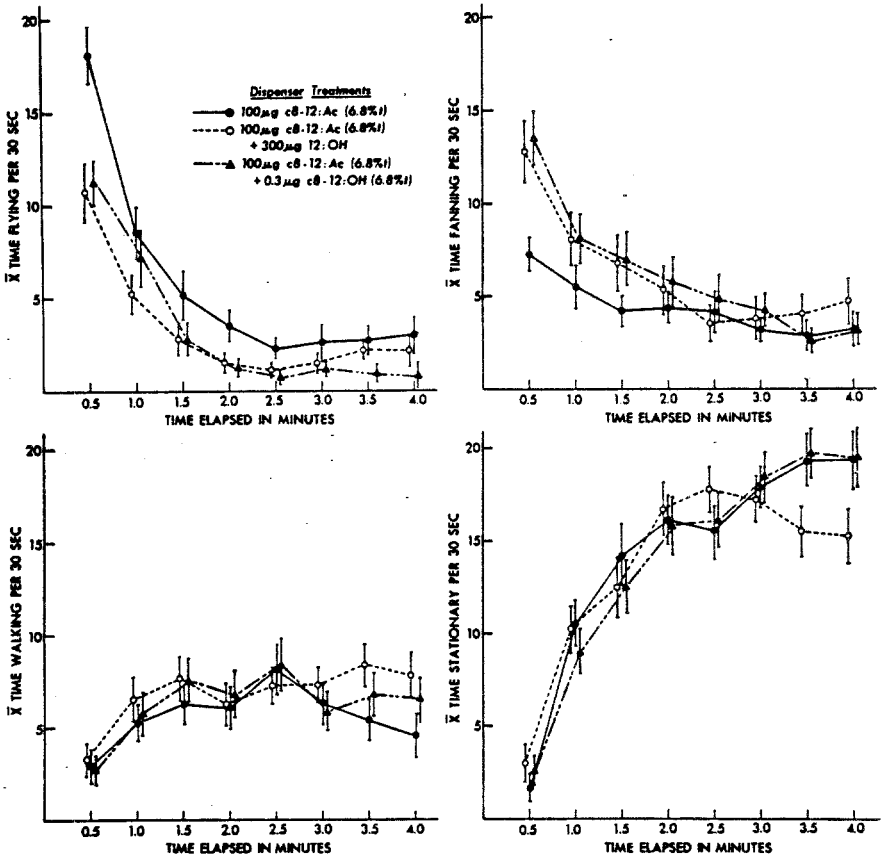

FIG. 1. Comparisons of the behavioral responses of males to blends during the first 4 min confinement after upwind attraction. Bars enclose the standard errors of the means for 30-sec intervals.

TABLE 2. TEMPORAL ANALYSES OF BEHAVIOR DURING THE FIRST 30 SEC ELAPSED AFTER UPWIND ORIENTATION

Stimulus	During first 30 sec		Initial behavior	
	Time flying ($\bar{X}$)	Time fanning/ walking ($\bar{X}$)	Time 1st flying ($\bar{X}$)	Time 1st fanning/ walking ($\bar{X}$)
100 μg c8–12:Ac (6.8% t8–12:Ac)	18.10	7.32	15.00	3.71
100 μg c8–12:Ac (6.8% t8–12:Ac) + 300 μg 12:OH	10.79***[a]	12.86**[a]	5.07**[a]	6.93
100 μg c8–12:Ac (6.8% t8–12:Ac) + 0.3 μg c8–12:OH (6.8% t8–12:Ac)	11.32***[a]	13.48***[a]	10.67	8.90**[a]

[a] **, *** differ from $\bar{X}$ of c8–12:Ac (6.8% t8–12:Ac) at $P < 0.01$ and $P < 0.001$, respectively, according to the t test.

30 sec of observations (Table 2) c8–12:Ac (6.8% t8–12:Ac) alone elicited flying or hovering behavior for a significantly longer time than these compounds plus 12:OH or c8–12:OH (6.8% t:–12:OH). During the first 30 sec the mean time of males fanning or fanning concurrent with walking was enhanced by the presence of 12:OH or c8–12:OH (6.8% t8–12:OH). These same trends are evident if one contrasts the mean times of the first occurrence (initial behavior, columns 3 and 4 of Table 2) of these behavioral responses during the initial 30 sec of observations.

For the three chemical mixtures presented, several trends are evident during the remaining 3.5 min of observations. The relative time males engaged in flying, fanning, or fanning while walking decreases concurrent with increases in time spent walking or not moving. There are no significant ($P < 0.05$) differences among any of the treatment means for the last 3.5-min observation period when behavioral modalities are compared at the same elapsed times. Such time-dependent changes may indicate a degree of habituation (alteration of the pheromone response threshold) within 30–60 sec from the time of attraction.

Effect of Dispenser Charges of 1 to 3000 μg c8–12:Ac (6.8% t8–12:Ac)

Observations of male behavior within 1 m of the 60-cm-radius table top baited with various charges of c8–12:Ac (6.8% t8–12:Ac) showed varying responses in several behavioral catagories (Table 3). Intermediate dosages effected quantitatively significant increases in frequencies of male landing and

TABLE 3. MALE BEHAVIOR NEAR 60-CM-RADIUS METAL ARENAS BAITED WITH 1 TO 3,000 μG OF *cis*-8-DODECENYL ACETATE (c8–12:Ac) WITH 6.8% t8–12:Ac ISOMER

μg c8–12:Ac (6.8% *trans*)	No. males observed	% Landing on table top[a]	% Fanning on table top[a]	% Displaying on table top	Orientation time, sec ($\bar{X}$ ± SE)[b]	Fanning time, sec ($\bar{X}$ ± SE)	Closest approach, cm($\bar{X}$ ± SE)[b]
1	14	36 cde	0 d	0	3.2 d ± 0.5	0	55.4 a ± 1.9
3	25	24 de	0 d	0	6.6 c ± 0.9	0	57.5 a ± 1.0
10	36	53 bc	22 b	8	14.9 a ± 3.2	8.0 ± 1.0	43.2 b ± 3.9
30	43	65 ab	16 bc	0	17.6 a ± 2.3	8.7 ± 0.5	44.7 b ± 2.9
100	54	69 ab	17 bc	0	14.1 a ± 1.7	2.9 ± 0.2	45.1 b ± 2.5
300	67	37 cd	1 d	0	9.9 b ± 1.1	0	55.9 a ± 0.9
1,000	55	40 cd	5 cd	0	7.5 b ± 0.6	3.7 ± 0.3	56.0 a ± 1.0
3,000	53	17 e	4 cd	0	5.1 c ± 0.4	1.5 ± 0.1	58.7 a ± 0.6
100 + 300 μg 12:OH	46	80 a	48 a	2	16.5 a ± 2.4	6.5 ± 1.1	36.9 c ± 3.2

[a] Percentages in the same column followed by the same letter do not differ at the 5% level when compared according to a 2 × 2 test of independence using the *G* statistic with Yates' correction.

[b] Means in the same column followed by the same letter do not differ at the 5% level according to the *t* test. Orientation time includes all categories within 0.5 m of the table top. In tabulating closest approach to the dispenser, males flying within 0.5 m of the table top but not landing were scored as approaching within 60 cm. The remaining approaches were by males walking on the table top.

TABLE 4. MALE BEHAVIOR NEAR 60-CM ARENAS BAITED WITH 10 μG *cis*-8-DODECENYL ACETATE (6.8% t8–12:Ac) PLUS DODECYL ALCOHOL (12:OH)

μg 12:OH	No. males observed	% Landing on table top[a]	% Fanning on table top[a]	% Displaying on table top[a]	Orientation time, sec ($\bar{X}$ ± SE)[b]	Fanning time, sec ($\bar{X}$ ± SE)[b]	Closest approach, cm ($\bar{X}$ ± SE)[b]
—	37	68	27	3	15.0 ± 1.8	7.3 ± 0.7	47.5 ± 2.5
1	40	88*	73**	8	20.7 ± 3.1	9.3 ± 1.6	33.6*** ± 3.2
3	39	77	36	23*	23.7** ± 2.6	16.5** ± 2.0	36.7* ± 3.9
10	33	94**	83**	42**	23.6* ± 3.1	15.9* ± 1.9	19.9*** ± 3.9
30	40	80	45	15	18.6 ± 2.7	11.4 ± 1.4	35.3** ± 3.6
100	30	83	43	30*	25.4 ± 5.1	18.3** ± 2.5	28.5*** ± 4.3

[a] Percentages in the same column compared to the treatment lacking 12:OH according to a 2×2 test of independence using the G statistic with Yates' correction: * indicates $P < 0.05$, ** $P < 0.01$.

[b] Means in the same column compared to the treatment lacking 12:OH according to a t test: * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Orientation time includes all categories of behavior within 0.5 m of the table top. In tabulating closest approach to dispenser, males flying within 0.5 m of the table top but not landing were scored as approaching within 60 cm. The remaining approaches were by males walking on the table top.

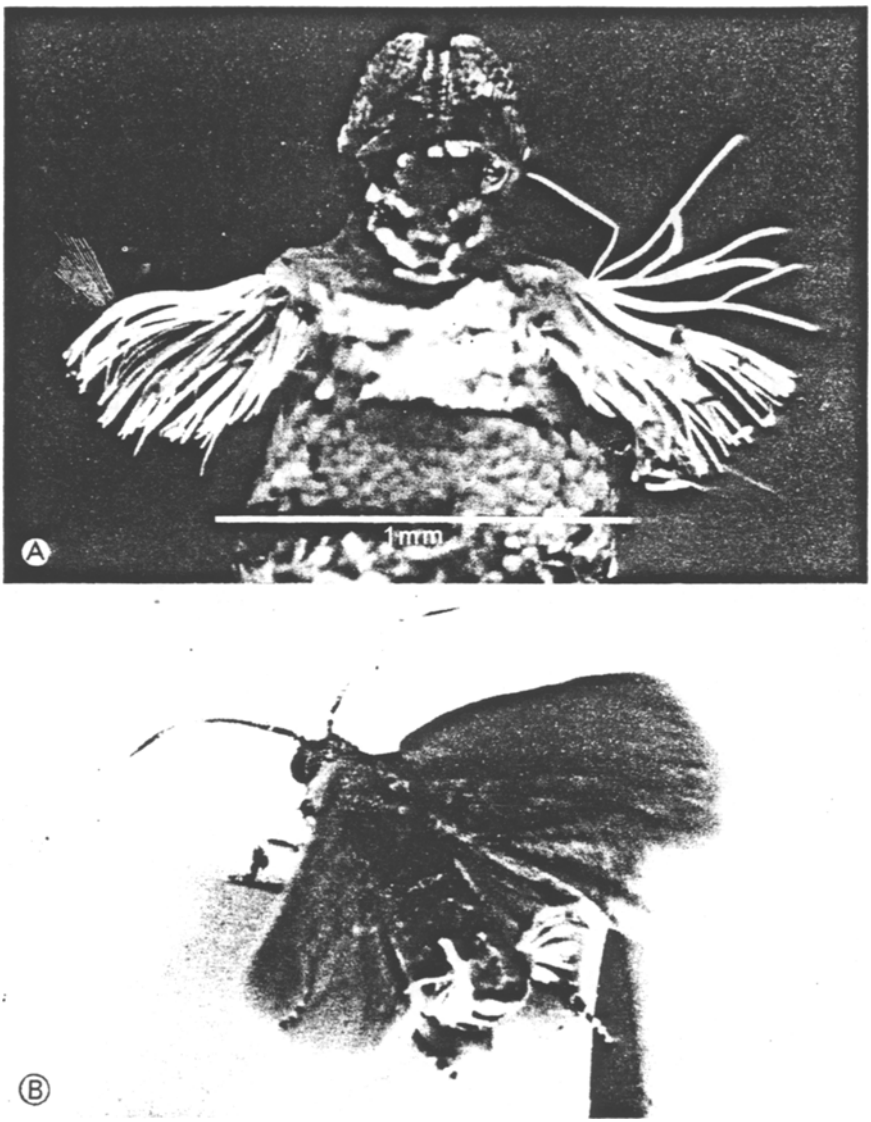

FIG. 2. (A) Male abdominal tip with hairpencils forcibly extruded. (B) Laboratory male everting his hairpencils on a rubber septum charged with 10 μg *cis*-8-dodecenyl acetate (6.8% *trans*) and 10 μg dodecyl alcohol. Electronic flash duration was 1/1000 sec.

(*Facing p.* 485)

walking concurrent with wing fanning, in mean times of orientation (flight or walking on the table top) and wing fanning, and in the closest approach to the chemical dispenser while walking on the table top. Since the lowest dispenser dosages (1 μg) would emit relatively reduced amounts of attractant, the lowered mean orientation time and close approach to this dispenser is probably related to the visual cues presented by the experimental observation table. Otherwise, continued upwind anemotaxis toward the chemical dispenser would be expected.

Effect of 12:OH with 10 μg c8–12:Ac (6.8% t8–12:Ac)

Addition of 300 μg of 12:OH to 100 μg of c8–12:Ac (6.8% t8–12:Ac) produced responses generally similar to those evoked by 100 μg c8–12:Ac (6.8% t8–12:Ac) alone, although the simultaneous emission of 12:OH elevated the frequency of landing and produced a closer approach to the chemical dispenser. Since 10 μg of attractant on the dispenser was the lowest charge level that elicited increased responses in the tabulated behavioral parameters, male responses to a combination of this charge plus 1–100 μg of 12:OH were examined in another experiment (Table 4). The response values for 10 μg of attractant alone in this test correlate very closely with those found in the previous test (Table 3). Concurrent release of 12:OH produced significant increases in the frequencies of landing on the observation table and wing fanning while walking, in mean times of orientation and fanning, and in closest approach to the chemical dispenser. Hairpencil display behavior (Figure 2)—found in a low percent of males in the previous observations—was noted in 42% of the males (51% of those landing) observed in the 10 μg attractant:10 μg 12:OH treatment.

Hairpencil display in general involved wing fanning while walking to within 1 to 2 cm of the rubber septum dispenser. Continuing to fan their wings males then turned away from the septum and everted their hairpencils 1–8 times, followed by a turning to face the septum, repeating this sequence until the male would abruptly fly away. Males would often crawl onto the septum, curl their abdominal tips toward the septum, or push the septum with their heads before departing. The temporal patterns of hairpencil eversions and turns are listed in Table 5. All dispenser dosages of 12:OH tested appeared to evoke similar patterns of precopulatory display.

Mating Behavior: The Role of Hairpencilling

Mating behavior was observed in the laboratory in a 0.15 × 1.5-m clear plastic wind tunnel with a wind speed of ca. 12 m/min. Males were released 1.2 m downwind of a calling female. Responsive males walked upwind while

TABLE 5. BEHAVIOR OF MALES ENGAGED IN HAIRPENCIL DISPLAY (CONCURRENT WITH WING FANNING)[a]

10 μg c8–12:Ac (6.8% t)+μg 12:OH	No. males displaying	Time display, sec ($\bar{X} \pm$ SE)	No. pencil eversions ($\bar{X} \pm$ SE)	No. eversions prior to turn ($\bar{X} \pm$ SE)	No. turns ($\bar{X} \pm$ SE)
0	1	2.0	4.0	2	2
1	3	7.7	8.0	3.2	2.5
3	9	14.9±1.4	11.4±1.8	3.7±0.7	3.0±0.6
10	14	12.6±1.8	9.6±1.2	3.1±0.8	4.1±0.6
30	6	15.5±1.1	15.0±0.7	3.8±0.8	5.2±0.6
100	9	10.9±1.9	7.9±1.2	3.2±0.8	3.3±0.5

[a] These are the males of Table 4, column 5.

fanning their wings. In all (n = 18) successful copulations, male hairpencil eversion preceded the males' attempts to couple. Males walked to within 1–2 cm of the female, turned 180° away, and everted the hairpencils toward the females' anterior or sides while continuing to fan their wings. During 14 of 18 sequences of hairpencil extrusion displays, the female either turned toward the males' posterior, or walked rapidly toward the male, or both. In some instances the females' run toward the male brought their antennae into apparent contact with the males' abdominal tip, whereas in others no tactile stimulus preceded the males' 180° whirl and subsequent curling of the posterior of his abdomen preceding a copulatory attempt. Initial experiments indicate that an olfactory cue is involved in eliciting female orientation, since methylene chloride extracts of a single male's hairpencils when presented on filter paper within 1–2 cm of a calling female generally elicit walking toward the stimulus. It is probable that the hairpencils also act as an aphrodisiac, producing female acquiescence to copulatory attempts (Birch, 1974).

DISCUSSION

In the Lepidoptera the ubiquitous nature of communication systems involving two or more chemicals has been elucidated only recently, and so it is hardly surprising that little is known of the behavioral function of individual chemical components. Apparent attractancy of isolated natural or synthetic compounds has been considered by most investigators as the prime behavioral response evoked by so-called attractant pheromones, when indeed pheromone-mediated upwind anemotaxis has been demonstrated (Kennedy and

Marsh, 1974) in but a few species. Single compounds or combinations thereof which produce a trap catch have been regarded as being intrinsically attractive when, as Kennedy (1972) has pointed out, the behavioral effect could be of quite a different nature, such as eliciting landing when in the vicinity of a trap. Similarly, nonattractive chemicals that increase trap catch (so-called synergists) and those that decrease trap catch (so-called inhibitors or anti-attractants) when released simultaneously with attractants could mediate close-range behavior rather than upwind anemotaxis.

In *G. molesta* the present data indicate that the combination of c8–12:Ac and t8–12:Ac elicits upwind orientation. In concert these two compounds are attractants in the sense of evoking apparent upwind anemotaxis. Three related chemicals, PrO–8:OH, 12:OH and c8–12:OH, increase male trap catches although they do not appear to possess intrinsic attractiveness. In the case of 12:OH the effects appear unrelated to long-range searching behavior. In the presence of c8–12:Ac and t8–12:Ac, 12:OH apparently evokes a repertoire of precopulatory behavior: close approach to the chemical emitter, landing, and wing vibration, either while stationary or concurrent with walking, and hairpencil extrusion in precopulatory display. Similar responses in landing and wing vibration and increases in trap catches were evoked by c8–12:OH at certain low ratios, whereas at high ratios trap catch was decreased. Such dual effects of c8–12:OH on trap catch are quite similar to those reported by Baker et al. (1975) for the alcohols corresponding to the *Playtnota stultana* (Walsingham) attractant pheromone, *cis*- and *trans*-11-tetradecenyl acetates (17:83). When the alcohols were present as 0.2–2% of the attractant pheromone mixture, the trap catch was elevated; at >20% the catch was suppressed. In *P. stultana* it is not known if the mechanism responsible involves modification of close-range behavior. In *G. molesta* the modification of close-range orientation is apparently related to increases in the catch on sticky traps baited with c8–12:Ac, t8–12:Ac plus 12:OH. Hence, in *G. molesta* the term attractant "synergist" is clearly inappropriate to the behavior evoked by 12:OH.

When c8–12:Ac with ca. 7% t8–12:Ac was presented at 1–3000 μg per rubber septum dispenser, charges of 10, 30, and 100 μg elicited the closest mean approaches to the dispensers and the longest mean searching times (Table 3). Such a concentration-dependent response pattern appears analogous to the behavior of two noctuid moth species in which the male catches were reported to be optimal at certain discrete emission rates of *cis*-7-dodecenyl acetate (Kaae et al., 1973).

Males approaching the arena's edge frequently engaged in "casting" flight for 10 or more seconds within 1–20 cm of the arena's edge. The flight pattern undulated both laterally and vertically as much as 30 cm, and the precision of orientation would appear to be altered from the more direct

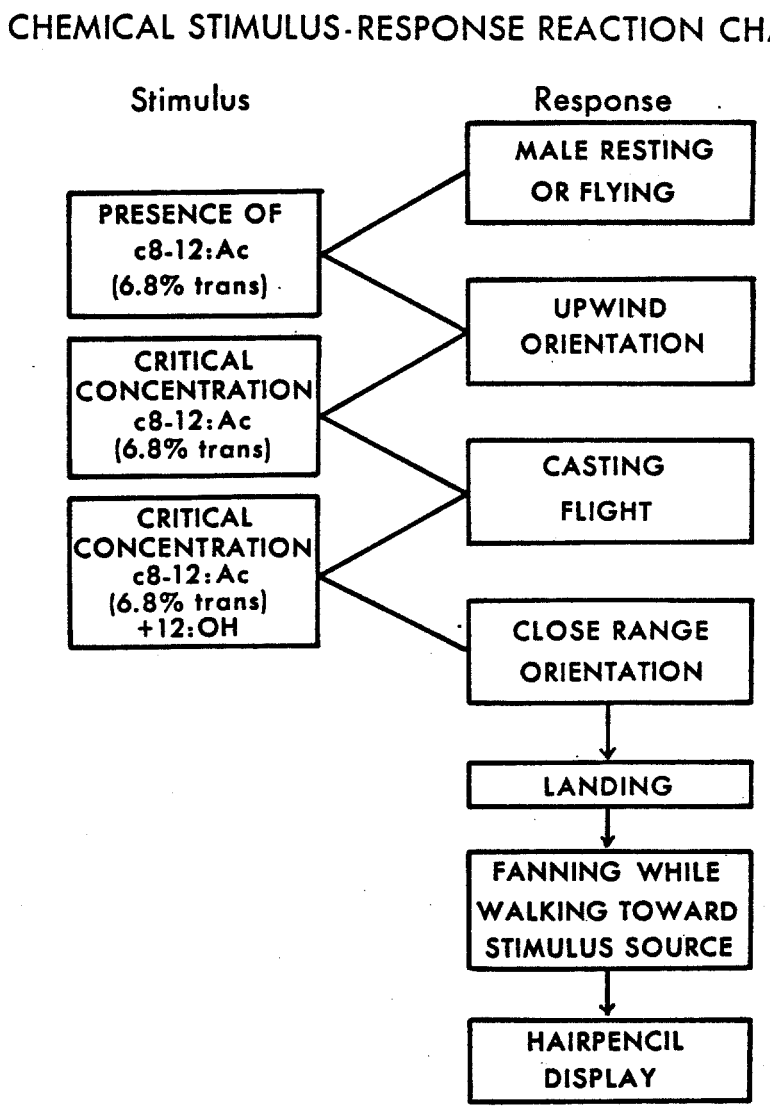

FIG. 3. Stimulus–response reaction chain. The behavior is indicated on the right and the chemical stimulus mediating the response is presented on the left.

flight path taken in upwind orientation. While the role of vision was not assessed directly, the presence of the arena surface is probably related to the mean closest approach to the chemical dispensers: males often flew for extended periods within several centimeters of the arena's edge.

The mean time spent within 20 cm of the arena, frequency of landing on the arena's surface, the mean time of wing fluttering concurrent with walking, and mean closest approach to the chemical dispenser were all elevated in response to the simultaneous emission of 12:OH in the presence of critical concentration of c8–12:Ac and t8–12:Ac over the emission of c8–12:Ac and t8–12:Ac alone. Extrusion of male hairpencils in precopulatory display ritual was evoked by 12:OH. Interestingly, such mediation of hairpencil display by a particular chemical component has not been reported previously, although George (1965) observed hairpencil extrusion in natural pheromone-stimulated *G. molesta* males. Indeed, the very existence of male brush organs within the Tortricoidea is not well documented.

The accumulated observations suggest that in *G. molesta* upwind anemotaxis is governed by a two-chemical blend, whereas close-range orientation and precopulatory display is effected by the addition of a third component. This mechanism contrasts with the hierarchy of behavior concept in which successive steps of the pheromone response sequence are elicited by increasing pheromone concentrations (Schwinck, 1955). This latter sort of pheromone-regulated behavior would be most likely in those species utilizing a communication system based on one chemical. The sequence of events in *G. molesta* as presently envisaged is summarized in the chemical stimulus–response reaction chain presented in Figure 3.

As yet only c8–12:Ac is known to be present in the pheromone-producing female abdominal tip of *G. molesta* (Roelofs et al., 1969), although it is reasonable to suppose on these results that additional components are involved in the natural mating communication system of this species. Notwithstanding, the finding that a specific chemical component can modify close-range orientation and precopulatory behavior has importance to the mating disruption technique, in which the pheromone or pheromone-related chemicals are dispersed throughout the air to prevent mating. Obviation of long-distance mate finding may not prevent mating (Cardé et al., 1975*b*) since attraction and mating behavior each may be modulated by a precise blend and concentration of components.

The complexity of manipulating pheromonal communication has been pointed out by Rothschild (1974) in disruption trials with *G. molesta* in Australia. Dodecyl acetate (12:Ac) emitted simultaneously with an attractant blend of c8–12:Ac containing ca. 3–7% t8–12:Ac from a single dispenser locus depressed male trap catch relative to the attractant blend alone (Roelofs et al., 1973; Roelofs and Cardé, 1974*a*; Rothschild, 1974). However, atmos-

pheric permeation with 12:Ac released from spaced evaporator stations elevated the catches of nearby traps baited with the attractant blend alone (Rothschild, 1974). When permeating the atmosphere, 12:Ac may habituate or modify the close-range behavioral repertoire normally elicited by additional components such as 12:OH. This habituating effect on close-range behavior could result in increased trap catches with traps baited only with the attractant blend.

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SOME CHEMICAL CONSTITUENTS OF THE SCENT OF THE STRIPED SKUNK (*Mephitis mephitis*)[1]

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Abstract—The malodorous, volatile portion of the scent or musk of the striped skunk (*Mephitis mephitis*) contains *trans*-2-butene-1-thiol, 3-methyl-1-butanethiol, and *trans*-2-butenyl methyl disulfide but no 1-butanethiol.

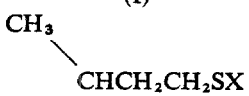
Key Words—skunk, musk, thiols, disulfides, mustelidae, *Mephitis mephitis*, *trans*-2-butene-1-thiol, 3-methyl-1-butanethiol, *trans*-2-butenyl methyl disulphide.

INTRODUCTION

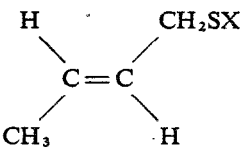
1-Butanethiol (I) is widely believed to cause the vile odor of striped skunk scent (Verts, 1967). We investigated the volatile portion of the scent and found it contained three malodorous compounds: *trans*-2-butene-1-thiol (II), 3-methyl-1-butanethiol (II), and *trans*-2-butenyl methyl disulfide (IV), but no thiol (I).

$CH_3CH_2CH_2CH_2SH$

(I)



(III), X = H
(VI), X = 2, 4-$(NO)_2C_6H_3$



(II), X = H
(IV), X = SCH_3
(V), X = 2, 4-$(NO_2)_2C_6H_3$
(VII), X = CH_3

[1] This work is taken from the M.S. thesis of D.T.B., University of New Hampshire, 1974.

METHODS AND MATERIALS

The nuclear magnetic resonance spectra were obtained using JEOL MN-100 and Varian A-60 spectrometers. The infrared absorption spectra were obtained using a Perkin-Elmer model 337 grating infrared spectrophotometer. The boiling points and melting points are uncorrected and are in degrees Celsius. The gas–liquid chromatographic (GLC) analyses and preparative purification of volatile compounds were achieved using an Aerograph A-90-P3 chromatograph.

Collection and Separation of the Volatile Scent Components

The scent was obtained from Mr. Bob Hoyt of Southwick, Massachusetts, who removed the fluid from live male and female striped skunks (*Mephitis mephitis*). The mixture, which separated into an aqueous and an oily phase with some fatty-appearing material at the interface, was stored frozen. The oily layer was distilled at 0.2 mm from a flask, initially cooled in liquid nitrogen, by gradually raising the bath temperature to 40°. A clear, malodorous liquid was collected in a receiving flask cooled in liquid nitrogen. A yellow-orange, viscous oil of much less odor remained behind. GLC of the distillate on a 6-ft × 1/4-in glass column packed with 15% Apiezon-M on Chromosorb-P 60/80 gave 3 fractions which were labeled IIa, IIIa, and IVa. At a column temperature of 75°, their retention times were 23.7, 41.7, and 150.0 min, respectively, and at 120°, 16.8, 28.8, and 55.2 min, with peak areas of 4:3:3, respectively. The three major components were collected, but a fourth trace component emerging after IVa was not investigated.

Characterization of Components IIa, IIIa, and IVa

The three natural components, IIa, IIIa, and IVa, were identified by comparison of their NMR and IR spectra with those obtained from synthetic samples, IIb, IIIb, and IVb. Identical spectra were obtained for each pair, IIa and IIb, IIIa and IIIb, and IVa and IVb. The NMR spectral parameters are given below. Using 1-chloro-2,4-dinitrobenzene, thiols IIa and IIIa were converted to solid sulfide derivatives which did not depress the melting point of authentic samples prepared from IIb and IIIb.

Moreover, GLC analysis of I, IIa, IIb, IIIa, and IIIb individually and in mixtures showed that I, II, and III could easily be separated from one another while IIa and IIb could not nor could IIIa and IIIb (see below).

Gas–Liquid Chromatographic Analyses

Various mixtures of thiols I, II, and III were analyzed by GLC on 12-ft ×

1/4-in Teflon-lined aluminum columns packed with 20% SE-52 on Chromosorb-P 60/80 (runs 1–6) or 10% diisodecylphthalate on Chromosorb-P 60/80 (runs 7 and 8). The results listed in the order of run number, compounds, retention times in min, and column temperature were: 1, I, IIa, IIIa, 13.6, 14.5, 22.5, 50°; 2, IIa, IIIa, 14.6, 22.8, 49°; 3, I, 13.6, 50°; 4, I, IIIb, 13.8, 23.6, 46°; 5, IIa, IIIa, IIIb, 14.8, 23.5, 46°; 6, IIIb, 23.5, 46°; 7, IIb, 5.7, 70°; 8, IIa, IIb, IIIa, 5.7, 8.2, 70°.

2-Butene-1-thiol (IIb)

Thiol IIb was prepared according to the procedure of Birch and McAllan (1951): IR (neat) 965 cm^{-1} (*trans* C = C); NMR ($CDCl_3$) δ 1.40 (t, 1, $CH_2S\underline{H}$), 1.64 (d, 3, $C\underline{H}_3CH{=}C$), 3.16 (m, 2, $C{=}CHC\underline{H}_2SH$), 5.60 (m, 2, $C\underline{H}{=}C\underline{H}$).

3-Methylbutane-1-thiol (IIIb)

Concentrated sulfuric acid (142.8 g, 1.42 mol) was added slowly with stirring to 48% hydrobromic acid (500 g, 6.17 mol) in a 1-liter flask. 3-Methyl-1-butanol (211 g, 2.39 mol) was added in one portion followed by 21.8 g (0.22 mol) of concentrated sulfuric acid. The reaction mixture was refluxed for 3 hr and then distilled until the distillate was clear. The organic layer was separated and washed successively with water, an equal volume of concentrated hydrochloric acid, 5% sodium bicarbonate, and water, and finally dried over magnesium sulfate. The crude product was fractionally distilled: bp 117–120°, lit. bp 122° (Heilbron, 1965, Vol. 1, p. 460); 128 g, 51.5% yield.

1-Bromo-3-methylbutane (52.0 g, 0.500 mol) and thiourea (38.0 g, 0.500 mol) were dissolved in 95% ethanol (250 ml), and the solution was heated on a steam bath for 3 hr. Sodium hydroxide (30 g in 300 ml of water, 0.75 mol) was added, and the resulting mixture was heated for 2 hr on a steam bath. The layers were separated and the aqueous layer acidified with 10% sulfuric acid and extracted with 75 ml of benzene. The organic layers were combined, washed twice with water, and then dried over sodium sulfate. The crude product was obtained by fractional distillation and purified by preparative GLC using a glass column 6-ft × 1/4-in, 20% SE-52 on Chromosorb-P 60/80, at a column temperature of 55° (Heilbron, 1965, Vol. 4, p. 2142). NMR ($CDCl_3$ δ 0.90 (d, 6, $(C\underline{H}_3)_2CH$), 1.45 (m, 4, $C\underline{H}C\underline{H}_2CH_2S\underline{H}$), 2.53 (q, 2, $C\underline{H}_2SH$).

trans-2-Butenyl 2,4-dinitrophenyl sulfide (V)

Thiol IIb (4.4 g, 0.05 mol) was added to an equimolar amount of sodium

hydroxide (2.00 g, 0.05 mol) in 20 ml of absolute ethanol. The solution was stirred for 2 hr. The solvent was then removed on a rotatory evaporator. 1-Chloro-2,4-dinitrobenzene (9.68 g, 0.048 mol) was added to a dimethylformamide (DMF) solution of the mercaptide, and the reaction was stirred with heating for 10 hr. The mixture was poured into 200 ml of ice water containing 98 g of 25% sodium hydroxide. The resulting mixture was stirred for 1 hr at −13°. The solids were collected, recrystallized from ethanol, and dried under reduced pressure: mp 97.5–98.5°, lit. mp 98.5–99° (Birch and McAllan, 1951). Thiol IIa was similarly treated to give VI, mp 97.5–98.5°. The mixture mp gave no depression.

3-Methylbutyl-2,4-dinitrophenyl sulfide (VI)

Thiol IIIb (0.50 ml, 4.2 mmol), sodium hydroxide (0.16 g, 4.0 mmol), and absolute ethanol (10 ml) were stirred until the solids were dissolved. An excess of thiol was used to ensure complete reaction of the sodium hydroxide. The solvent was then removed using a rotatory evaporator, and the resulting mercaptide was dissolved in DMF. 1-Chloro-2,4-dinitrobenzene (0.80 g, 4.0 mmol) was added with stirring. The DMF solution was heated to reflux with stirring for 5 hr. The reaction mixture was then poured into 20 g of ice water containing 9.6 g of a 25% solution of sodium hydroxide and was stirred for 1 hr at −13°. The solids were collected and recrystallized from ethanol, mp 77–77.5°, lit. mp 59° (Bost et al., 1932); NMR ($CDCl_3$) δ 0.99 (d, 6, $(C\underline{H}_3)_2CH$), 1.71 (m, 3, $C\underline{H}C\underline{H}_2C\underline{H}_2$), 3.07 (t, 2, $CH_2C\underline{H}_2S$), 7.58 (d, 1, 6-Ar$\underline{H}$), 8.35 (q, 1, 5-Ar$\underline{H}$), 9.00 (d, 1, 3-Ar$\underline{H}$).

Analysis. Calculated for $C_{11}H_{14}N_2O_4S$: C, 48.88; H, 5.21; N, 10.36. Found: C, 49.01; H, 5.21; N, 10.29.

Thiol IIIa was treated similarly to give VII, mp 77–78°. The mixture mp gave no depression.

trans-*2-Butenyl methyl disulfide (IVb)*

S-Methyl thiophthalimide (0.1 g, 0.51 mmol) and thiol IIb (49.8 μl, 0.51 mmol) were refluxed in anhydrous benzene (3 ml) for 6 hr during which time a white solid precipitated (Harpp et al., 1970). The reaction mixture was centrifuged and the liquid drawn off. The disulfide was obtained by preparative GLC on a 12-ft × 1/4-in Teflon-lined aluminum column, 10% diisodecylphthalate on Chromsorb-W 60/80, 70°. NMR ($CDCl_3$) δ 1.70 (d, 3, $C\underline{H}_3CH{=}C$), 2.32 (s, 3, $C\underline{H}_3SS$), 3.46 (d, 2, $SC\underline{H}_2CH{=}C$), 5.58 (m, 2, $C\underline{H}{=}C\underline{H}$).

DISCUSSION

Swarts (1862), working in collaboration with F. Wohler, investigated the scent of the "North American" skunk and found it contained 16% sulfur. Earlier work by Lassaigne was mentioned, though not referenced.

But it was the work by Aldrich (1896) on *Mephitis mephitis* which led to the belief that 1-butanethiol (I) was responsible for the repulsive odor of skunk scent or musk. He separated the oily portion of the scent into two volatile and one nonvolatile fractions by distillation at atmospheric pressure. The lower boiling fraction was characterized by conversion to and elemental analysis of mercury (R_2Hg) and lead (R_2Pb) salts. His experimental values are shown in Table 1 together with values calculated for R equal to butyl (C_4H_9) and to butenyl (C_4H_7). His results are ambiguous because the C and H analyses of both the lead and mercury salts are closer to the values calculated for R = C_4H_7 while the Pb analyses agree better with the value calculated for R = C_4H_9. Aldrich did not characterize the higher boiling fraction or pot residue, but he did prepare 3-methyl-1-butanethiol and noted its similarity in odor and reactions to both volatile fractions.

In our work, done with the advantage of modern instruments, we analyzed the volatile portion of the organic phase of the scent. It is this part which has the characteristic odor of the defensive spray. After a preliminary distillation, we separated the material into three substances by GLC and identified them spectroscopically and chemically as thiols II and III and disulfide IV.

NMR spectroscopy readily identified the thiols, and this identification was confirmed by comparison of their NMR and IR spectra to those obtained

TABLE 1. ELEMENTAL ANALYSES OF SUPPOSED 1-BUTYL MERCAPTIDES

	Found[a]	Calculated for $(C_4H_9S)_2Pb$	Calculated for $(C_4H_7S)_2Pb$
C	25.45 25.31	25.00	25.18
H	3.95 3.89	4.68	3.67
Pb	53.22 53.02	53.64	54.30
		Calculated for $(C_4H_9S)_2Hg$	Calculated for $(C_4H_7S)_2Hg$
C	25.90	25.40	25.62
H	3.88	4.76	3.73

[a] Data from Aldrich (1896).

from synthetic samples, as well as by derivative formation. Our mp of 77–78° for VI, the derivative of III, did not agree with the literature value of 59°. However, the NMR spectra and elemental analysis of VI did agree with the proposed structure. Either the literature mp is in error or else it refers to another crystal modification.

Disulfide IV was identified by comparison of its NMR and IR spectra to those obtained from an authentic sample. The NMR spectrum of IV clearly differed from the spectrum of *trans*-2-butenyl methyl sulfide (VII).

GLC of various mixtures of I, II, and III easily separated thiol I from thiols IIa and IIIa. Since we did not observe a peak corresponding to I, this thiol was not present in our sample of scent. Thiols IIa and IIIa were not separable from their synthetic counterparts IIb and IIIb, thus offering additional support for their structure.

Neither our work nor Aldrich's supports the presence of 1-butanethiol (I) in the scent of the striped skunk. Beckmann (1896), however, reported I in the scent of the teledu (*Mydaus marchei Huet*), a mustelid of the Philippines. Fester and Bertuzzi (1937) steam distilled the scent of a South American skunk (*Conepatus suffocans*) and obtained an oil. They concluded on the basis of an elemental analysis that it contained II and its disulfide, but their experimental and calculated values differed by an unacceptable 2%. Stevens (1945) isolated a small amount of dicrotyl sulfide from *Mephitis mephitis* scent after first precipitating the thiols with mecuric chloride. Although we did not detect this sulfide, it may have been relatively nonvolatile or else present in very low concentration.

While we have identified the malodorous compounds in striped skunk scent, the nature of the nonvolatile and aqueous-phase components is not known nor is the biochemical origin of II, III, and IV.

Acknowledgments—We thank Mr. Bob Hoyt and Mr. S. Rae Mackay, III, for procuring a sample of striped skunk scent, Prof. D. N. Harpp for a sample of *S*-methyl thiophthalimide, and the Central University Research Fund for financial assistance.

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PHENOLS AS PHEROMONES OF IXODID TICKS: A GENERAL PHENOMENON?

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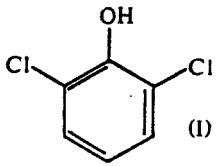
Abstract—Extracts of female *Rhipicephalus appendiculatus* Neumann and *R. pulchellus* Neumann, vectors or potential vectors of East Coast fever, have yielded phenol and *p*-cresol. The same phenols have been found in five additional species of hard ticks, three of which also contained 2,6-dichlorophenol. Salicylaldehyde appeared as another phenolic component in four of these species. On the basis of this information and of the rather limited literature available, it appears that ixodid ticks may generally utilize simple phenols as attractants. Behavioral observations suggest a role of the phenols in the meeting of the sexes.

Key Words—ticks, ixodid ticks, pheromone, *Rhipicephalus appendiculatus*, *Rhipicephalus pulchellus*, phenol, cresol, 2,6-dichlorophenol, salicylaldehyde.

INTRODUCTION

Since the first pheromone (bombykol) was chemically characterized (Butenandt et al., 1959), research on chemical communication has made increasingly rapid progress. Insect sex attractants have received the most attention; the list of chemically defined sex pheromones is long and constantly growing (MacConnell and Silverstein, 1973). Much less is known about other arthropod groups. Thus, while references have been made to the presence of sex pheromones in several species of ixodid ticks (Berger et al., 1971; Chow et al., 1972), it was not until 1973 that 2,6-dichlorophenol (I) was reported to serve

as the female pheromone of the lone star tick, *Amblyomma americanum* (L.) (Berger, 1972).



(I)

We now report the chemical characterization of compounds which appear to serve as female pheromones from two species of hard ticks, *Rhipicephalus appendiculatus* Neumann and *R. pulchellus* Neumann, which are of major economic importance, since they serve as vectors (or potential vectors) of East Coast fever, a protozoan infection lethal to cattle in East Africa (Arthur, 1962). Evidence of the attractivity of females to males in these species will be presented elsewhere. The isolation, bioassay, and characterization of the compounds responsible for the attractivity is described below.

METHODS AND MATERIALS

Virgin females (*R. appendiculatus*, ca. 7000), which are most attractive to males on the sixth day of feeding, were fed on rabbit ears for 6 days prior to their removal and freezing. It was found that simply washing the intact, frozen bodies with ether gave an extract which was active in the following bioassay. A glass T-tube (2-cm stem, 6-cm top, and 7 mm ID) is placed upright over a single virgin male which had fed on a rabbit for 8–10 days. In this state, *Rhipicephalus* males exhibit maximal response toward the females. The tick washing or other test sample, dissolved in ether (BDH, AnalaR

TABLE 1. FIRST CHOICE OF T-TUBE ARM CONTAINING FILTER PAPER TREATED WITH ETHEREAL EXTRACT OR OTHER SOLUTIONS, BY 6–10-DAY FED *R. appendiculatus* MALES

Sample	Microliters of solution			
	0.1	1	10	100
Original tick washings (from female)	76/100	70/100	77/100	88/100
Phenol (2 ng/μl) and *p*-cresol (0.57 ng/μl)	77/100	76/100	77/100	83/100
Phenol (2 ng/μl)	40/50	39/50	40/50	44/50
p-Cresol (0.57 ng/μl)	40/50	37/50	42/50	45/50
Ether	—	24/50	28/50	28/50
Clean filter papers	(26/50)			

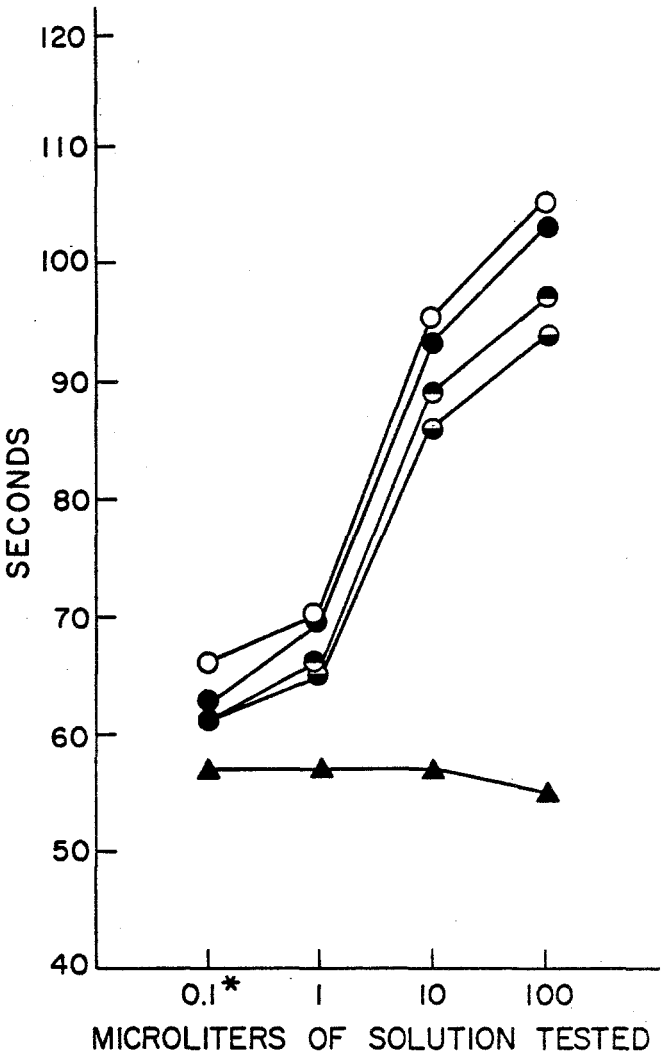


FIG. 1. This graph shows the average number of seconds within a 120-sec test period that *R. appendiculatus* males spent in the arm of the T-tube containing tick extracts or synthetic pheromone solutions, plotted against the volume of solution tested. ○, original tick washings (100 ticks each point); ●, solution containing 2 ng phenol and 0.57 ng *p*-cresol per microliter of ether (50 ticks each point); ◒, solution containing 2 ng of phenol per microliter of ether (50 ticks each point); ◓, solution containing 0.57 ng of *p*-cresol per microliter of ether (50 ticks each point); ▲, ether control. * One microliter of ether in a 1:10 dilution of stock solutions.

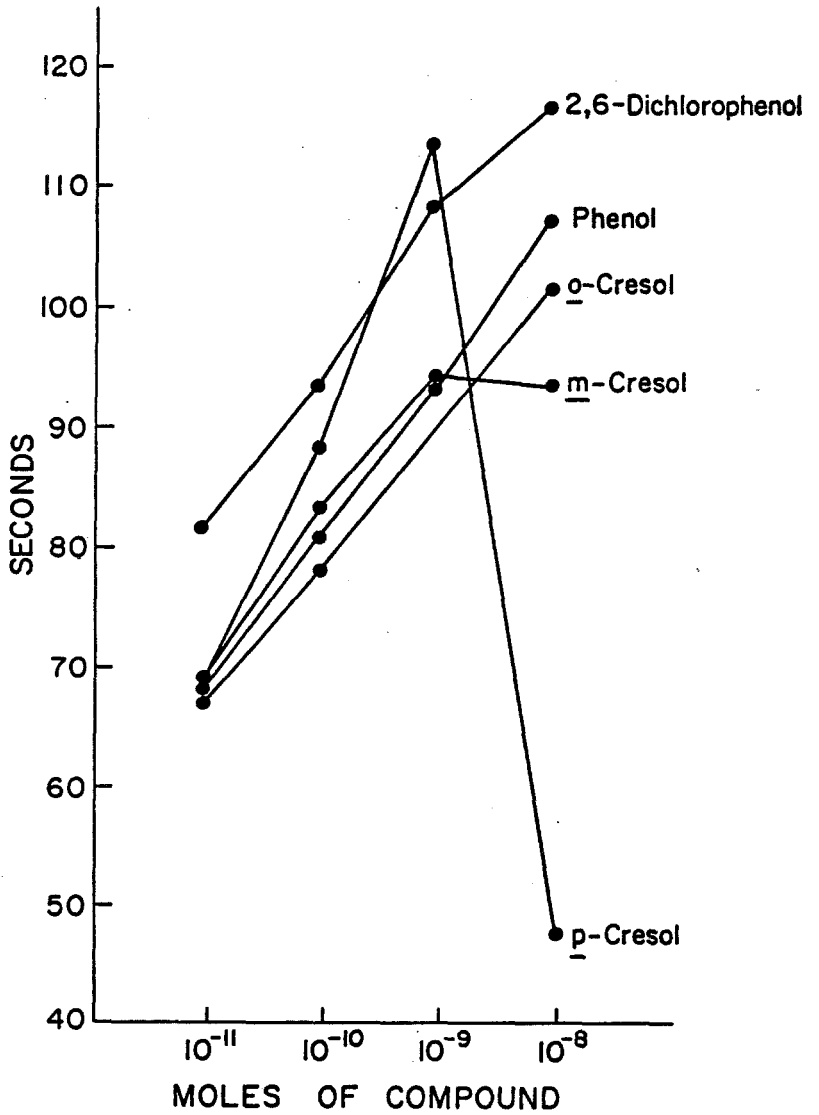
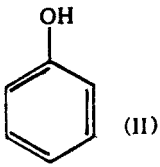


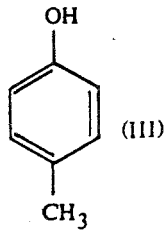
FIG. 2. A plot of the average time (in a 120-sec test period) that 50 *R. appendiculatus* males spent in the arm of a T-tube containing a variety of phenols, as a function of the amount of phenol used.

Grade), is applied to a small circle of filter paper (3 cm diameter). After evaporation of the ether, the filter paper is made into a plug which is inserted into one arm of the T tube, while the other arm is plugged with untreated filter paper. The male climbs up the tube, reaches the horizontal portion, and then enters one arm or the other; the first choice of the male (Table 1) is recorded. In addition, for each experiment the number of seconds out of 120 sec which the male spends on the side of the sample-containing arm is recorded (see Figures 1 and 2; at least 50 male ticks were used for each point shown).

Extraction of the initial ethereal female washing with 1 N sodium hydroxide left an inactive organic phase; acidification and ether extraction of the aqueous phase yielded an active, acidic fraction. Further washing with aqueous sodium bicarbonate, to remove carboxylic acids, left a biologically active ether solution which should contain weak, organic acids. Gas chromatographic examination of this material showed the presence of only two major, volatile components.[3] These had retention times indistinguishable from those of phenol (II) and *p*-cresol (III), and direct gas chromatographic–mass spectral comparisons[4] with authentic samples of II and III provided unambiguous identification of both compounds. In two such isolation experiments, the *p*-cresol recovered amounted to ca. 3.5 ng/female, while the phenol



(II)



(III)

content ranged from 4.5 to 13 ng. (Whether this variability reflects selective losses during the extraction procedure because of phenol's greater water solubility and volatility compared to those of *p*-cresol or an intrinsic variability in the production of II and III is still undetermined.)

RESULTS AND DISCUSSION

The data summarized in Table 1 indicate that the first choice of males in the T-tube assay favored that side of the T-tube which contained filter paper

[3] Gas chromatographic analyses were carried out on a Hewlett-Packard 204 instrument using 5% Carbowax 20M as the stationary phase.

[4] Analyses were performed on a Finnigan 1015 D GC-MS utilizing 5% Carbowax 20M as the stationary phase.

TABLE 2. AMOUNT OF PHENOL AND *p*-CRESOL PRODUCED PER TICK (IN NANOGRAMS) BY DAY OF FEEDING

Day	Number of ticks	Phenol	*p*-Cresol
0	3000 virgin females	none detected	none detected
2	1300 virgin females	1	0.2
4	1500 virgin females	2.1	2.7
6	1000 virgin females	3.8	4.2
8	950 virgin females	4.2	3.3
10	850 virgin females	8.6	6.2
12	800 virgin females	11	5.8
8	600 mated females	3.2	21.8
6	3000 virgin males	none detected	none detected

treated either with female washings, a mixture of II and III, or these phenols individually. Furthermore, once the choice was made, the males tended to remain in the arm containing the phenolic component(s).

Figure 1 presents the average time males spent on the side of a test sample, again in response to (1) the original female washings, (2) a mixture of II and III, and (3) solutions of II and III alone. From the results presented in Table 1 and Figure 1, we conclude that the mixture of II and III can account for the attractivity shown by the natural extract in our T-tube bioassay. Furthermore, in contrast to observations on some insect species whose pheromones are mixtures (MacConnell and Silverstein, 1973), the individual phenolic components also show comparable activity.[5]

Washings from unfed females did not contain significant amounts of II and III, implying either that unfed females do not yet contain these compounds or that they do not release them. The washings from fed males also lacked these phenols. Two days after initiation of feeding, however, washings from females begin to show small amounts of the phenolic compounds. The quantities increased with the feeding period and reached their peak when the mated females were fully engorged (Table 2). Thus mating does not terminate phenol production. It may be relevant to mention here that ticks are not monogamous; multiple insemination has been described in several species (Arthur, 1962).

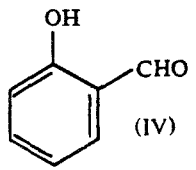
Preliminary studies of the structural specificity of this chemically very simple pheromone system have indicated a surprising lack of receptor discrimination. For example both *o*-cresol and *m*-cresol elicit a positive response (see Figure 2), and even the *A. americanum* pheromone, I, whose activity, size, and shape all differ markedly from those of II and III, proved

[5] See page 509 for note added in proof.

attractive to *R. appendiculatus* males. In view of this result, a special effort was made to ascertain whether *R. appendiculatus* females produce I itself. However, no 2,6-dichlorophenol could be detected by gas chromatographic analysis (5% Carbowax 20M) in the extracts of either *R. appendiculatus* or *R. pulchellus* virgin females. That this phenol was not lost in the extraction procedure was established, since small quantities of I could be recovered and detected almost quantitatively in a control experiment.

Following the above-described procedures, active washings of *R. pulchellus* virgin females were also found to contain II and III (in a 1:5 ratio). These compounds again accounted for all of the observed pheromonal activity in the T-tube assay.

Finally, washings of 6-day fed *R. simus* Koch and *R. compositus* Neumann females were found to contain II and III, while those of *Amblyomma americanum*, *A. variegatum* (Febricius), and *Hyalomma truncatum* (Koch) showed the presence of I, II, and III. In addition, the four last-mentioned species showed an additional phenolic component whose GLC retention time was shorter than that of phenol itself, which was identified as salycilaldehyde (IV) on the basis of direct GLC and mass spectral comparisons with an



(IV)

authentic sample. These analytical results are summarized in Table 3. No bioassays were carried out with these additional species. Recently reported studies on *R. sanguineus* (Latrelle) and *Boophilus microplus* (Canostrini) (Chow et al., 1972) also implicate as yet uncharacterized phenols as female pheromones.

In view of the number of species producing phenols, the apparent lack of receptor specificity, and the observations of interspecific matings among ixodid ticks (Oliver et al., 1972; Gladney and Dawkins, 1973), it may well be that the pheromonal vocabulary of these arachnids is restricted to this group of simple aromatic compounds. We believe that these phenols play a role in the orientation of the sexually mature male toward the feeding female. In *Metastriata* species most females rarely change an attachment site once feeding begins, while males become motile after initial feeding and become stationary again upon contacting a female (Oliver, 1974). We observed that *R. appendiculatus* males after 6–10 days of feeding try to copulate with anything that is about the same size and shape as a partially fed female. Thus a chemical messenger emitted by the feeding female might be of great help in the proper orientation of the male. Unfed males, which do not mate, as well

TABLE 3. PHENOLIC COMPOUNDS[a] IN HARD TICKS (IN NANOGRAMS/6-DAY FED FEMALE TICK)

Species	No. washed	Salicyl-aldehyde	Phenol	*p*-Cresol	2,6-Dichloro-phenol
Ripicephalus appendiculatus	7000	—	4.5–13	3.5	—
R. pulchellus	2700	—	1.8	10	—
R. simus	4700	—	3.8	5.0	—
R. compositus	1750	0.3[b]	3.2	1.1	—
Amblyomma americanum	1900	0.3[b]	4.6	2.9	2.2
A. variegatum	523	3.6	52	47	16.
Hyalomma truncatum	459	7.5	67	4.4	2.2

[a] Identified by comparison of GLC retention times and mass spectra with those of authentic samples. Compounds are listed in order of increasing retention times on a 5% Carbowax 20M column.
[b] Identified by GLC retention time only.

TABLE 4. RESPONSE OF *R. appendiculatus* TO SOLUTIONS CONTAINING 2 NG PHENOL AND 0.57 NG *p*-CRESOL PER MICROLITER ETHER (SECONDS OUT OF 120 SPENT IN THE TREATED ARM)

	Microliters of solution			
	0.1	1	10	100
Unfed females	not done	61 ± 25	65 ± 34	86 ± 38
Unfed males	61 ± 7.8	63 ± 9.7	67 ± 5.8	86 ± 7
Fed males	62 ± 6.7	69 ± 6.2	93 ± 6.2	103 ± 9.0

as unfed females exhibit only slight attraction to II and III (Table 4). The preferential attraction of the mature male, taken along with the fact that females produce phenols only when they are feeding, suggest that these compounds can be designated as sex pheromones of *R. appendiculatus* and *R. pulchellus*.

Acknowledgments—The cooperation and encouragement of Prof. T.R. Odhiambo is gratefully acknowledged. We thank Miss Surita Bambrah for carrying out the bioassays. We are indebted to the United Nations Development Programme and to the National Institutes of Health (Grant No. AI-12020) for partial support of this research.

NOTE ADDED IN PROOF

In more recently carried out bioassays, phenol alone no longer appeared to be highly attractive. Nevertheless, the attractivity of phenol+*p*-cresol mixtures, of *p*-cresol alone, and of 2,6-dichlorophenol for *R. appendiculatus* males could be confirmed. The reason(s) for this variability in the ticks' behavior remains undetermined.

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BOOK REVIEW

PHEROMONES. Frontiers of Biology, Vol. 32. *Martin C. Birch (Ed.).* Amsterdam and New York: North-Holland and American Elsevier, 1974. 495 pp. $42.50.

The biotic community is a dynamic system of interacting plant and animal populations in which organisms are continuously subjected to some sort of pressure from other species. In this complex interaction of an organism with its environment the organism must often give signals and receive information from other organisms to insure its survival and the preservation of the species. This transfer of information between organisms may occur through one or several sensory modes depending on their relative degree of physiological development.

In the past 15 years it has been determined that chemical communication may play a major role in the survival of organisms ranging from bacteria to primates. Chemical communication in nature is of two general types: between species (interspecific) and within species (intraspecific). It is the group of intraspecific chemical signals named "pheromones" by Karlson and Butenandt (1959) that we are dealing with in this book.

Birch and his coauthors have written a book composed of three sections dealing primarily with the biology of pheromonal communication in insects and vertebrates. Section I, consisting of 13 chapters dealing with insect pheromones, discusses gland structure, pheromone perception, orientation mechanisms, control of reproductive behavior, reproductive isolation, speciation, and aggregation related to pheromone systems in Lepidoptera, Scolytidae, and some Coleoptera. The final chapters of this section discuss the importance of pheromone systems in social insects.

Section II, consisting of 7 chapters dealing generally with vertebrate pheromones, discusses the use of pheromonal communication in the reproduction, aggression, social dominance, territorial marking, various forms of scent marking, recognition, and alarm signaling of fish, amphibia, small animals and primates. This section concludes with a theoretical discussion of the possibility of human pheromones.

Section III is dedicated to an examination of the use of pheromones in the management, survey, and control of animal populations. The discussion is slanted primarily toward field trials with insects and concludes with a short discussion on mammals.

This book provides the most comprehensive treatment of the subject of chemical communication systems currently available and is essential reading

for all scientists who are interested in chemical communication systems. The book is well organized and adequately covers the current pool of knowledge on insect and vertebrate pheromones. Occasionally the interpretation of literature reflects more closely an author's own research interests than the general interpretation of the scientific community, but this is to be expected in a multiauthor book.

It is unfortunate that a section on chemical communication systems in microorganisms and plants was not provided. Many interesting ideas and papers exist on chemoreception, orientation mechanisms, behavior, and evolution of pheromone systems in these organisms. It is probable that the study of these systems will increase in number and importance during the next few years.

Although most of the topics have been reviewed previously, the book will still become influential because it presents an integrated overview of a discipline. Most specialists will be pleased with the book and refer to it repeatedly in the future.

FRED E. REGNIER

ERRATUM

Table 4 of the recent paper "Mutual Inhibition of the Attractant Pheromone Response by Two Species of *Ips* (Coleoptera: Scolytidae)" by M. C. Birch and D. L. Wood [*J. Chem. Ecol.* 1(1), 1975, p. 108] inadvertently read 4.50 for the number of *I. pini* trapped in Test 1; the number should have read 45.0.

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