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FEMALE SEX PHEROMONE OF THE MELONWORM, *Diaphania hyalinata* (LEPIDOPTERA: PYRALIDAE), AND ANALYSIS OF MALE RESPONSES TO PHEROMONE IN A FLIGHT TUNNEL[1]

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Abstract—Ten C_{16} chain-length compounds were identified from heptane extracts of ovipositors of female melonworm, *Diaphania hyalinata* (L.). The major constituents of the extracts were (*E*)-11-hexadecenal and (*E,E*)-10,12-hexadecadienal [(*E,E*)-10,12-16:Ald] and the alcohols and acetates of these olefins were found in trace amounts (<2%). Extracts also contained traces of (*E,Z*)- and (*Z,Z*)-10,12-16:Ald, hexadecanal, and 1-hexadecanol. Analysis of the behavioral responses of males to synthetic mixtures of these compounds and responses to ovipositor extracts in a flight tunnel showed that a synthetic mixture of the 10 compounds elicited a behavioral repertoire from males that was indistinguishable from that elicited by ovipositor extract. Flight tunnel studies also indicated that six of the 10 compounds probably represent the essential components of the female's sex pheromone.

Key Words—*Diaphania hyalinata, D. nitidalis*, melonworm, pickleworm, Lepidoptera, Pyralidae, sex pheromone, insect behavior, flight tunnel, 10,12-hexadecadienal, 11-hexadecenal.

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INTRODUCTION

Larvae of the melonworm, *Diaphania hyalinata* (L.), feed almost exclusively upon the foliage of pumpkin and other cucurbits, and it is rarely considered a pestiferous insect. The impetus for studying the sex pheromone of *D. hyalinata* was an interest in the comparative sex pheromone chemistry of *D. hyalinata* and *D. nitidalis* (Stoll). The two species occur sympatrically and exhibit similar life cycles and habits; however, *D. nitidalis* is considered a serious pest because it feeds upon and damages the fruits of cucurbits. The results reported here define the chemistry of pheromone-like compounds from the melonworm female and male behavioral responses to synthetic mixtures of the female-borne compounds. These findings were pivotal to elucidation of the *D. nitidalis* sex pheromone (Klun et al., 1986) because compounds discovered here aided in the detection of minute amounts of similar compounds in *D. nitidalis* and the combined findings provide a basis for comparative insight into the pheromone communication systems of the two species.

METHODS AND MATERIALS

Insects. Insects used in this study were reared on pinto bean diet (Robinson et al., 1979) at the Vegetable Insects Laboratory in Charleston, South Carolina. Pupae were shipped to Beltsville, Maryland, and pupae and adults of each sex were kept in separate enviornmental chambers that were maintained under a reverse photoperiod of 16:8 light–dark, 26°C light, 20°C dark; 60% relative humidity. Adults were provided a 10% sucrose solution.

The ovipositors (terminal abdominal segments containing the pheromone gland) of 2- to 3-day-old females at 5–6 hr of scotophase (the time of their sexual activity in nature: K.D. Elsey, personal communication) were excised and transferred to heptane (ca. 5 μl/ovipositor). The tissue was soaked for ca. 30 min, and then the solvent was drawn away from the tissue using a 50-μl syringe. The behavioral response of males to the ovipositor extracts signaled the presence of female sex pheromone, and these extracts served as a resource for chemical analyses.

Chemicals and Analytical Methods. The combined extracts of several hundred melonworm ovipositors were analyzed by open-tubular capillary chromatography (OTCC) using 60 m × 0.25 mm (ID) polar and nonpolar (SE-30) columns and by OTCC–mass spectrometry using instruments and operating conditions described by Klun et at.(1982). The ovipositor extracts were derivatized by successive treatment with $NaBH_4$ and acetic anhydride–pyridine (9:1) (Klun et at., 1982), fractionated by preparative gas–liquid chromatography (Schwarz et al., 1983), and the chromatographic fractions were ozonized and

the ozonolysis products analyzed by OTCC (Klun et al., 1982) to establish sites of unsaturation in the compounds.

The monounsaturated and saturated C_{16}-chain-length compounds used in this study were purchased. All 10,12-hexadecadienes were stereoselectively synthesized in our laboratory using the methods described below.

(Z,Z)-10,12-hexadecadienal. Synthesis of this compound was patterned after the preparation of (Z,Z)-9,11-hexadecadien-1-ol reported by Svirskaya et al. (1980). Thus, 10-undecyn-1-ol was coupled in a Cadiot-Chodkiewicz reation with 1-pentyne to produce 10,12-hexadecadiyn-1-ol. The latter compound was reduced stereospecifically to (Z,Z)-10,12-hexadecadien-1-ol with dicyclohexylborane. Oxidation of the alcohol with pyridinium chlorochromate (Corey and Suggs, 1975) afforded the desired (Z,Z)-10,12-hexadecadienal.

(E,Z)-10,12-Hexadecadienal. This compound was prepared by the reaction scheme reported for the same compound by Hall et al. (1980).

(E,E)-10,12-Hexadecadienal. Pure (E,E)-10,12-hexadecadien-1-ol was obtained by low-temperature recrystallization from pentane of the equilibrium mixture of all four isomers of this alcohol obtained after treatment of (E,Z)-10,12-hexadecadien-1-ol with 0.5% thiophenol at 100°C (Henrick et al., 1975). Pyridinium chlorochromate oxidation (Corey and Suggs, 1975) then afforded the desired aldehyde.

Each of the compounds used in bioassays was purified by argentation high-pressure liquid chromatography (Klun et al., 1982), and they were >95% pure based upon OTCC.

Flight Tunnel, Test Procedures, and Experimental Design. The flight tunnel was 3 m × 60 cm, horseshoe-shaped in cross-section, and made from Plexiglas. Two air conditioners, with a combined capacity of 42,000 BTU, provided the source of air that was conditioned in a chamber (12 m^3 provided with supplemental heating and humidification) at the head of the flight tunnel. The air was blown from the chamber by a fan, driven by a 0.75 HP variable-speed motor, through a Varicel filter and cheesecloth screen into the tunnel. the downwind end of the tunnel was fitted with an 8-mesh stainless-steel screen having a 7-cm-diam. circular hole at the center of the tunnel's cross-section 16 cm from the tunnel floor. The opening was fitted with a collar that held a Plexiglas tube (20 cm long × 6.5 cm diam.) that was used for release of individual males. The release tube was fitted with a spring-mounted lid on the upwind end and a cheesecloth cover on the other. Air flow from the tunnel was directed into a hood and exhausted to the outside of the building. Three sliding windows, located along the side of the tunnel, provided access to the interior of the tunnel across its length. Overhead illumination was provided by red incandescent bulbs that were rheostat-regulated to 2.5 lux. A laminar air flow was maintained through the tunnel at 50 cm/sec and 20.5 ± 1.5°C. The position of the stimulus-plume generated in the tunnel was determined by observing the path of

smoke generated from a burning incense stick. The observation indicated that the plume traversed the length of the tunnel in a narrow band and exited the tunnel through the male-release tube at the downwind end of the tunnel.

Males, 2–3 days old, were used in the bioassays after they had been conditioned for ca. 30 min in the flight-tunnel room. Males were used once and discarded. Tests were carried out at 5–6 hr of scotophase. Each male was allowed to settle on the cheesecloth at the downwind end of the release tube, and observations were commenced when the lid was removed from the tube.

Based on previous observations of male response to the extracts of female glands and virgin females in the tunnel, six behavioral categories were chosen for quantitative analysis. These were (1) Activation (a dichotomous variable), characterized by onset of wing fanning and rapid walking, each male was allowed a maximum of 2 min to express the activation response; (2) time to activation, represented elapsed time from the removal of the lid to start of wing fanning; (3) upwind flight in plume, a dichotomous variable, represented the percentage of insects that traversed the length of the tunnel and arrived at the stimulus source; (4) flight time, the elapsed time from start of flight to arrival at the source; (5) time near source, the time a male spent hovering near the source with extended claspers and making frequent antennal and tarsal contacts with the pheromonal stimulus; (6) return to source (dichotomous), represented those males that hovered at the source, flew away, reentered the plume, and returned to the source. A male was allowed a maximum of 2 min to return to the source. The test chemicals and ovipositor extracts were applied to a 4 × 0.5-cm filter paper strip that was suspended 16 cm above the tunnel floor from a narrow clip 20 cm from the upwind end of the tunnel. The quantity of ovipositor extracts applied to the paper was adjusted so that the quantity of female-derived compounds and the quantity of synthetic compounds applied to the paper were equivalent (according to OTCC).

The experimental design for the behavioral tests was a randomized complete-block design replicated 24 times over 24 days. All the variables involving time measurements in test I were analyzed by the nonparametric M-rank procedure because the data did not meet the assumptions for homogeneities of variances. The M-rank procedure is a generalization of the Friedman's test, which can be used for unbalanced data and a randomized complete-block design (Benard and Van Elteren, 1953). The computer program used was a supplemental procedure of the Statistical Analysis System statistic programs (Sarle, 1981). The dichotomous response variables were analyzed by the analysis of variance (Cochran, 1950). Significance was tested by χ^2 analysis, and the means were ranked using Duncan's multiple-range test.

In test II, three 2-day-old, virgin females or filter paper strips treated with synthetic pheromonal components were placed in a 5-cm × 3-cm-diam. stainless-steel screen cage that was suspended by the clip at the upwind end of the

TABLE 1. COMPOUNDS IDENTIFIED FROM OVIPOSITOR EXTRACTS OF FEMALE MELONWORM[a]

Compound number	Composition (%)	Compound (abbreviation)
1	50.4	(*E*)-11-Hexadecenal (*E*-11-16:Ald)
2	5.2	Hexadecanal (16:Ald)
3	0.3	(*E,Z*)-10,12-Hexadecadienal (*E,Z*-10,12-16:Ald)
4	0.5	(*Z,Z*)-10,12-Hexadecadienal (*Z,Z*-10,12-16:Ald)
5	39.1	(*E,E*)-10,12-Hexadecadienal (*E,E*-10,12-16:Ald)
6	1.8	(*E*)-11-Hexadecen-1-ol (*E*-11-16:OH)
7	0.4	1-Hexadecanol (16:OH)
8	2.0	(*E,E*)-10,12-Hexadecadien-1-ol (*E,E*-10,12-16:OH)
9	0.1	(*E*)-11-Hexadecen-1-ol acetate (*E*-11-16:OAc)
10	0.1	(*E,E*)-10,12-Hexadecadien-1-ol acetate (*E,E*-10,12-16:OAc)

[a] Percentage composition values are averages derived from analyses of the combined extracts of 62 females on polar and nonpolar capillary columns.

tunnel. Male responses to these treatments were compared by scoring male responses in the six behavioral categories. The amount of (*E*)-11-hexadecenal in each treatment of tests I and II was fixed at 252 ng, and the amounts of other compounds in the various treatments were proportional to the percentage compositions found in the female extracts (Table 1). The test was replicated 36 times over 36 days. Data were analyzed statistically using the same procedures described for test I.

Studies of the responses of feral melonworm males to pheromonal stimuli were attempted, but field populations of the insect were too low to allow acquisition of any meaningful data. Therefore, we had to rely on the flight-tunnel assay to assess the impact of the female compounds on male sexual behavior.

RESULTS AND DISCUSSION

Chemistry. Analyses of the ovipositor extracts of the females revealed the presence of 10 pheromone-like compounds (Table 1). The identities of these compounds were deduced from the coincidence of retention times with authentic reference compounds that were fully resolved on the capillary columns and by comparison of mass spectral fragmentation patterns with the reference compounds. Reduction of the ovipositor extract components with $NaBH_4$ followed by acetylation of the resulting alcohols yielded a mixture of five C_{16} acetates. These acetates had OTCC retention times coincident with (*E*)-11-16:OAc;

16:OAc; (*E,Z*)-, (*Z,Z*)-, and (*E,E*)-10,12–16:OAc, respectively. The (*E*)-11–16:OAc and (*E,E*)-10,12–16:OAc were major components of the mixture. This result was consistent with the analyses of the underivatized extract which showed that the corresponding C_{16} aldehydes were major components of the extract (Table 1). The mixture of acetates was fractionated by preparative chromatography; *E*-11–16:OAc and 16:OAc (fraction 1) and the 10,12–16:OAc isomers (fraction 2) were collected and ozonized. OTCC analysis of ozonized fraction 1 on polar and nonpolar columns showed the presence of two compounds: unreacted 16:OAc and 11-acetoxyundecanal. Ozonized fraction 2 yielded a single compound having OTCC retention times coincident with 10-acetoxydecanal on polar and nonpolar OTCC. These results confirmed that the site of unsaturation in the monoenes of the extract was 11 and that the site of conjugation in the dienes was 10,12.

Flight Tunnel Tests. Replicated observations (24/treatment) of responses of individual males to the ovipositor extracts in the flight tunnel (Table 2, treatment M) revealed the following: Males became active within 8 sec after exposure to the pheromone and subsequently took flight. Plume-oriented upwind flight was completed by 83% of the males tested. The average flight time from the release-tube to the stimulus source was 21.3 sec. During the upwind flight, males displayed a helical-like flight pattern as they repeatedly crossed the pheromone plume over 2/3 of the flight path. The last 1/3 of the flight was most often a straight-line dash in the pheromone plume to the stimulus source. After reaching the source, the males hovered with their claspers fully extended and made repeated antennal and tarsal contacts with the paper strip. Males also landed on the strip and made copulatory strikes (abdominal curving with claspers extended) at the paper strip. The average time spent by the males near the stimulus source was 33 sec, after which they flew away downwind and then began upwind flight as they casted widely across the width of the tunnel. Seventy-five percent of the males that exhibited this behavior reentered the plume and returned to the source.

Data in Table 2 (test I) show that treatment B, which lacked 16:Ald and 16:OH, was not significantly different from treatment A in any of the behavioral categories and elicited significantly better response in time to activation category compared to female extracts. Treatments C, D, E, G, and L (blank) were significantly less effective than female extracts in two or more of the behavioral categories of the test. These treatments lacked (*E*)-11–16:Ald (*compound* 1, Table 1), (*E,E*)-10,12–16:Ald (5), (*E,Z*)-10,12–16:Ald (3), and (*E,Z*)- and (*Z,Z*)-10,12–16:Ald (4), respectively. Results showed that (*E*)-11–16:Ald (1) and (*E,E*)-10,12–16:Ald (5) were absolutely essential for pheromonal activity; male responses to treatments (C and D) lacking either of these compounds were statistically equivalent to the blank treatment. Thus, these compounds are essential pheromonal components, but they must be sensed in context with other compounds to elicit optimal behavioral reactions from the males.

TABLE 2. RESPONSES OF ADULT MELONWORM MALES IN A FLIGHT TUNNEL TO PERMUTATIONS OF COMPOUNDS, EXTRACTS OF FEMALE, AND VIRGIN FEMALES

Treatment and compounds deleted from the mixture of ten[a]	Behavioral category [least-squares mean percentage response or time (sec)]					
	Act. (%)	Time to act.	Flt. upw. in plume (%)	Flt. time to source	Time at source	Return to source (%)
Test I (N = 24)[b]						
A. None	100 a	6 ab	96 a	22 ab	31 bc	70 a
B. 2,7	100 a	3 a	100 a	19 a	42 a	63 a
C. 1,2,7	33 b	45 d	0 e	—	—	—
D. 2,5,7	13 c	50 d	0 e	—	—	—
E. 2,3,7	88 a	13 cd	63 c	25 b	19 c	21 b
F. 2,4,7	88 a	10 bc	67 c	22 ab	32 ab	60 a
G. 2,3,4,7	88 a	7 ab	29 d	25 b	7 c	52 ab
H. 2,6,7	92 a	11 cd	71 bc	20 ab	34 ab	64 a
I. 2,7,8	100 a	3 a	96 a	19 a	49 a	79 a
J. 2,7,9	100 a	12 cd	67 c	24 ab	30 bc	74 a
K. 2,7,10	100 a	7 ab	88 ab	20 ab	37 ab	74 a
L. Blank	17 c	38 d	0 e	—	—	—
M. Female extracts	92 a	9 bc	83 abc	22 ab	36 ab	75 a
Test II (N = 36)						
B. 2,7	100	3 b	100	25 a	28 a	67 b
N. 2,7,8,10	100	2 a	100	23 a	45 a	83 a
M. Female extracts	100	2 a	100	23 a	40 a	89 a
O. 3 females	100	2 a	100	25 a	44 a	97 a

[a] Compounds, designated by number, are defined in Table I. Means followed by a common letter within each test in each column are not statistically different at 0.05% level of probability. Abbreviations: Act. = activation, flt. upw. = flight upwind.
[b] N = Number of males tested against each treatment.

(*E,Z*)-10,12-16:Ald (3) proved important to maintenance of male behavioral activity in the vicinity of the pheromonal stimulus; when it was deleted from the stimulus (E) males spent less time at the source and they returned to it less frequently (E vs. A, B, or M). Deletion of (*Z,Z*)-10,12-16:Ald (4) from the stimulus (F) had a detrimental effect on percent flight upwind category in comparison to A and B, but not M. When the *Z,Z* and *E,Z* isomers were simultaneously deleted from the stimulus (G), the expected negative effects attendant with deletion of either isomer were greater than anticipated. Response scores were impacted negatively in the percent flight upwind category as well as in the time at source category. This result indicates that the two dienes have an interactive effect on male behavior and that male perception of them involves a contextual relationship.

Although results of test I indicated that compounds 1, 3, 4, and 5, (Table 1) were behaviorally important components of the female's sex pheromone, a replicated ($N = 6$) test of the four-component mixture in the flight tunnel showed that it would not elicit upwind flight in the stimulus plume. Test I data showed that treatments containing compounds 1, 3, 4, and 5 plus at least three other compounds (6, 8, 9) always produced response scores that were either equivalent to or greater than those generated by female ovipositor extracts. Inspection of data for treatments H, I, J, and K and inference led us to the conclusion that a combination of compounds 1, 3, 4, 5, 6, and 9 represented the minimum set of compounds required for elicitation of a complete repertoire of male response in all behavioral categories of the assay. Test II (Table 2) confirmed the validity of this inference. The assay showed that the 1, 3, 4, 5, 6, and 9 combination of compounds elicited responses from males that were statistically equivalent to the responses generated by female extracts or virgin females. The mixtures of the eight olefins (treatment B) identified from the female extracts was slightly less effective than all other treatments in the time to activation and percent return to source categories. This result may reflect the limits of precision for the assay, since treatment B did not differ from female extracts in Test I.

Thus, the chemical data and the behavioral studies in the flight tunnel indicate that the melonworm female sex pheromone may comprise a complex set of six compounds:(*E*)-11-16:Ald, (*E,Z*)-10,12-16:Ald, (*Z,Z*)-10,12-16:Ald, (*E,E*)-10,12-16:Ald, (*E*)-11-16:OH, and (*E*)-11-16:OAc. The copious amounts of (*E,E*)-10,12-16:Ald found in the melonworm female alerted us to the possibility that a similar compound might be found congenerically, and this cognizance proved indispensable in identification of the pickleworm female sex pheromone. The four other compounds (2, 7, 8, and 10; Table 1) identified in the melonworm female extracts did not have any detectable behavioral significance in the assay; however, they appear to be related to the pheromonally active set in a biosynthetic sense.

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