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IDENTIFICATION OF MAJOR COMPONENTS OF THE FEMALE PHEROMONE GLANDS OF *EUCHAETES EGLE* DRURY (ARCTIIDAE)

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ABSTRACT. Extracts of the female pheromone glands of *Euchaetes egle* were analyzed using gas chromatography, infrared spectroscopy and mass spectroscopy. Identified compounds of the pheromone gland extract include a simple alkane (2-methyloctadecane), one diene (Z,Z-6,9-heneicosadiene), two trienes (Z,Z,Z-3,6,9-eicosatriene, Z,Z,Z-3,6,9-heneicosatriene), and one tetraene (Z,Z,Z-1,3,6,9-heneicosatetraene). The primary compound was found to be Z,Z,Z-3,6,9-heneicosatriene. Male sensitivity to these compounds was determined using an electroantennogram assay, and Z,Z,Z-1,3,6,9-heneicosatetraene elicited the greatest male response. Trienes are found in pheromone glands of the Arctiidae, Geometridae, Lymantriidae, and Noctuidae, suggesting that the use of these in compounds in pheromone glands is a plesiomorphic character for Arctiidae.

Additional key words: pheromones, mating behavior, Arctiidae.

In Lepidoptera, reproductive isolation among species can be achieved via temporal, chemical, or behavioral mechanisms. In moth systems, temporal and chemical isolation mechanisms are particularly important (Roelofs & Cardé 1974, Meyer 1984). Temporal isolation is achieved by variations in flight times. Chemical isolation is achieved by variations in the female pheromone blend, which is emitted from glands located in terminal abdominal segments (Baker 1985, Linn & Roelofs 1989).

Lepidopteran female pheromone glands vary in structure, ranging from a simple band of glandular tissue to eversible sacs or folds (Jefferson et al. 1971, Percy & Weatherston 1974, Percy-Cunningham & MacDonald 1987). Female pheromone glands in arctiids usually are paired, air-filled tubular invaginations that open dorsally between the eighth and ninth abdominal segments (Meyer 1984, Conner et al. 1980). The glands have two layers: an outer cellular layer and an inner cuticular layer (Fig. 1). The cuticular layer is then elaborated into internal cuticular spines (Fig. 2) (Conner et al. 1980, Yinn et al. 1991). To release the pheromone, arctiid females rhythmically protrude the eighth and ninth segments (Conner et al. 1985).

Female pheromone blends are typically composed of straight-chained hydrocarbons, 16 to 22 carbons in length. These compounds can contain

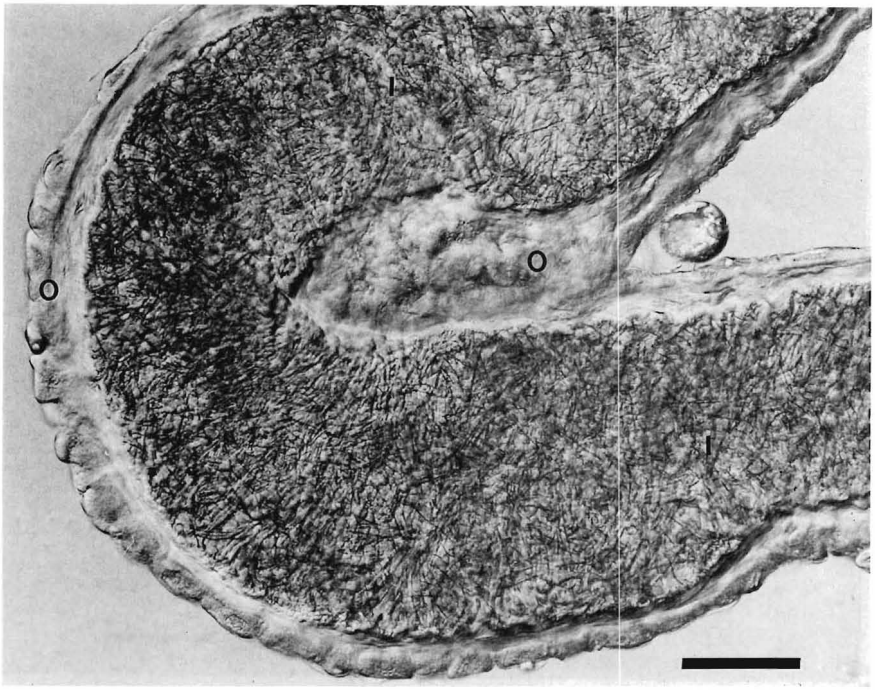

FIG. 1. Portion of the *E. egle* female pheromone gland, viewed using differential interference contrast (DIC) microscopy (O = outer cellular layer, I = inner cuticular layer, scale bar = 100 μm).

a variety of functional groups, such as alcohols, aldehydes, acetate esters, epoxides and ketones (Leonhardt 1985, Mayer & McLaughlin 1991). In members of the Arctiidae, the major female pheromone components are trienes, triene derivatives, or 2-methylalkanes (Mayer & McLaughlin 1991). In this study, we examined the pheromone blend of *Euchaetes egle* Drury, the milkweed tussock moth, and determined male electroantennogram response to identified major components of the female's pheromone blend.

MATERIALS AND METHODS

Insects. Adult female *E. egle* were collected by James Adams in Dalton, Georgia. These gravid females were shipped to Wake Forest University, Winston-Salem, North Carolina. Eggs from these females and subsequent generations were also reared on *Asclepias syriaca*.

Female pheromone identification. Pheromone glands were extracted from nine adult females before the calling cycle, and placed in

FIG. 2. Lumen of the *E. egle* female pheromone gland, viewed using DIC microscopy (S = cuticular spines, scale bar = 100 μm).

100 μl of methylene chloride. No internal standard was used. Glands were extracted overnight. This extract was subjected in 1 μl aliquots to gas chromatography/infrared spectroscopy/mass spectrometry on a Hewlett Packard (HP) 5890 GLC coupled with both an HP 5965A infrared detector and an HP 5970 mass selective detector. Components of the pheromone blend were separated on a DB1 fused silica capillary column, measuring 60 m × 0.32 mm × 0.5 mm thickness. The initial temperature was held at 100° C for 2 min and then increased to 240° C at 2.5°C/min.

The *E. egle* pheromone blend was compared to a set of nine standards, that were subjected to the conditions given above. These standards were: (1) Z,Z-6,9-heneicosadiene (> 99% pure); (2) Z,Z,Z-1,3,6,9-heneicosatetraene (> 95% pure); (3) Z,Z,Z-3,6,9-octadecatriene (> 99% pure); (4) Z,Z,Z-3,6,9-eicosatriene (92% pure); (5) Z,Z,Z-3,6,9-heneicosatriene (99% pure); (6) Z,Z,Z-3,6,9-docosatriene (94% pure); (7) 2-methyloctadecane (99% pure); (8) 2-methylheneicosane (75% pure);

and (9) 2-methyldocosane (82% pure). Standards were analyzed as a 1:1:1:1:1:1:1:1:1 blend. Standards were run in concentrations that bracketed all extract concentrations.

Z,Z,Z-1,3,6,9-heneicosatetraene was undetectable using the methods described above due to its instability at high temperatures (Jain et al. 1983). Because of this instability, the pheromone and standard blends were also analyzed using an HP 5790A gas chromatograph with an on-column injection port and a flame ionization detector. An Ultra II (5% phenyl methyl silicone) column was used for this analysis (25 m, 0.32 ID, 0.52 mm film thickness). Carrier flow was set at 5.0 ml/min. The initial temperature was 50°C, which was held for 5 min. and then increased 5°/min until it reached 280°C. Extracts of pheromone glands and the nine-component standard blend were injected as 1 µl aliquots and compared. The percentage of each sample in the extract was also calculated by dividing the abundance of the individual compound by the total abundance of all compounds.

Electroantennograms. Electroantennograms (EAG's), as described in Rodgers (1991), were used to determine male responsiveness to possible components of the female pheromone blend of *E. egle*. Antennae were removed from 1 to 2 day old males (n = 7). The proximal end of the antenna was placed in a Petri dish containing EAG saline (NaCl 7.5 g/l, $CaCl_2$ 0.21 g/l, KCl 0.35 g/l, $NaHCO_3$ 0.2 g/l) (Rodgers 1991). Several segments were removed from the distal end of the antenna. The remaining distal end was placed in contact with a input electrode. The input electrode, via a high impedance electrode (Tektronix model 013-0071-00), was connected to a Tektronix 5115 storage oscilloscope. A continuous charcoal filtered airstream (1800 ml/min) was passed over the antenna. Two ml puffs of air were passed through Pasteur pipettes containing filter paper impregnated with one of nine pheromone standards (10 µg dissolved in 100 µl of methylene chloride) and a negative control (methylene chloride). These puffs were injected into the air stream, through an opening in the air supply tube.

Responses to the following nine standards were measured: (1) Z,Z-6,9-heneicosadiene; (2) Z,Z,Z-3,6,9-octadecatriene; (3) Z,Z,Z-3,6,9-eicosatriene; (4) Z,Z,Z-3,6,9-heneicosatriene; (5) Z,Z,Z-3,6,9-docosatriene; (6) Z,Z,Z-1,3,6,9-heneicosatetraene; (7) 2-methyloctadecane; (8) 2-methylheneicosane; (9) 2-methyldocosane. Standards were presented in random order. Antennal responses were measured twice per antenna for all standards and the control (the order of presentation of the stimuli being reversed for the second set of measurements). These responses were then averaged, and compared across all males using one way analysis of variance (ANOVA) and a Scheffe F-test.

TABLE 1. Summary of compounds found in the female pheromone blend of *Euchaetes egle*. Letters refer to compounds in total ion chromatogram, compound X to subsequent analysis, — = trace amount.

Compound	GC retention time	Identification/Comments	Amount in Blend (%)
A	81.5 min	Mass spectrum indicates an alkane	—
B	96.6 min	Parent ion, mass spectrum indicates a branched alkane	—
C	98.0 min	Infrared and mass spectra match **2-methyloctadecane**; presence also confirmed by subsequent gas chromatography.	23.4
D	108.0 min	Infrared and mass spectra indicates an alkane	—
E	109.8 min	Mass spectrum is similar to that of **Z,Z,Z-3,6,9- eicosatriene**; presence confirmed by subsequent gas chromatography	2.7
F	128.5 min	Mass spectrum matches **Z,Z-6,9-heneicosadiene**; presence confirmed by subsequent gas chromatography	4.3
G	130.0 min	Infrared and mass spectra match **Z,Z,Z-3,6,9-heneicosatriene**; presence confirmed by subsequent gas chromatography	37.5
H	157.4 min	Mass spectrum indicates an alkane	—
I	163.1 min	Mass spectrum corresponds to spectrum of **Z,Z,Z-1,3,6,9-heneicosatetraene**, may be a 22-or 23-carbon tetraene	—
X	—	Retention time in subsequent gas chromatography matches **Z,Z,Z-1,3,6,9-heneicosatetraene**	14.8

RESULTS

Female pheromone identification. A total ion chromatogram of the *E. egle* pheromone blend extract revealed nine compounds. Of these, three (C, F, G) could be matched to standards (Table 1). The retention time for compound E was close to that of Z,Z,Z-3,6,9-eicosatriene, and the compound had a fragmentation pattern indicative of trienes (Descoins et al. 1986). However, the parent ion of compound E had a mass/charge ratio of 281, while the parent ion of Z,Z,Z-3,6,9-eicosatriene had a mass/charge ratio of 275. Because compound E was found in trace amounts in the total sample, an infrared spectrum could not be obtained. Compound I did not match any standards tested, but showed a mass spectrum similar to that of Z,Z,Z-1,3,6,9-heneicosatetraene (Jain et al. 1983). The other four unidentified components (A, B, D, H) correspond to alkanes of varying chain lengths (McLafferty 1973).

A subsequent analysis using on-column injection, Ultra II 5% phenyl methyl silicone column revealed compounds that had matching retention times to the following standards (relative percentage of compound in the total sample): 2-methyloctadecane (23.4%), Z,Z-6,9-heneicosadiene (4.3%), Z,Z,Z-3,6,9-eicosatriene (2.7%), and Z,Z,Z-3,6,9-heneicosatriene (37.5%). Additionally, 2-methyldocosane (1.7%) and Z,Z,Z-

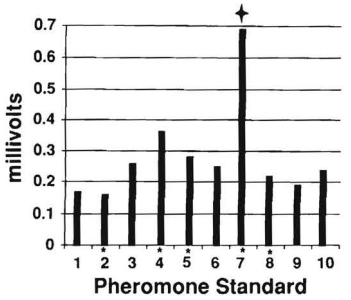

FIG. 3. Mean electrical response of male antennae to female pheromone standards and a control (1–10) (n = 7). * = compound found in pheromone blend extract, ✦ = significantly different from control, 1 = Control, 2 = Z,Z-6,9-heneicosadiene, 3 = Z,Z,Z-3,6,9-octadecatriene, 4 = Z,Z,Z-3,6,9-eicosatriene, 5 = Z,Z,Z-3,6,9-heneicosatriene, 6 = Z,Z,Z-3,6,9-docosatriene, 7 = Z,Z,Z-1,3,6,9-heneicosatetraene, 8 = 2-methyloctadecane, 9 = 2-methylheneicosane, 10 = 2-methyldocosane.

3,6,9-docosatriene (1.8%) were also found. This analysis also revealed an additional peak (Compound X; Table 1) at 30.61 minutes, which matches the retention time of Z,Z,Z-1,3,6,9-heneicosatetraene (14.8%).

Electroantennograms. Electrical responses of male antennae were significantly higher to Z,Z,Z-1,3,6,9-heneicosatetraene (7) than to the control (1) ($P < 0.05$). Responses to other standards, including those compounds found in the *E. egle* pheromone gland extract, were not significantly different than responses to the control (Fig. 3).

DISCUSSION

It should be noted that the compounds identified are components of the extract of a pheromone gland and that this extract may include pheromonal precursors as well as true pheromone components. Compounds found in the pheromone gland extracts of *E. egle* are similar to pheromonal compounds of other arctiids. Z,Z,Z-3,6,9-heneicosatriene, is found in *Arctia villica* L.(Einhorn et al. 1984), two species of *Creatonotos* (Bell & Meinwald 1986, Wunderer et al. 1986), *Halysidota leda* Druce (C. Descions pers. comm.), *Paraeuchaetes pseudoinsulata* (Walker) (Schneider et al. 1992), *Phragmatobia fuliginosa* L. (C. Descions pers. comm.), *Syntomeida epilais* Walker (C. Descions pers. comm.), and *Utetheisa ornatrix* L. (Conner et al. 1980, Jain et al. 1983). The triene, Z,Z,Z-3,6,9-eicosatriene, is a component of the pheromone blend of *Paraeuchaetes pseudoinsulata* (Walker) (Schneider et al. 1992). Trienes are found in the pheromone glands of Geometridae, Lymantriidae, and Noctuidae (Mayer & McLaughlin 1991). This distribution suggests that triene use is the ancestral condition for Arctiidae, and perhaps much of Noctuoidea.

The other components found in the *E. egle* gland extract are also found in other arctiid pheromone blends. A secondary diene component, Z,Z-6,9-heneicosadiene, is present in pheromone blends of members of *Creatonotos* (Bell & Meinwald 1986, Wunderer et al. 1986), *Paraeuchaetes pseudoinsulata* (Walker) (Schneider et al. 1992), and *Utetheisa ornatrix* L. (Jain et al. 1983). Alkanes, such as 2-methylalkanes, are found in the female pheromones of many species of *Holomelina* and in *Pyrrharctia isabella* (J.E. Sm.) (Roelofs & Cardé 1977).

EAG's performed on *E. egle* males (Fig. 3) show that the compound (7), Z,Z,Z-1,3,6,9-heneicosatetraene, elicits the greatest response. It is also found as a secondary component in the pheromone blends of *Arctia villica* L. (Einhorn et al. 1984), and two species of *Utetheisa* (C. Descoins pers. comm., Jain et al. 1983). The tetraene may act as the species specific cue, guiding *E. egle* males to appropriate mates.

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LITERATURE CITED

Baker, T. C. 1985. Chemical control of behavior, pp. 621–672. *In* G. A. Kerkut & L. I. Gilbert (eds.), Comprehensive insect physiology, biochemistry, and pharmacology. Pergamon Press, New York, vol. 9.

Bell, T. W. & J. Meinwald. 1986. Pheromones of two arctiid moths (*Creatonotos transiens* and *Creatonotos gangis*): chiral components from both sexes and achiral female components. J. Chem. Ecol. 12:385.

Conner, W. E., T. Eisner, R. K. VanderMeer, A. Guerrero, D. Ghiringelli & J. Meinwald. 1980. Sex attractant of an arctiid moth (*Utetheisa ornatrix*): a pulsed chemical signal. Behav. Ecol. Sociobiol. 7:55–63.

Conner, W. E., R. P. Webster & H. Itagaki. 1985. Calling behavior in arctiid moths: the effects of temperature and wind speed on the rhythmic exposure of the sex attractant. J. Insect Physiol. 31:815–820.

Descoins, C., B. Lalanne-Cassou, D. Malosse, & M. L. Milat. 1986. Analyse de la phéromone sexueel émise par la femelle vierge de *Moica latipes* (Guénéé) (Noctuidae, Catocalinae de Guadeloupe, Antilles, Françaises). C. R. Acad. Sc. Paris 302:509–512.

Einhorn, J., B. Boniface, M. Renou & M.-L. Milat. 1984. Etude de la phéromone sexuelle de *Arctia villica* L. (Lepidoptera: Arctiidae). C. R. Acad. Sci. (Paris) 298:573.

Jain, S. C., D. E. Dussourd, W. E. Conner, T. Eisner, A. Guerrero & J. Meinwald. 1983. Polyene pheromone components from an Arctiid moth (*Utetheisa ornatrix*): characterization and synthesis. J. Org. Chem. 48:2266.

Jefferson, R. N., L. L. Sower & R. E. Rubin. 1971. The female sex pheromone gland of the pink bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). Ann. Entomol. Soc. Am. 64:311–312.

Leonhardt, B. A. 1985. Insect communication codes: isolation and identification of pheromones. Anal. Chem. 57:1240A–1249A.
Linn, C. E. & W. L. Roelofs. 1989. Response specificity of male moths to multicomponent pheromones. Chem. Senses 14:421–437.
Mayer, M. S. & J. R. McLaughlin. 1991. Handbook of insect pheromones and sex attractants. CRC Press, Boca Raton, Florida.
McLafferty, F. W. 1973. Interpretation of mass spectra. W. A. Benjamin, Inc., Reading, Massachusetts.
Meyer, W. L. 1984. Sex pheromone chemistry and biology of some arctiid moths (Lepidoptera: Arctiidae): enantiomeric differences in pheromone perception. unpublished M. S. Thesis, Cornell University, Ithaca, New York.
Percy, J. E. & J. Weatherston. 1974. Gland structure and pheromone production in insects, pp 11–34. *In* M. C. Birch (ed.), Pheromones. North-Holland Publ. Co., London.
Percy-Cunningham, J. E. & J. A. MacDonald. 1987. Biology and ultrastructure of sex pheromone-producing glands, pp 27–75. *In* G. D. Prestwich & G. J. Blomquist (eds.), Pheromone biochemistry. Academic Press, Inc., New York.
Rodgers, M. R. 1991. Sex attraction in *Cycnia tenera* Hbn. (Lepidoptera: Arctiidae): chemical characterization of the pheromone blend and description of the pheromone gland. Unpubl. M. S. Thesis. Wake Forest University, Winston-Salem, North Carolina.
Roelofs, W. L. & R. T. Cardé. 1974. Sex pheromones in the reproductive isolation of lepidopterous species, pp. 96–114. *In* M. C. Birch (ed.), Pheromones. American Elsevier Publishing Company, Inc., New York.
Roelofs, W. L. & R. T. Cardé. 1977. Hydrocarbon sex pheromone in tiger moths (Arctiidae). Science 171:684–686.
Schneider, D., S. Schulz, R. Kittmann & P. Kanagaratnam. 1992. Pheromones and glandular structures of both sexes of the weed-defoliator moth *Paraeuchaetes pseudoinsulata* Rego Barros (Lep., Arctiidae). J. Appl. Entomol. 113:280–294.
Wunderer, H., K. Hansen, T. W. Bell, D. Schnieder & J. Meinwald. 1986. Sex pheromones of two Asian moths (*Creatonotos transiens, C. gangis*; Lepidoptera-Arctiidae): behavior, morphology, chemistry and electrophysiology. Exp. Biol. 46:11.
Yinn, L. R. S., C. Schal & R. T. Cardé. 1991. Sex pheromone gland of the female tiger moth *Holomelina lamae* (Lepidoptera, Arctiidae). Can. J. Zool. 69:1916–1921.

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