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SEX PHEROMONE OF THE COMMON SHEEP MOTH, *Hemileuca eglanterina*, FROM THE SAN GABRIEL MOUNTAINS OF CALIFORNIA

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Abstract—The sex pheromone of *Hemileuca eglanterina* from the San Gabriel Mountains, California, was determined to be a combination of *E*10,*Z*12-hexadeca-10,12-dien-1-yl acetate, *E*10,*Z*12-hexadeca-10,12-dien-1-ol, and *E*10,*Z*12-hexadeca-10,12-dienal. Ratios of the compounds in extracts of female pheromone glands varied around a mean of 100 : 48 : 1.1 of the acetate, alcohol, and aldehyde, respectively. Field trials with synthetic compounds indicated that the optimum ratio of alcohol to aldehyde was 10 : 1 and that this ratio was more critical than the ratio of either compound to the acetate. A synthetic blend of 100 : 10 : 1 acetate–alcohol–aldehyde was effective at attracting male moths in the field. Additional compounds found in both extract and aeration samples failed to significantly increase trap catches of male moths, although some of these minor components elicited responses from male moth antennae in coupled gas chromatography–electroantennography studies.

Key Words—*Hemileuca eglanterina*, *E*10,*Z*12-hexadeca-10,12-dien-1-yl acetate, *E*10,*Z*12-hexadeca-10,12-dien-1-ol, *E*10,*Z*12-hexadeca-10,12-dienal, sex pheromone.

INTRODUCTION

Hemileuca is the most diverse genus in the Saturniidae of the United States and Canada, with 18 species currently recognized (Tuskes et al., 1996). These attractive moths are variable in color, and they have received a great deal of attention

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from collectors. As a result, considerable information on their biology and distribution is available, despite few of the species being of economic importance. Most species are diurnal and locally abundant during their short flight seasons, but they are difficult to catch as they are strong fliers and seldom land.

There have been a number of observations of interspecific cross-attraction (Tuskes, 1984; Tuskes et al., 1996). For example, *H. eglanterina* (Boisduval) males are attracted to females of *H. electra* (Wright) (Tuskes et al., 1996). These two species are not sympatric and do not interact under natural conditions. However, *H. eglanterina* females attract males of *H. nuttalli* (Strecker) (Collins and Tuskes, 1979), a species with which *H. eglanterina* is sympatric throughout Idaho, Nevada, Utah, and portions of several other states, raising the question of how these two species minimize interactions that would result in wasted reproductive effort, particularly as the life-span of the nonfeeding adults is short (a few days).

There are several possible mechanisms that may contribute to reproductive isolation between *H. eglanterina* and *H. nuttalli*. For example, differing diurnal activity patterns were suggested as an isolating mechanism for a population at Monitor Pass, Sonora County, California (Collins and Tuskes, 1979), with males of *H. eglanterina* favoring the morning hours and *H. nuttalli* males flying in late afternoon. These authors also suggested that differing pheromone chemistry may be important because attraction was not symmetrical; *H. nuttalli* males were attracted to *H. eglanterina* females, but not the reverse. The objective of the study reported here was to determine the composition of the sex pheromone of *H. eglanterina*, with the ultimate goal of using this information to investigate pheromone based mechanisms contributing to reproductive isolation between *H. eglanterina* and its sibling species, *H. nuttalli*.

METHODS AND MATERIALS

Insects

Insects were obtained from the Blue Ridge, San Gabriel Mountains, Los Angeles County, California, USA. Larvae were collected from May through July, either on larval hosts [*Prunus emarginata* L., *Symphoricarpos spp.* (Dougl.) Walp., and *Ceanothus* spp. L.], or while wandering before pupation (CAUTION: larvae have stinging spines). Larvae were reared on bouquets of cut host material in screen-covered 40-liter glass aquaria. Mature, wandering larvae were placed in plastic boxes containing peat as a pupation substrate where they were allowed to spin cocoons and pupate. Pupae were kept in screen cages in the shade outdoors to allow natural coordination of emergence. This population requires two years to complete development under natural conditions, but may emerge in one

year if collected as early instars and reared under warmer temperatures (approximately 27°C). Emerging adults were collected daily and were either used for studies immediately or held in 6- × 8-cm screen cages inside sealed plastic bags with a damp towel at 10–15°C for one to two weeks. Cooling was necessary due to the short life-span (less than one week under normal conditions) of the nonfeeding adults. Unmated females generally ceased calling and began depositing infertile eggs within four days of eclosion at 22°C. Virgin females were used for preparation of pheromone extracts, while males were used for electroantennographic studies.

Extraction of Sex Pheromone Glands

Sex pheromone glands were removed from virgin calling females of 1–4 days of age. The gland was extruded by applying gentle pressure to the abdomen near the tip to force eversion of the ovipositor, at which point it and the terminal abdominal segments were excised with small scissors into a 0.25-ml conical glass insert containing ~30 μl pentane. The glands were extracted for 20 min; and then the solvent was transferred to a clean insert, with two rinses with one drop of pentane. Extracts were concentrated as needed by passive evaporation prior to analysis. If not used immediately, extracts were stored at −20°C.

Aeration of Calling Females

Pheromone collection was attempted from three individual calling females (<2 days old) for approximately 90 min in the late afternoon under a combination of natural and fluorescent lighting at 21°C, as follows. A 0.25-ml Kimble glass conical vial insert was modified by grinding off the tip with a diamond grinding wheel until there was a small opening (approx. 1.5–2 mm). A small amount of Porapak Q (precleaned with ether in a Soxhlet extractor) was placed in the narrow end and held in place with ether-extracted glass wool plugs. This collector was inserted into a 1/4-in. brass Swagelok union with a Teflon ferrule, with the other end of the union connected to a vacuum source via a flowmeter (flow 60 ml/min). The chamber consisted of a 10 cm long × 3 cm diameter glass tube tapered at one end to 1/2 in. so that Swagelok fittings could be used to connect it to an air source. A coarse sintered glass frit 5 cm from the open end divided the chamber in half. The air supply side of the chamber was filled with activated charcoal, while the moth was allowed to hang from the sintered glass frit on the other side. An air flow of ~400 ml/min was used to continuously sweep the aeration chamber. The collector was positioned directly below the everted sex pheromone gland of a calling female moth (which could be seen clearly during calling) to sample the moth effluent.

Gas Chromatography

GC analyses were conducted with Hewlett-Packard 5890A or 5890 Series II gas chromatographs (H-P, Palo Alto, California), with helium carrier gas, in splitless mode. Columns and programs used to analyze composite extracts from multiple females included: HP Ultra-1 (H-P; 30 m × 0.25 mm ID, 0.17-μm film, 100°C for 1 min, then 10°C/min to 275°C, hold for 45 min), DB-225 (J&W Scientific, Folsom, California; 30 m × 0.26 mm ID, 0.25-μm film, 100°C for 1 min, then 10°C/min to 220°C, hold for 45 min), DB-1701 (30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, then 5°C/min to 275°C, hold for 45 min), and DB-Wax (30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, then 10°C/min to 250°C, hold for 45 min). Extracts from individual females were analyzed on a DB-5 column (30 m × 0.32 mm ID, 0.25-μm film, programmed as for the H-P Ultra-1 column above). Tentative identifications were made by comparison of compound retention times in extracts to those of synthetic standards.

Coupled Gas Chromatography-Electroantennography (GC-EAD)

Aliquots of female extracts, aerations, and synthetic standards were analyzed by splitless coupled GC-EAD, with an H-P 5890 series II GC equipped with a DB-5 column (30 m × 0.32 mm ID, 0.25-μm film; J&W Scientific), programming from 100°C at 0 min, then 10°C/min to 250°C, and holding for 20 min, and with an injector temperature of 250°C. The column effluent was split equally with a press-fit Y-connector (J&W Scientific) into two branches of 0.25 mm uncoated fused silica tubing, with one branch going to the GC's flame ionization detector (FID), and the other being directed through a heated transfer line (275°C) to a 1-cm-diameter glass stimulus delivery tube. The capillary effluent was diluted with humidified air (400 ml/min) and passed over the male moth antennal preparation (see below). The moth antenna was suspended between glass capillary electrodes filled with insect physiological saline (modified Locke's saline, 7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1 liter distilled water) (Humason, 1972), with electrical contact to the custom-built multistage amplifier made with AgCl-coated silver wires. The amplifier and FID outputs were simultaneously recorded on a matched pair of H-P 3394 recording integrators.

Male antennae were removed by using fine forceps to firmly hold the scape and pull the antenna free of the head without crushing the scape. The terminal rami and tip of the antenna were removed so that the end of the antenna could be placed into the saline-filled recording electrode. The antenna was positioned on the amplifier mount at the end of the delivery tube such that the plane of the antennal branches was perpendicular to the airflow from the stimulus delivery tube. A single antennal preparation could be used for several hours with periodic additions of saline.

Coupled Gas Chromatography–Mass Spectrometry

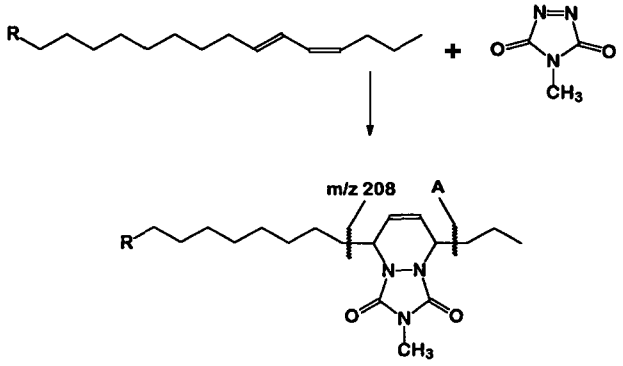
EI mass spectra (70 eV) were taken with an H-P 6890 gas chromatograph interfaced to a 5973 mass selective detector. A 30 m × 0.25 mm ID HP-5MS column was used (H-P), programmed at 50°C for 1 min, then 10°C/min to 250°, and holding for 20 min. Compounds in the insect extract were identified by comparison of retention times and mass spectra with those of authentic standards (see below). A composite sample from five females obtained in July 1997 was analyzed.

Formation of Adducts with 4-Methyl-1,2,4-triazoline-3,5-dione (MTAD) (Scheme 1)

A combined extract from five pheromone glands was concentrated to ~5 μl, then diluted with 20 μl of CH_2Cl_2. A 10-μl aliquot was transferred to a Kimble 0.25-ml glass conical vial insert and treated with 2.5 μl of a solution of MTAD in CH_2Cl_2 (1.2 mg/ml). The faint pink solution was concentrated under a gentle stream of nitrogen to <5 μl, and a 1-μl aliquot was analyzed by splitless GC-MS (injector 300°C, temperature program: 100°C at 0 min, then 15°C/min to 300°C for 20 min; solvent delay was 8 min; instrument and column are as described above).

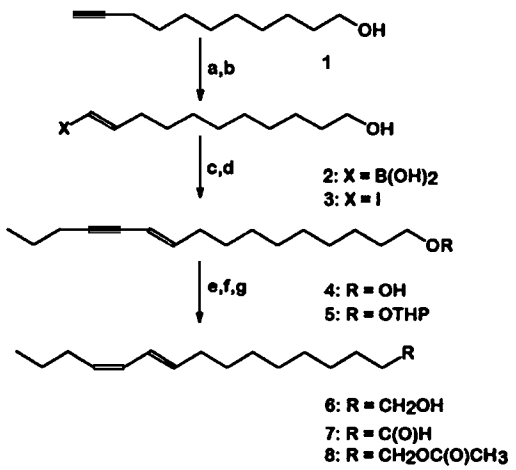
Synthesis of Pheromone Components

Proton NMRs were recorded on a General Electric QE 300 instrument (300 MHz) in $CDCl_3$ solution. EI mass spectra (70 eV) were taken with an H-P



R = CH_2OAc, A = m/z 350
R = CH_2OH, A = m/z 308
R = CHO, A = m/z 306

SCHEME 1.



a) i. Catecholborane, ii. water; b) NaOH, I_2; c) Pentyne, Pd^{+2}, CuI, diisopropylamine; d) Dihydropyran, H^+; e) i. Dicyclohexylborane, ii. AcOH, iii. MeOH, H^+; f) PDC; g) AcCl, pyridine

Scheme 2.

5970B mass selective detector interfaced to a 5890 GC fitted with a DB5-MS column (20 m × 0.2 mm ID, J&W Scientific). Air- and/or water-sensitive reactions were carried out in oven-dried glassware under an N_2 atmosphere. Tetrahydrofuran (THF) was purified by distillation from sodium–benzophenone ketyl under argon. Flash chromatography was carried out with 230–400 mesh silica gel (Aldrich Chemical, Milwaukee, Wisconsin).

Synthesis of E10,Z12-Hexadeca-10,12-dien-1-ol (E10,Z12–16:OH) (Scheme 2).

(E1)-1-Iodoundecen-11-ol (3). A mixture of 10-undecyn-1-ol **1** (4.9 g, 29.2 mmol; Farchan Labs, Gainesville, Florida), catecholborane (7.0 ml, 66 mmol; Aldrich), and THF (10 ml) were stirred at 70°C overnight under N_2. After cooling to room temperature, 180 ml water was added, and the mixture was stirred 4 hr. The resulting white slurry was cooled in an icebath, filtered, and the solids rinsed three times with 50 ml icewater. The sticky white boronic acid **2** (5.83 g) was dried under aspirator vacuum and used without further purification. NMR: δ 5.78 (br. d, 1H, 18 Hz), 5.42 (br. d, 1H, J = 18 Hz), 3.64 (t, 2H, J = 6.5 Hz), 2.15 (m, 2H), 1.6–1.2 (m, 14H).

The dried (*E*1)-11-hydroxy-1-undecenylboronic acid **2** (2.9 g, 13.7 mmol) was taken up in a mixture of ether (40 ml) and 1.5 M NaOH (60 ml) and stirred vigorously for 30 min. The mixture was cooled to 0°C, and iodine (3.82 g, 15

mmol) in 15 ml ether was added dropwise over 30 min. The mixture was stirred an additional 30 min at 0°C, then quenched by addition of a few mls of saturated aq. Na_2SO_3. The layers were separated and the aqueous phase extracted with hexane. The combined organic phases were washed with dilute aq. Na_2SO_3 and brine and then concentrated and purified by flash chromatography (22 × 5 cm), eluting with 22.5% EtOAc in hexanes, yielding 2.6 g of iodoalcohol **3** as a pale yellow oil, which solidified upon refrigeration. NMR: δ 6.51 (dt, 1H, J = 14.4, 7.0 Hz), 5.98 (br. d, 1H, J = 14.2 Hz), 3.64 (t, 2H, J = 6.5 Hz), 2.05 (br. quart, 2H, J = 6.8 Hz), 1.55 (m, 2 H), 1.5–1.22 (m, 12H). MS: m/z 180 (31), 167 (23), 109 (28), 95 (77), 81 (53), 67 (66), 55 (89), 41 (100).

E10-Hexadecen-12-yn-1-ol (4). To a dry 250-ml three-neck flask under N_2 were added sequentially 0.25 g bis(triphenylphosphine)palladium(II) chloride, 0.25 g CuI, iodoalcohol **3** (2.5 g, 8.4 mmol), and 1-pentyne (1.36 g, 20 mmol) in 25 ml of THF. Diisopropylamine (3 g) was then added dropwise with stirring, and the reaction mixture warmed and precipitated brown–black solids. The slurry was stirred for 1 hr, then hexane (100 ml) was added, and after stirring for 5 min, the mixture was filtered with suction. The brown solution was washed twice with sat. aq. NH_4Cl, dried, and concentrated. MS: m/z 236 (3), 208 (1), 207 (1), 193 (2), 179 (4), 121 (12), 107 (18), 94 (21), 93 (37), 91 (45), 79 (100), 67 (29), 55 (34), 41 (44).

E10,Z12-Hexadeca-10,12-dien-1-ol (6). The crude enynol **4** was taken up in ether, treated with 2 ml dihydropyran and a few crystals of *p*-toluenesulfonic acid (PTSA), and the mixture stirred overnight. The ether solution was then washed twice with dilute $NaHCO_3$, dried, concentrated, and purified by flash chromatography (20 × 5 cm) eluting with 7.5% EtOAc in hexanes, yielding 2.1 g of the protected alcohol **5** as a yellow oil. NMR: δ 6.05 (dt, 1H, J = 15.8, 7.1 Hz), 5.46 (dt, 1H, J = 16.0, 1.6 Hz), 4.58 (br t, 1H, J ~ 4.6 Hz), 3.87 (m, 1H), 3.74 (dt, 1H, J = 10.5, 6.5 Hz), 3.50 (m, 1H), 3.38 (dt, 1H, J = 9.6, 6.6 Hz), 2.27 (td, 2H, J = 7.1, 1.8 Hz), 2.07 (br. quart, 2H, J ~ 6.9 Hz), 1.9–1.47 (m, 10 H), 1.43–1.22 (m, 12H), 0.98 (t, 3H, J = 7.5 Hz).

Cyclohexene (2.0 ml, 20 mmol) was added dropwise over 10 min to a solution of borane–dimethylsulfide complex (1 ml, 10 mmol) in THF (12 ml) at 0°C under N_2. The mixture was warmed to room temperature, stirred 2 hr, cooled again to 0°C, and the crude enynol THP ether **5** (2 g, ~6.3 mmol) was added dropwise. The solution was allowed to warm slowly to room temperature, monitoring the disappearance of **5** by TLC. The mixture was cooled to 0°C and glacial acetic acid (4 ml) was added dropwise. It was warmed to room temperature and stirred overnight, then cooled to 0°C in a Dry Ice–acetone bath while 5 M NaOH (15 ml) and 30% H_2O_2 were added sequentially (EXOTHERMIC!). The mixture was diluted with water and extracted with pentane. The pentane extracts were dried and concentrated, taken up in MeOH (50 ml) with a few crystals of PTSA, and stirred overnight to remove the THP group. The mixture was worked up by

addition of 1 g $NaHCO_3$, removal of most of the MeOH by rotary evaporation, and partitioning of the residue between water and hexane. The hexane extracts were washed with brine, dried, concentrated, and purified by flash chromatography, yielding 1.04 g of *E*10,*Z*12-hexadecadienol **6**, >98.9% pure by GC. NMR: δ 6.31 (ddt, 1H, J = 14.3, 11.1, 1.1 Hz), 5.97 (br. t, 1H, J = 10.8 Hz), 5.66 (dt, 1H, J = 15.0, 7.0 Hz), 5.32 (dt, 1H, J = 10.8, 7.6 Hz), 3.65 (t, 2H, J = 6.6 Hz), 2.2–2.05 (m, 4H), 1.62–1.25 (m, 16H), 0.93 (t, 3H, J = 7.4 Hz). MS: m/z 238 (11), 149 (2), 135 (5), 121 (8), 109 (15), 96 (30), 95 (29), 82 (36), 81 (50), 79 (32), 67 (100), 55 (37), 41 (49).

E10,Z12-Hexadeca-10,12-dienal (E10,Z12–16:Ald) (7). Dienol **6** (0.44 g, 1.85 mmol) was stirred overnight with pyridinium dichromate (1.1 g, 3 mmol) in 5 ml CH_2Cl_2. The mixture was poured into 50 ml hexane, stirred for 5 min, then filtered. After concentration, the residue was flash chromatographed (4% ether in hexane) and Kugelrohr distilled (oven temp. ~120°C, 0.07 mm Hg), yielding the pure aldehyde. The NMR and mass spectral data closely matched literature values (Samain and Descoins, 1979; Bestmann et al., 1981). The final product was diluted in heptane to a concentration of 10 mg/ml to inhibit trimerization, BHT was added as a antioxidant, and the solution was stored at −20°C until needed.

E10,Z12-Hexadeca-10,12-dien-1-yl Acetate (E10,Z12–16:Ac) (8). Acetyl chloride (0.5 ml) was added dropwise to a solution of dienol **6** (150 mg, 0.6 mmol) and pyridine (1 ml) in dry ether (50 ml), and the mixture was stirred overnight at room temperature. Then 0.5 M $NaHCO_3$ (50 ml) was added and the mixture stirred for 15 min. The layers were separated, and the aqueous phase was extracted with hexane. The combined organic phase was washed with 0.1 M HCl and brine, dried, concentrated, and Kugelrohr distilled (oven 150°C, 0.07 mm Hg), yielding 160 mg of the acetate. The NMR and mass spectral data agreed with literature values (Samain and Descoins, 1979; Bestmann et al., 1981), and the GC and MS data perfectly matched those obtained from an authentic standard obtained from Darwin Reed (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada).

10,12-16:OH Isomers. Aliquots (80 mg) of a sample of the mixed geometric isomers of 10,12–16:OH, generated by scrambling the double bonds of *E*10,*Z*12–16 : OH with aqueous $NaNO_2$ and HNO_3 (Sonnet, 1974), were separated by passage through a 1.2-m × 6-mm-ID column of macroporous ion exchange resin (Lewatit SP 1080, Ag^+ form, E. Merck, Gibbstown, New Jersey), eluting with MeOH (1.5 ml/min) (Houx et al., 1974) and monitoring the effluent with a Dynamax RI-2 refractive index detector (Rainin Instruments, Emeryville, California). Fractions that were >99% isomerically pure were combined, concentrated, and taken up in hexane. The hexane solutions were dried over anhyd. Na_2SO_4, then concentrated and Kugelrohr distilled (oven temp. ~155°C, 0.03 mm Hg).

Field Trials

Field trials were conducted at the Blue Ridge site where larvae had been obtained during July and August of 1993 through 1996 (see above). Initially, Pherocon 1C traps were used (Trécé Inc., Salinas, California), but these proved inadequate due to the large size of the moths and the numbers attracted. Consequently, traps of our own design were used, consisting of a 150-mm-diameter plastic funnel (1 pint funnel, No. 5016, Contico Automotive Corp., St. Louis, Missouri) resting upon a 3.2-mm-mesh wire screen cylinder (14.5–15 cm OD by 20–25 cm high). The spout was removed from the funnel, leaving a 45- to 50-mm opening at the bottom. A 32-gauge wire was wrapped around a tab on the lip of the funnel and bent so that the lure could be suspended over the center of the funnel. The wires at the bottom of the screen cylinder were bent inward so that a 150-mm plastic Petri dish could be rested inside the cylinder as a bottom and weighted with rocks or soil for stability.

Lures consisted of 11-mm red rubber septa (Wheaton Scientific, Millville, New Jersey), individually labeled with a treatment number and loaded with heptane solutions (100 μl) of test compounds, plus two drops of 10 mg/ml butylated hydroxytoluene (BHT) in heptane as an antioxidant. Once the treatment solutions had soaked into the septa, the lures were impaled on No. 6 bronzed fishhooks and stored in 20-ml glass vials for transport. Lures were kept in a freezer or an ice-chest when not in use. No one set of lures was used for more than five days.

A single trial consisting of three blocks located at different points along the ridge. Traps were spaced approximately 10 m apart, and blocks were 600 m or more apart. Two to five trap counts were made at one-day intervals, with the traps being cleaned out and rerandomized after each reading. Counts were made early in the morning or, more often, late in the day after flight had ceased.

Statistics

For the statistical analysis of field trials, elimination of the effect of day was accomplished by pooling the day counts for each treatment in a given block. This sum was then transformed ($\sqrt{x} + 0.5$) prior to conducting a two-way ANOVA, followed by linear and quadratic contrasts. If significant effects were detected, then the Student-Neuman-Keuls (SNK) test was used to separate means (SAS Institute 1996).

RESULTS

Analysis of Female Extracts. Analysis of female pheromone gland extracts by coupled GC-EAD demonstrated that male moth antennae responded strongly to three compounds, with smaller, less consistent responses to several other com-

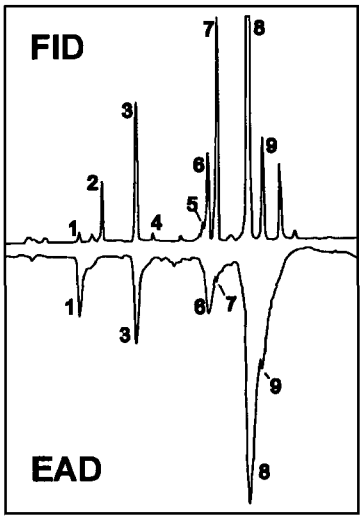


FIG. 1. Coupled gas chromatogram–electroantennogram of male *H. eglanterina* (Boisduval) antenna stimulated by a female extract. Column used was DB-5 (30 m × 0.32 mm ID, 0.25-μm film, program used was 100°C for 1 min, then 10°C/min to 275°C, held for 45 min). Identifications of peaks: 1, *E*10,*Z*12–16 : Ald; 2, 16 : OH; 3, *E*10,*Z*12–16 : OH; 4, *E*10,*E*12–16 : OH; 5, *Z*10–16 : Ac; 6, *E*- and *Z*11–16 : Ac; 7, 16 : Ac; 8, *E*10,*Z*12–16 : Ac; and 9, *E*10,*E*12–16 : Ac.

ponents (Figure 1). These compounds, and several others present in the extracts, were identified by GC and GC-MS (Table 1). The mass spectrum of the most abundant of the antennal stimulatory compounds (and the component eliciting the strongest antennal response) was consistent with that of a conjugated 16-carbon diene acetate, with a comparatively strong molecular ion (m/z 280, 20% of base peak, possible molecular formula $C_{18}H_{32}O_2$), and a small but distinct fragment at m/z 220 (2%, loss of acetic acid). Furthermore, the spectrum closely matched a database spectrum of *E*10,*Z*12–16 : Ac (NIST/EPA/NIH Mass Spectral Database 1992). The compound from the extract was confirmed to be *E*10,*Z*12–16 : Ac in several ways. First, the position of the conjugated diene system was confirmed by formation of the adduct with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) (Young et al., 1990). The EI mass spectrum of the resulting derivative gave a molecular ion at m/z 393 (5%), and diagnostic fragments at m/z 350 (19%) and 208 (100%) from cleavage of the alkyl groups on the carbons α to the two nitrogens (Scheme 1), unequivocally fixing the diene in the 10,12 position. Second, the *E*10,*Z*12 stereochemistry was unambiguously proven by comparison of retention times on five columns of differing polarity (HP Ultra-1, DB5-MS, DB-1701, DB-225, DB-Wax) with those of synthetic

TABLE 1. COMPOUNDS, METHODS OF IDENTIFICATION, AND AMOUNTS RELATIVE TO *E*10,*Z*12–16 : Ac IN EXTRACTS AND AERATIONS OF FEMALE *Hemileuca eglanterina* FROM SAN GABRIEL MOUNTAINS

Compound	Identification methods used[a]		Amount in single female extracts relative to *E*10,*Z*12–16 : Ac[b]				Amount in	
	GC	GC-MS	Mean	SD	Range	% detected[c]	Combined extract[d]	Aeration
*E*10,*Z*12–16 : Ac	X	X	100			100	100	100
*E*10,*Z*12–16 : OH	X	X	48.4	52.5	6.0–260	96	30	30
*E*10,*Z*12–16 : Ald	X	X	1.1	0.8	0.3–3.3	56	0.9	
*E*10,*E*12–16 : Ac	X	X	10.4	2.9	4.8–15.1	100	7.4	4.3
*E*10,*E*12–16 : OH	X	X	4.6	1.0	1.7–21.8	37	1.4	
E/*Z*11–16 : Ac	X	X	10.8	4.9	5.2–25.2	93	8.8	
*Z*10–16 : Ac	X		2.1	0.7	1.2–3.1	22	1.6	
16 : Ac	X	X	53.2	43.9	16.4–200	100	35	114
16 : OH	X	X	57.5	71.6	4.1–285	96	11	

[a]GC = retention time matches on at least three columns (see materials and methods) with authentic standards. GC-MS = Mass spectral match with authentic standards.
[b]Means of 27 female extracts.
[c]Percent of female extracts with detectable amounts.
[d]Combined extract from six females.

standards. Standards of the other three 10,12–16 : Ac isomers (synthesized by and obtained from D. Reed, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada), had retention times different from *E*10,*Z*12–16 : Ac on four of the five GC columns (Table 2). Furthermore, GC-EAD of the isomers of 10,12–16 : Ac indicated that *E*10,*Z*12–16 : Ac elicited the largest response from male antennae (mean mV ± SD of 10 males tested *EZ*, 0.66 ± 0.17; *ZE*, 0.39 ± 0.10; *ZZ*, 0.28 ± 0.06; and *EE*, 0.26 ± 0.07; one-way ANOVA, $F = 24.4$, $df = 39$, $P < 0.0001$ followed by SNK, $\alpha = 0.05$).

The second EAD-active compound (Figure 1, peak 3) gave a comparatively strong molecular ion at *m*/*z* 238 (16% of base peak at *m*/*z* 67), 42 amu less than *E*10,*Z*12–16 : Ac, and consistent with the corresponding alcohol, *E*10,*Z*12–16 : OH. This tentative identification was confirmed from the mass spectrum of the MTAD adduct [molecular ion at *m*/*z* 351 (9%), and diagnostic fragments at *m*/*z* 208 (100%) and 308 (36%), Scheme 1]. The geometry of the diene was confirmed by retention time comparisons with synthetic standards of the four isomers on five columns. Furthermore, the mass spectrum exactly matched that of an authentic standard of *E*10,*Z*12–16 : OH.

The third compound eliciting consistent EAD activity, while being present in only small amounts (Figure 1, peak 1), was identified by combination of several data. First, the selected ion monitoring profile of eight ions (236, 109, 96, 95, 82, 81, 67, 55) and the retention time matched those of an authentic stan-

TABLE 2. RETENTION TIMES (MINUTES) OF ISOMERS OF 10,12–16 : Ald, 10,12–16 : OH, AND 10,12–16 : Ac ON CAPILLARY GC COLUMNS OF DIFFERING POLARITY[a]

Compound and isomer	Columns (increasing polarity →)				
	Ultra-1	DB-5	DB-1701	DB-225	DB-Wax
(10,12)–16 : Ald					
ZE	9.30	10.70	13.14	12.63	11.37
EZ	9.40	10.79	13.25	12.76	11.52
ZZ	9.51	10.92	13.35	12.85	11.63
EE	9.57	10.97	13.39	12.83	11.73
(10,12)–16 : OH					
ZE	10.02	11.37	13.83	13.46	13.53
EZ	10.11	11.45	13.92	13.56	13.64
ZZ	10.22	11.58	14.04	13.68	13.77
EE	10.28	11.62	14.07	13.65	13.85
(10,12)–16 : Ac					
ZE	11.31	12.66	14.79	13.77	12.81
EZ	11.40	12.74	14.88	13.87	12.94
ZZ	11.51	12.87	14.99	13.98	13.06
EE	11.57	12.91	15.02	13.95	13.15

[a]Temperature programs are described in Materials and Methods.

dard. Second, although the mass spectrum of the MTAD adduct did not show a molecular ion, it clearly showed diagnostic fragments at m/z 306 and 208, confirming the 10,12 position of the diene. Third, in retention time comparisons, only the *E*10,*Z*12–16 : Ald isomer of the four 10,12–16 : Ald isomers matched the retention time of the insect produced compound on all five GC columns (Table 2).

Confirmation of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald as the correct structures of the pheromone components was obtained from the strong antennal responses elicited from male moth antennae in coupled GC-EAD experiments and from the results of field trials (see below).

In addition to these three compounds, several other components in the pheromone gland extracts also elicited small responses from male antennae in some GC-EAD runs or were present is significant amounts (Figure 1, Tables 1 and 3). These compounds included 16 : OH, 16 : Ac, *E*10,*E*12–16 : Ac, *E*10,*E*12-16 : OH, *Z*10–16 : Ac, plus *E*- and *Z*11–16 : Ac, all of which were identified by comparison of retention times on multiple GC columns, and, with the exception of the *Z*10–16 : Ac, by comparison of mass spectra. For identification of the monoene acetates, the retention times were measured on DB-Wax of the series of *E* and *Z* isomers of monoene hexadecenyl acetates in which the double-bond position ranged from carbon 5 to 13. Comparison of retention times narrowed

TABLE 3. ELECTROANTENNOGRAPHIC RESPONSES (RELATIVE TO *E*10,*Z*12–16 : Ac) AND PERCENT OF MALE *Hemileuca eglanterina* ANTENNAE RESPONDING TO COMPOUNDS IN FEMALE EXTRACTS AND AERATION[a]

	Response to female extracts[b]			Aeration	
Compound	mean (%)	range (%)	% detected	Mean	SD
*E*10,*Z*12–16 : Ac	100		100	100	
*E*10,*Z*12–16 : OH	35	13–81	88	14	[c]
*E*10,*Z*12–16 : Ald	15	4–28	81	8	2.6
*E*10,*E*12–16 : Ac	43	21–56	50	50	18.1
E/*Z*11–16 : Ac	21	6–60	94	14	2.3
16 : Ac	17	12–31	31	11	[c]

[a]Percent of female extracts that elicited responses by male antennae.
[b]Responses from 10 males.
[c]Only detected by one of the two males used for the aeration sample.

the possibilities down to a few, one of which was *Z*11–16 : Ac. This assignment was corroborated by coinjection of *Z*11–16 : Ac with a composite female extract, resulting in an exact retention time match. Furthermore, using *Z*11–16 : Ac and 16 : Ac as references, comparison of retention times on multiple columns indicated that *Z*10–16 : Ac was present as well, because the relative areas of the *Z*11–16 : Ac to the *Z*10–16 : Ac peaks in the female extract were different on the two different columns, with the ratio being 2.8 : 1 on DB-Wax and 5.5 : 1 on DB-5. This discrepancy in ratios can be explained by the fact that on DB-Wax, *Z*10- and *E*11–16 : Ac coelute, whereas on DB-5, *E*11- and *Z*11–16 : Ac coelute. Thus, on DB-Wax, the *Z*10–16 : Ac peak is actually a composite with *E*11–16 : Ac and the *Z*11–16 : Ac peak is due to the single compound, while on DB-5, the situation is reversed, with the *Z*10–16 : Ac peak arising from a single component, and the *Z*11–16 : Ac peak now being a composite with *E*11–16 : Ac. Thus, all three isomers (*Z*11–16 : Ac, *Z*10–16 : Ac, and *E*11–16 : Ac), were tentatively determined to be in female extracts.

Only one of the three female aerations yielded detectable amounts of pheromone. GC peaks were obtained for *E*10,*Z*12–16 : Ac, *E*10,*E*12–16 : Ac, 16 : Ac, and *E*10,*Z*12–16 : OH in a 100 : 4.3 : 114 : 30 ratio (Table 1). Although the very small *E*10,*Z*12–16 : Ald and *E*/*Z*11–16 : Ac peaks were not integrated by the GC integrator, they were detected by EAD (Table 2), confirming that the compounds found in female gland extracts are actually released by calling females and that the ratios in the aeration samples were consistent with those found in gland extracts.

Of all the compounds from pheromone gland extracts or aeration samples that elicited GC-EAD responses, *E*10,*Z*12–16 : Ac was consistently the most abundant compound and elicited the largest responses from male anten-

nae. Estimates of the total amounts of *E*10,*Z*12–16 : Ac present in single female extracts ranged from 4 to 229 ng with a mean of 57 ng. For *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald, the mean ratio for 27 female extracts was 100 : 48 : 1.1 respectively (Table 1). For the remaining compounds identified (*E*10,*E*12–16 : Ac, *E*10,*E*12–16 : OH, *E*/*Z*11–16 : Ac, Z10–16 : Ac, 16 : Ac, and 16 : OH), the mean ratio was 10 : 2 : 11 : 2 : 53 : 58 respectively (expressed as a percentage of the *E*10,*Z*12–16 : Ac) for the 27 single female extracts (Table 1). Thus, *E*10,*Z*12–16 : Ac was considered the major component of the pheromone when optimizing the blend ratio in field trials, with the other components expressed as a percentage of this compound.

Field Trials. The first field trial tested the three most EAD-active compounds (*E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald) singly, in all binary combinations at two ratios (1 : 1 and 10 : 1), and in a ternary blend mimicking the blend found in a female gland extract (Figure 2). All traps baited with single components caught no moths, while several of the binary blends attracted a few moths. All baits that attracted moths contained *E*10,*Z*12–16 : Ac, indicating its role as a crucial component of the blend, whereas the roles of the other two components were less clear. None of the binary blends were as attractive as the three component blend (Figure 2), suggesting that all three compounds were necessary for optimal attraction.

Because of the short flight season of this species, optimization trials of the

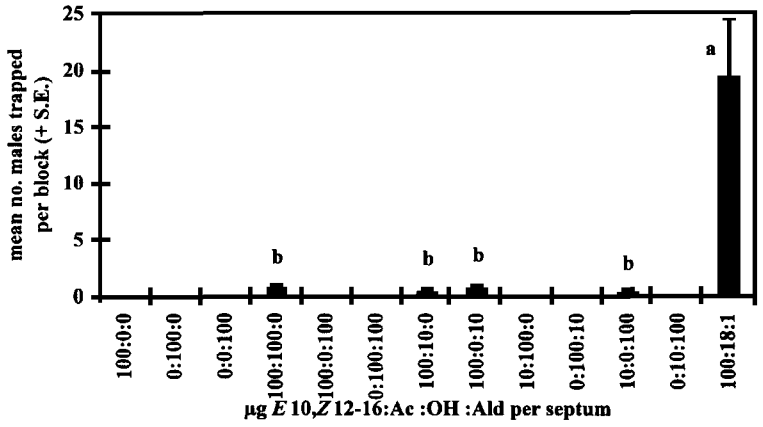


FIG. 2. Results of *Hemileuca eglanterina* (Boisduval) field trials of potential pheromone components as single components, binary blends at two different rates, and a ternary blend. The trial was conducted from July 20 to 22, 1993. Total number of moths trapped was 64. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for blend effect, $F = 20.6$; $df = 4, 8$; $P = 0.0003$.

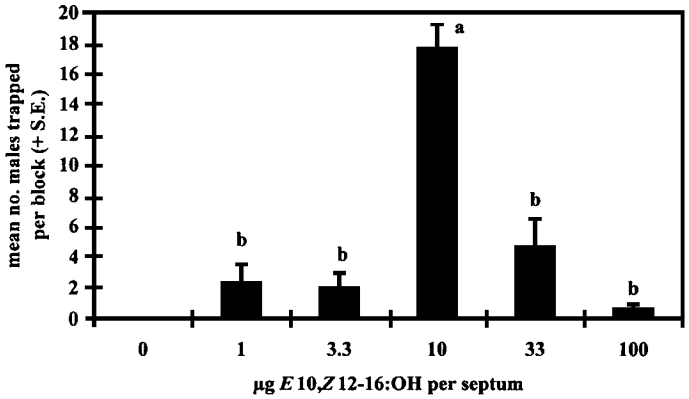


FIG. 3. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the *E*10,*Z*12–16 : OH rate, with *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : Ald held constant at 100 and 1 μg/septum, respectively. The trial was conducted from July 27 to 30 and August 3 to 4, 1993. Total number of moths trapped was 82. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : OH rate effect, $F = 10.5$; $df = 4, 8$; $P = 0.0028$; linear contrast, $F = 0.05$; $df = 1$; $P = 0.8286$; quadratic contrast, $F = 22.6$; $df = 1$; $P = 0.0014$.

ratios of *E*10,*Z*12–16 : Ald and *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ac were conducted concurrently. In each of the two tests, the dose of *E*10,*Z*12–16 : Ac was held constant (100 μg/septum), and either *E*10,*Z*12–16 : Ald or *E*10,*Z*12–16 : OH was added in varying amounts, bracketing the amounts found in female pheromone gland extracts. For the series with varying amounts of *E*10,*Z*12–16 : Ald, the *E*10,*Z*12–16 : OH was held constant at 33 μg/septum, while in the trial with varying amounts of *E*10,*Z*12–16 : OH, *E*10,*Z*12–16 : Ald was held constant at 1.0 μg/septum.

For the *E*10,*Z*12–16 : OH rate series, the 100 : 10 : 1 blend of *E*10,*Z*12–16 : Ac to *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald was most attractive (Figure 3). For the *E*10,*Z*12–16 : Ald rate test, the 100 : 33 : 3.3 rate attracted the most moths, although it was not statistically significantly better than the 100 : 33 : 10 or 100 : 33 : 1 blends (Figure 4). Comparing the best blends from each series, the 100 : 10 : 1 blend was significantly better than the 100 : 33 : 3.3 blend (*t* test, $t = 2.21$, $df = 22$, $P = 0.038$).

The fact that the two best blends both used a 10 : 1 ratio of the *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald suggested that the ratio of these minor components to each other might be more important than the ratio of either component to the major component, *E*10,*Z*12–16 : Ac. Two further tests were conducted to address this question. In the first, the *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald were held constant at 10 and 1 μg/septum, respectively, while

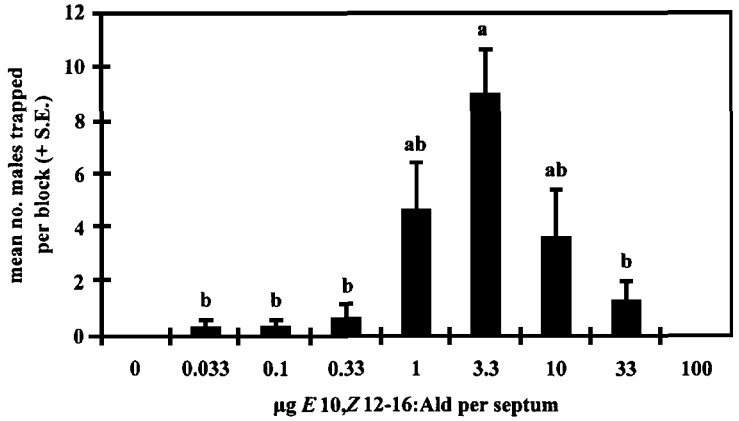


FIG. 4. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the *E*10,*Z*12–16 : Ald rate, with *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : OH held constant at 100 and 33 µg per septum, respectively. The trial was conducted from July 27 to 30 and August 3 to 4, 1993. Total number of moths trapped was 60. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ald rate effect, $F = 5.5$; $df = 6, 12$; $P = 0.0058$; linear contrast, $F = 8.2$; $df = 1$; $P = 0.0145$; quadratic contrast, $F = 10.0$, $df = 1$; $P = 0.0081$.

the dose of *E*10,*Z*12–16 : Ac was varied. The results indicated that increasing the amount of *E*10,*Z*12–16 : Ac in the blend increased the attractiveness of the lure (Figure 5), suggesting that the ratio of the major component to the minor components was not as important as its absolute dose.

To approach this from a different perspective, a second trial was conducted in which *E*10,*Z*12–16 : Ac was held constant and the amount of the two lesser components was varied, while keeping the ratio of those two components constant at 10 : 1. The 100 : 10 : 1 ratio attracted the most moths, but there was no significant difference between this treatment and a number of others, spanning more than a 100-fold range of ratios (Figure 6).

The final field trial addressed the roles of the other pheromone gland components that were present in extracts in significant amounts and/or that elicited small antennal responses in some GC-EAD replicates. In a subtractive bioassay of attraction, in which a "complete" reconstruction of the blend from the gland was compared to blends with single component deletions (Figure 7), only elimination of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, or *E*10,*Z*12–16 : Ald resulted in significantly lower trap catches than the complete blend. Thus, there was no statistical evidence that these other components either synergize or antagonize the response to the three major components.

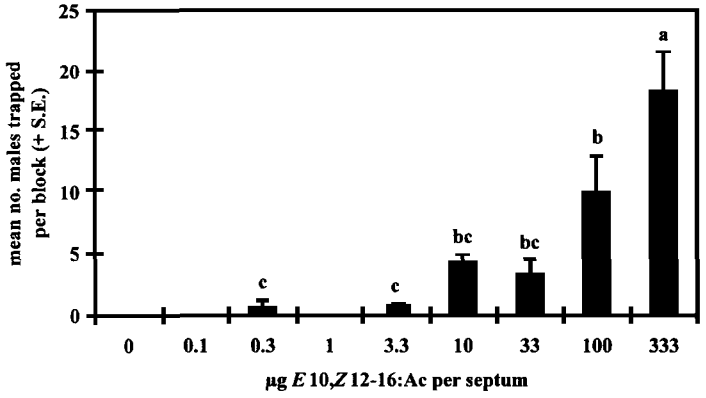


FIG. 5. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the *E*10,*Z*12–16 : Ac rate, with *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald held constant at 10 and 1 μg/septum, respectively. The trial was conducted from July 10 to 13, 1994. Total number of moths trapped was 112. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : Ac rate effect, $F = 14.3$; $df = 5, 10$; $P = 0.0003$; linear contrast, $F = 63.2$; $df = 1$; $P = 0.0001$; quadratic contrast, $F = 3.8$; $df = 1$; $P = 0.0792$.

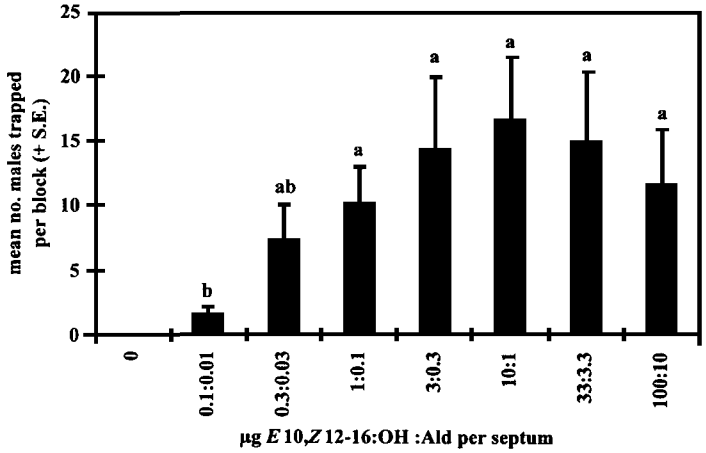


FIG. 6. Results of *Hemileuca eglanterina* (Boisduval) field trials for optimization of the minor components, when altered in tandem. The *E*10,*Z*12–16 : Ac rate was held constant at 100 μg/septum, while the combined dose of *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald, held at a 10 : 1 ratio, was varied. The trial was conducted from July 26 to 28, 1994. Total number of moths trapped was 230. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16 : OH/*E*10,*Z*12–16 : Ald rate effect, $F = 4.4$; $df = 6, 12$; $P = 0.0146$; linear contrast, $F = 15.6$; $df = 1$; $P = 0.0019$, quadratic contrast, $F = 10.1$; $df = 1$; $P = 0.0080$.

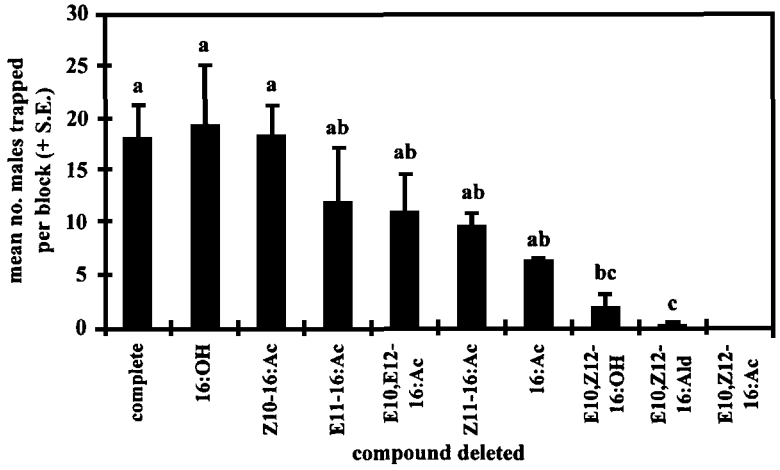


FIG. 7. Attractiveness to male *Hemileuca eglanterina* (Boisduval) of a reconstructed blend mimicking that obtained from female pheromone gland extracts versus blends containing single deletions from a complete blend. Amounts of materials (μg/septum): *E*10,*Z*12–16 : Ac, 100; *E*10,*Z*12–16 : OH, 30; *E*10,*Z*12–16 : Ald, 1; *E*10,*E*12–16 : Ac, 8; *Z*10–16 : Ac, 1.7; *Z*11–16 : Ac, 8.3; *E*11–16 : Ac, 1; 16 : Ac, 33; and 16 : OH, 11. The trial was conducted from July 21 to 24, 1996. Total number of moths trapped was 289. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for additive effect, $F = 7.3$; $df = 8, 16$; $P = 0.0004$.

DISCUSSION

The MTAD adducts proved to be useful in the identification of the three major pheromone components, for several complimentary reasons. First, the adducts were easy to form in quantitative yield by simply treating the pheromone extract with an aliquot of MTAD in CH_2Cl_2. Second, the increased molecular weight of the resulting adducts moved them to a region of the chromatogram that was relatively free of interference. Third, the molecular ions of the adducts were easily discernible for the alcohol and the acetate (and for standards of the aldehyde), providing confirmation of the molecular weights of the original compounds. Fourth, the diagnostic fragments locating the diene position were unmistakable, with one, the fragment at m/z 208, being the base peak in the spectrum. Fifth, the adducts were more reliably detected at lower concentration than the parent compounds because the adduct fragmentations were concentrated into a few diagnostic ions. Furthermore, because these ions were of distinctive and high molecular weights, they could be easily discerned from background interference. Overall, this method may be even more useful than the isobutane chemical ion-

ization method of determining diene positions (Doolittle et al., 1985) because the latter method gives diagnostic fragments of variable size and does not work well with acetates.

Previous field studies suggested that *Hemileuca* spp. may use both differing daily flight periods and differing pheromone chemistries to achieve reproductive isolation (Collins and Tuskes, 1979; Tuskes et al., 1996). Differences in sex pheromone chemistry have been postulated based on field observations of the relative attractiveness of different populations and species. However, to date, the sex pheromone of only a single species of North American saturniid moth has been reported (*E*6,*Z*11–16 : Ald), that of *Antherea polyphemus* (Cramer), (Kochansky et al., 1975), despite the biology of a number of species being quite well known. The only previous indication of the utilization of 10,12-hexadecadiene compounds as sex pheromone components in the Saturniidae is the observation that males of *Amphion nessus* (Cramer) were attracted to a female *Anisota virginiensis* (Drury) (Willaims, 1985). Bombykal, *E*10,*Z*12–16 : Ald, is known to attract males of *Amphion nessus floridensis* (B.P. Clark) (Landolt et al., 1989), thus suggesting that *A. virginiensis* may be producing this compound.

The major sex pheromone component of *H. eglanterina*, *E*10,*Z*12–16 : Ac, is the major component of the sex pheromone of *Rondotia menciana* Moore (Bombycidae) (Dai et al., 1987, 1988a) and, in combination with the *EE* isomer, has been used for mass-trapping (Dai et al., 1988b). Thus, our study is only the second time this compound has been identified as a moth sex pheromone and the first instance as a sex pheromone for the family Saturniidae. There is evidence that isomers of 10,12–16 : Ac are components of sex pheromones for moths in the family Sphingidae, with *Z*10,*E*12–16 : Ac having been identified as an attractant and eliciting antennal responses from males of *Pachysphinx modesta* (Harris) and *Smerinthus cerisyi* Kirby, and evidence, albeit scant, suggesting that *E*10,*Z*12–16 : Ac may be part of the sex pheromone of *Paonis exceactus* (J. E. Smith) (Reed et al., 1987). Furthermore, extracts of the pyralid moth *Diaphania hyalinata* (L.) are reported to contain trace amounts of *E*10,*E*12–16 : Ac, but it does not appear to be important for attraction (Raina et al., 1986). Our ongoing studies indicate that *E*10,*Z*12–16 : Ac may be widespread as a pheromone component in the genus *Hemileuca* (McElfresh and Millar, personal observation).

This is the first report of *E*10,*Z*12–16 : OH, or bombykol, as a sex pheromone component for a saturniid moth. Since its original isolation from *Bombyx mori* (L.) (Butenandt et al., 1959), bombykol has been identified from the congeneric *B. mandarina* Moore (Kuwahara and My-Yen, 1977) and from the pyralid moth *Notarcha derogata* (F.) (Himeno and Honda, 1992). However, the role of bombykol in the latter species is unclear because neither bombykol nor the *E*10,*E*12–16 : OH isomer were necessary for attraction; aldehydes were the major attractive constituents of the pheromone blend. *E*10,*E*12–16 : OH has now

been identified in *B. mori* female extracts, although its importance to the insect has not been established (Kasang and Schneider, 1978), and in *B. mandarina*, where it did not appear to affect attraction of male moths (Kuwahara et al., 1984). In fact, in all species in which bombykol has been found to date, including *H. eglanterina*, the *E*10,*E*12–16 : OH isomer has also been found, but there is not yet any mention of the other two stereoisomers, *Z*10,*E*12–16 : OH and *Z*10,*Z*12–16 : OH, in the sex pheromone literature (Arn et al., 1995).

In contrast to *E*10,*Z*12–16 : Ac and *E*10,*Z*12–16 : OH, *E*10,*Z*12–16 : Ald (bombykal) is a relatively common lepidopteran pheromone component, having been reported from moths in several families. It was first identified from female extracts of *B. mori* (Kasang et al., 1978). Soon afterwards it was identified as the major component for the sphinx moth, *Manduca sexta* (Johansson) (Starratt et al., 1979). A number of other sphingid species also have been attracted to lures containing *E*10,*Z*12–16 : Ald, including *Sphinx drupiferarum* (J. E. Smith) and *Hyles gallii* (Rottenburg) (Reed et al., 1987), and *Amphion floridensis* (= *Amphion nessus floridensis* B. P. Clark) (Landolt et al., 1989). Two genera of pyralid moths possess this compound, *Notarcha* and *Diaphania*. In *Notarcha*, both *N. basipunctalis* (Brem.) and *N. derogata* use bombykal as their major pheromone component, but the former also possesses significant amounts of the *E*10,*E*12–16 : Ald isomer, which appears to act as an isolating mechanism between the two species (Honda et al., 1994). For *Diaphania nitidalis* (Stoll) and *D. hyalinata* (L.), bombykal was present in small amounts in female extracts, and in wind-tunnel tests the compound was necessary to duplicate the entire range of male behaviors involved in attraction [*D. hyalinata* (Raina et al., 1986) and *D. nitidalis* (Klun et al., 1986)]. *D. hyalinata* also possessed significant amounts of the *E*10,*E*12–16 : Ald isomer and traces of the *Z*10,*Z*12–16 : Ald, both of which appeared to be important in wind-tunnel studies (Raina et al., 1986). Furthermore, it has been suggested that *E*10,*Z*12–16 : Ald is important in reproductive isolation of two noctuid species, *Earias insulana* (Boisduval) and *E. vittella* (F.), both of which use *E*10,*E*12–16 : Ald as their major pheromone component (Cork et al., 1988). Addition of *E*10,*Z*12–16 : Ald to a multicomponent blend increases attraction of *E. insulana* and decreases attraction of *E. vittella*. In addition to those mentioned above, moth species in the families Schreckensteiniidae and Gracillariidae are known to be attracted to isomers of 10,12–16 : Ald (Arn et al., 1995).

Conducting field trials for optimization of the *E*10,*Z*12–16 : OH and *E*10,*Z*12–16 : Ald ratios concurrently resulted in the interesting observation that the ratio of *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald appeared to be more critical than the ratios of either one to the major component, *E*10,*Z*12–16 : Ac. A shift of 10-fold or less from the optimum ratio of the two minor components resulted in significant reductions in trap catches (Figure 3 and 4). In contrast, when the dose of the two minor components was varied as a unit with the

ratio of *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald fixed at 10 : 1, and the amount of *E*10,*Z*12–16 : Ac was held fixed, there was no statistical difference between ratios tested over a range in excess of 100 fold (Figure 6).

In the final trial of a reconstructed blend versus blends with single deletions, only deletion of the three major components in the blend caused a statistically significant reduction in trap catches (Figure 7). However, the limited number of replicates and the variability in catch may have obscured subtle effects because single deletions of 16 : Ac, *Z*11–16 : Ac, *E*11–16 : Ac, and *E*10,*E*12–16 : Ac did appear to lower trap catches by as much as twofold. Thus, we hesitate to exclude the possibility that one or more of these minor components may indeed contribute to the efficacy of the blend. More sensitive tests, such as detailed analysis of male flight behaviors in a wind tunnel, and more detailed examination of the behavior exhibited by the responding males, as was employed in pheromone identifications for *Diaphania nitidalis* and *D. hyalinata*, for example (Klun et al., 1986; Raina et al., 1986), may reveal a role for these components.

The optimum blend of synthetic pheromone on a rubber septum was 100 : 10 : 1 *E*10,*Z*12–16 : Ac to *E*10,*Z*12–16 : OH to *E*10,*Z*12–16 : Ald (Figure 6), which differed somewhat from the mean ratio found in single female extracts (100 : 48 : 1.1, Table 1). The increased quantity of *E*10,*Z*12–16 : OH in the gland extracts may reflect that the alcohol is a potential precursor of both the corresponding acetate and the aldehyde. Furthermore, the mean gland content of *E*10,*Z*12–16 : OH was influenced by a single extract that possessed 2.5 times as much *E*10,*Z*12–16 : OH as *E*10,*Z*12–16 : Ac, further suggesting that *E*10,*Z*12–16 : OH may be a precursor or storage form of the two other pheromone components. Removal of this extract from the calculations reduced the mean content of *E*10,*Z*12–16 : OH for single female extracts from 48% to 40% of the *E*10,*Z*12–16 : Ac and halved the range from 6–260% to 6–129% (Table 1). The female aeration had somewhat less *E*10,*Z*12–16 : OH (30% of the amount of *E*10,*Z*12–16 : Ac), but was well within the range found with the single female extracts (Table 1). Much of this variation may be a reflection of the physiological state of the moth (e.g., when the gland extract was made in relation to when the moth began calling).

In summary, the female-produced sex attractant pheromone blend for *H. eglanterina* consists of *E*10,*Z*12–16 : Ac, *E*10,*Z*12–16 : OH, and *E*10,*Z*12–16 : Ald. Several other compounds found in the extracts elicited antennal responses but did not appear to be important in field trials. These compounds are possible precursors or by-products and may elicit antennal responses as a result of their structural similarity to pheromone components. It is possible that these compounds are important to other populations of this moth or to other species. Further studies of the sex pheromones of other members of the genus and of intraspecific differences in the pheromones of geographically isolated populations are in progress.

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