Novel Lepidopteran Sex Pheromone Components From *Marmara gulosa* (Lepidoptera: Gracillariidae)

J. STEVEN MCELFRESH,[1] JARDEL A. MOREIRA,[1] ELIZABETH E. GRAFTON-CARDWELL,[1] DAVID H. HEADRICK,[2] JOHN M. HERATY,[1] MARTA GUILLÉN,[1] AND JOCELYN G. MILLAR[1,3]

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ABSTRACT *Marmara gulosa* Guillén & Davis (Lepidoptera: Gracillariidae) is a sporadic pest of citrus and a number of other crops in southern and central California. Coupled gas chromatographic-electroantennogram detection analyses of headspace volatiles collected by solid phase microextraction from virgin female moths revealed at least four related compounds in the extracts that elicited significant antennal responses from antennae of male moths. These compounds were identified as (8*E*,10*E*)-tetradecadien-1-ol, and the corresponding aldehyde, acetate, and formate ester, representing the first report of a formate as a lepidopteran pheromone component. The four compounds were consistently found in headspace volatiles collected from virgin female moths from different regions of the state and from *M. gulosa* collected from different host plants (citrus and squash). Repeated field trials determined that the formate ester alone was as attractive or more attractive than any blend of the formate with one or more of the remaining compounds. Although large numbers of moths were caught in some field trials, trap catches were not consistent. Thus, the pheromone may be useful for detection of the moth and setting an initial biofix, but it remains unclear whether the pheromone can be used as a reliable and accurate tool for monitoring densities of *M. gulosa* populations.

KEY WORDS SPME, (8*E*,10*E*)-tetradecadien-1-yl formate, (8*E*,10*E*)-tetradecadien-1-ol, (8*E*,10*E*)-tetradecadienal, (8*E*,10*E*)-tetradecadien-1-yl acetate

Marmara gulosa Guillén & Davis (Lepidoptera: Gracillariidae) is a sporadic pest of citrus and other crops in southern and central California and Arizona, with grapefruit historically being the most susceptible citrus crop (Atkins 1961). *M. gulosa* larvae form long serpentine mines between the epidermal layers of the peel, and they feed on sap extracted from damaged cells within the mines (Guillén et al. 2001). Mining by a single larva can damage up to 25% of the fruit surface, rendering the fruit unacceptable for the fresh fruit market (Guillén et al. 2003). Larvae remain protected for almost their entire development within the mines (Atkins 1961, Guillén et al. 2001), limiting the effectiveness of chemical control (Grafton-Cardwell et al. 2003). There are seven generations per year (O'Neal 2007) in the central San Joaquin Valley of California, and typically three to four generations cause damage to citrus crops.

In southern California, *M. gulosa* has been a cyclical pest, and only periodically damages >5% of citrus fruit. In the central San Joaquin Valley, *M. gulosa* was a minor pest of grapefruit for many years, but since 1999 it has expanded its host range to include pummelo; several thin-skinned navel orange varieties, including 'Fukumoto', 'Thompson Improved', 'Powell', and 'Atwood'; and it has attacked these and other crops, such as walnuts, grapes, cotton, and beans to a greater extent than observed previously (Godfrey et al. 2003; Grafton-Cardwell et al. 2003).

Although there are several endemic parasitoids that attack *M. gulosa* and closely related gracillariid moths (Guillén 1999, Godfrey et al. 1999, Godfrey and Mayhew 2001, Gates et al. 2002), they have not provided reliable control of *M. gulosa* infestations in susceptible citrus varieties nor prevented significant losses (10–80%) of marketable fruit. Current methods of detection and sampling for *M. gulosa* consist of visual inspections of fruit for the presence of the characteristic larval mines. However, control measures need to be applied before mining affects the marketability of fruit. Thus, a pheromone-based method of sampling *M. gulosa* populations by using trap catches of adult male moths might provide a method for timing and so maximizing the efficacy of insecticide treatments.

Within the subfamily Gracillariinae, sex pheromones have been identified for only three species, with sex attractants identified serendipitously for another six species during pheromone screening trials targeting other lepidopteran species (Witzgall et al. 2008). The compounds that have been identified vary in structure from very common lepidopteran pheromone components, such as (Z)-11-hexadecenal, to unique and more complicated structures, such as

[1] Department of Entomology, University of California, Riverside, CA 92521.
[2] College of Agriculture, California Polytechnic State University, San Luis Obispo, CA 93407.
[3] Corresponding author, e-mail: jocelyn.millar@ucr.edu.

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(4*E*,6*E*,10Z)- and (4*E*,6Z,10Z)-hexadecatrienyl acetates (Witzgall et al. 2008). The goal of the work described here was to identify the sex pheromone of *M. gulosa* and to explore whether the pheromone could be used for monitoring this pest species.

Materials and Methods

Insects. Insects used in solid phase microextraction (SPME; see below) collection of volatiles from live females for the identification of sex pheromone components were obtained from field infested "Marsh Red" grapefruit *Citrus* x *paradisi* Macfadyen from commercial orchards in the Coachella Valley, Riverside Co., CA, in 2001 through 2002, and reared using methods described in Guillén et al. (2007). Insects used for comparisons of populations, using SPME to collect pheromone from live females, came from the above-mentioned location and two additional sites: University of California Riverside, Agricultural Operations (collected from table queen squash in October and November 2002; *Curcurbita pepo* L.); and Kearney Agricultural Center, Parlier, Fresno Co., CA (collected from bell pepper in October and November 2002; *Capsicum annuum* L.). Insects used in making solvent extracts of female sex pheromone glands were from the Parlier population and were from a colony reared in August 2006, on zucchini squash (*C. pepo* variety 'Revenue', Syngenta Seeds Inc., Boise, ID) in the laboratory at 25 ± 3°C, and 30–40% ambient humidity, and a photoperiod of 12:12 (L:D) h. The Parlier collection was made after infestations of the new strain had become widespread in the San Joaquin Valley.

Insects were allowed to pupate either on the fruit or on paper toweling below the fruit. The paper toweling was cut into pieces with one to three cocoons and then placed in 49- by 11-mm shell vials. Vials containing pupae were capped with a foam plug and placed on their sides on a pleated wire rack inside a plastic box (32 cm length by 17.5 cm width by 10 cm height). Humidity inside the box was provided by a water-saturated cellulose sponge (17 by 20 by 0.3 cm dry) underneath the wire rack inside the plastic box. Vials were checked for emergence and the sex of adults determined by examining the terminal end of the abdomen (females possess an opening between the scales where the ovipositor is located, male terminalia are covered by scales). Adults were fed an 8% sugar-water solution on cellulose sponge cubes (≈3 mm square). Male moths were used for coupled gas chromatography-electroantennogram detection (GC-EAD) analyses, whereas females were used for collection of pheromone by adsorption of headspace volatiles from live virgin females. Voucher specimens of adult moths have been deposited in the Entomology Museum, Department of Entomology, University of California, Riverside.

Identification of Volatiles Collected from Female Moths. *Collection and Analysis of Pheromone.* SPME (Supelco, Bellefonte, PA) was used to collect pheromone compounds emitted by virgin female moths from Coachella grapefruit, Riverside squash, and Parlier squash populations. The SPME fiber (100-μm polydimethylsiloxane coating) was conditioned before use at 250°C for 10 min in a GC injector. One to 20 virgin female moths were placed in a cylindrical glass tube (4.5–9 cm by 1 cm diameter) fitted with an ≈1.3-mm internal diameter by 65-mm glass outlet tube. Swagelok fittings with Teflon ferrules were used to connect the tubes (0.5–0.125-in. [1.27–0.32 cm] Swagelok union). Another tube containing activated charcoal (50–200 mesh) and Soxhlet-extracted glass wool saturated with deionized water was placed upstream from the aeration chamber using a 0.5–0.5-in. (1.27–1.27 cm) Swagelok union with Teflon ferrules. A medical air supply line was attached to the upstream end of the humidifier (purifier) by using another Swagelok union (0.5–0.125 in. [1.27–0.32 cm]), with the flow regulated by either a 0–7 or 0–50 ml/min maximum Gilmont flowmeter. The apparatus was flushed with charcoal-purified medical air at 2.5–3.0 ml/min in early aerations, and from 5 to 11 ml/min in subsequent aerations, with the SPME fiber placed in the outlet tube of the vial to collect the emitted volatiles. Collections were started at ≈1700 hours and stopped at ≈0900 hours the next morning. During the daylight hours and at least every other night moths were fed an 8% sugar–water solution on 3-mm cubes of sponge placed on a small section of foil in the aeration chamber. Before beginning pheromone collections, the sponge and foil were removed. Aerations were conducted in the laboratory with ambient lighting during the day, and with the temperature ranging from 22 to 30°C.

Comparison of Headspace Collections and Solvent Extractions of Pheromone Glands. Sex pheromone glands of virgin females from the Parlier laboratory colony reared on squash were dissected and extracted for analysis. Virgin females were isolated individually in vials, which were placed in a light box at ≈21°C on a reversed photoperiod with lights out at 1335 hours (a photoperiod of 16:8 [L:D] h). Ovipositors were dissected from females 1–3 h into the scotophase by holding the moth with one pair of forceps, isolating the sex pheromone gland with another pair of fine forceps, and then cutting off the ovipositor with a razor blade and placing the dissected tissue into 50 μl of pentane in a 0.25-ml conical insert in a 3.5-ml vial. Ovipositors from groups of 24–101 females (corresponding to how many females were available) were combined and extracted for 1 h, and then the extract was transferred to a clean vial insert. Samples were stored at −20°C until analyzed.

Synthetic standards and the insect-produced volatiles collected on the SPME fiber were analyzed by coupled GC-EAD, using a Hewlett–Packard (HP) 5890 series II GC operated in splitless mode. Effluent from the column was split between two 1-m sections of deactivated fused silica tubing (0.25 mm i.d.). One branch was directed to the flame ionization detector, whereas the other branch was directed through a heated conduit (250°C) into an ambient temperature humidified airstream (≈500 ml/min), which passed

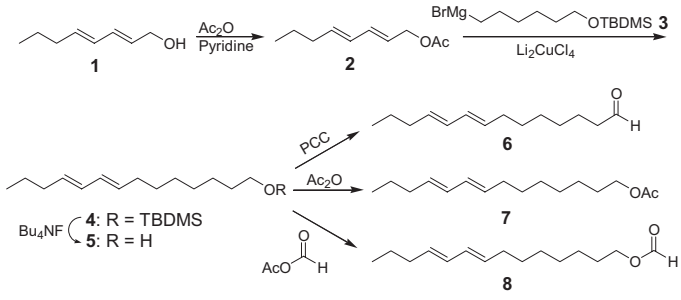

Fig. 1. Synthesis of the possible pheromone components 8*E*,10*E*-14:OH, 8*E*,10*E*-14:Ald, 8*E*,10*E*-14:formate, and 8*E*,10*E*-14:Ac.

over the antennal preparation. Males of the Coachella and San Joaquin strains were captured by the body using fine forceps and the head and antennae were removed by holding the head in place while pulling the body away. The terminal 1–3 antennal segments were removed using a razor blade before placing the base of the head into the indifferent electrode, whereas the two antennal tips were inserted into the recording electrode. Electrodes consisted of saline-filled (7.5 g of NaCl, 0.21 g of $CaCl_2$, 0.35 g of KCl, and 0.20 g of $NaHCO_3$ in 1 liter of distilled water) 2-mm o.d. glass capillary tubes, with chloridized silver wires or gold wires down the center of each electrode. GC and EAD traces were recorded simultaneously on a matched pair of HP-3394 recording integrators. Columns and programs used to analyze extracts included the following: DB-5 (30 m by 0.32 or 0.25 mm i.d., 0.25-μm film, 70°C for one or 5 min, or 100°C for 1 min, 5, 10, or 15 °C/min to 250 or 275°C for five or more min) and DB-WAX (30 m by 0.25 mm i.d., 0.25-μm film, 100°C/1 min, 5 or 10°C/min–250°C/5 or more min). Antennal preparations usually only lasted for approximately five injections or less, with deterioration in sensitivity over time.

SPME extracts also were analyzed by coupled GC-mass spectrometry (GC-MS). Electron impact mass spectra (70 eV) were taken with an HP 6890 gas chromatograph interfaced to a 5973 mass selective detector. A 30 m by 0.25 mm i.d. HP5-MS column was used in splitless mode, programmed from 40°C/1 min, 10°C/min–250°C/20 min, injector 250°C, and transfer line 280°C. The SPME fiber was desorbed for 30 s in the injection port with the split valve closed before starting the temperature program.

Synthesis of Pheromone Components (Fig. 1). Tetrahydrofuran (THF) was distilled from sodium-benzophenone ketyl before use. Unless otherwise stated, extracts of reaction mixtures were dried over anhydrous Na_2SO_4, and concentrated under partial vacuum on a rotary evaporator. All reactions were carried out in oven-dried glassware under an atmosphere of argon. Flash chromatography was performed using 230–400 mesh silica gel. NMR spectra were taken on a Varian INOVA 400 NMR spectrometer in $CDCl_3$ solvent, and mass spectra were taken with an HP 6890 gas chromatograph interfaced to an H-P 5973 mass selective detector as described above. Reaction yields have not been optimized.

Preparation of (2E,4E)-octadien-1-yl Acetate (2). (2*E*,4*E*)-octadien-1-ol **1** (5.00 g; 39.6 mmol; Bedoukian Research, Danbury CT) was dissolved in dry pyridine (8.0 ml), and a solution of acetic anhydride (8.10 g, 79.2 mmol) in dry pyridine (8.0 ml) was added at 0°C. The mixture was stirred for 5 h at room temperature and then poured onto ice (80 g) and extracted with hexane (5 by 50 ml). The organic layer was washed with brine, dried, and concentrated, and the crude product was Kugelrohr distilled (0.4 mmHg; 56–62°C), giving acetate **2** (6.24 g, 93.7% yield) as a colorless oil. ^{1}H NMR: δ 0.90 (t, 3H, J = 7.2 Hz), 1.41 (sext, 2H, J = 7.2 Hz), 2.06 (s, 3H), 2.03–2.12 (m, 2H), 4.57 (d, 2H, J = 6.8Hz), 5.64 (dt, 1H, J = 6.4, 15.2 Hz,), 5.75 (dt, 1H, J = 6.8, 15.2 Hz), 6.04 (dd, 1H, J = 10.8, 15.2 Hz), 6.26 (dd, 1H, J = 10.8, 15.2 Hz). ^{13}C NMR: δ 13.66, 21.00, 22.27, 34.67, 64.99, 123.81, 129.26, 135.08, 136.69, 170.86.

Preparation of (8E,10E)-Tetradecadien-1-ol (5). A 0.5 M solution of Grignard reagent (**3**) was prepared from (6-bromohexyloxy)-*tert*-butyldimethylsilane (5.00 g; 16.8 mmol) and magnesium turnings (0.49 g; 20.2 mmol) in THF (34 ml). This solution was added dropwise over 10 min to a cooled (−50°C) solution of acetate **2** (1.88 g; 11.2 mmol) in THF (15 ml) and Li_2CuCl_4 (0.48 mmol; 4.8 ml of 0.1 M solution in THF). The resulting mixture was allowed to warm to room temperature and stirred overnight. Saturated aqueous NH_4Cl (30 ml) was then added, and the mixture was extracted with hexane (4 by 50 ml). The organic layer was washed with brine, dried, and concentrated, and the crude product was used in the next step without purification.

Protected alcohol **4** (6.20 g of crude product) was dissolved in THF (9 ml), cooled to 0°C, and a solution of tetrabutylammonium fluoride (1 M; 25 ml) was added. The mixture was stirred at 0°C for 10 min and 5 h at room temperature. The reaction was quenched by addition of HCl (1 M; 50 ml) and extracted with hexane (4 by 50 ml). The combined organic layers were washed with water and saturated $NaHCO_3$, dried, and concentrated. The crude product was purified by flash chromatography (hexanes:ethyl acetate, 5:1) affording alcohol **5** (3.01 g). The product was

purified further by recrystallization from hexane at −20°C, affording pure **5** (81% recovery from recrystallization) in >99% chemical and isomeric purity. ^{1}H NMR: δ 0.90 (t, 3H, J = 7.2 Hz), 1.24–1.46 (m, 9H), 1.56 (m, 4H), 2.03 (q, 2H, J = 7.2 Hz), 2.05 (q, 2H, J = 6.8 Hz), 3.64 (t, 2H, J = 6.4 Hz), 5.50–5.63 (m, 2H), 5.94–6.05 (m, 2H). ^{13}C NMR: δ 13.71, 22.56, 25.62, 29.12, 29.25, 29.32, 32.54, 32.72, 34.68, 63.06, 130.38, 130.45, 132.23, 132.30. GC-MS: *m/z* 210 (M^+, 15), 149 (2), 135 (6), 121 (9), 109 (17), 95 (26), 81 (54), 67 (100), 55 (30), 43 (10), 41 (30).

Preparation of (8E,10E)-Tetradecadienal (6). 8*E*,10*E*-14:OH **5** (0.050 g; 0.24 mmol) was added to a suspension of pyridinium chlorochromate (0.108 g; 0.29 mmol) and powdered molecular sieve 4A (0.108 g) in dry CH_2Cl_2 (2.0 ml), and the mixture was stirred for 2 h. Dry ethyl ether (10 ml) was then added, and after stirring 5 min, the mixture was filtered through a pad of Celite and charcoal, rinsing well with ether. After concentration, the crude product was Kugelrohr distilled (0.50 mmHg; 65–69°C), giving 8*E*,10*E*-14:Ald **6** (0.034 g; 69% yield) as a colorless oil. ^{1}H NMR: δ 0.89 (t, 3H, J = 7.2 Hz), 1.24–1.45 (m, 8H), 1.56–1.68 (m, 2H), 2.03 (q, 2H, J = 6.8 Hz), 2.04 (q, 2H, J = 6.4 Hz), 2.41 (td, 2H, J = 2.0, J = 7.2 Hz), 5.48–5.62 (m, 2H), 5.94–6.04 (m, 2H), 9.75 (t, 1H, J = 2.0 Hz). ^{13}C NMR: δ 13.70, 21.99, 22.54, 28.85, 28.98, 29.14, 32.44, 34.67, 43.86, 130.39, 130.50, 132.04, 132.33, 202.88. MS: *m/z* 208 (M^+, 17), 151 (3), 137 (3), 123 (4), 109 (16), 95 (25), 81 (44), 67 (100), 55 (23), 41 (27).

Preparation of (8E,10E)-Tetradecadien-1-yl Acetate (7). Acetic anhydride (0.25 ml) was added dropwise to a solution of 8*E*,10*E*-14:OH **5** (0.020 g; 0.10 mmol) and pyridine (0.25 ml) in dry ethyl ether (2.0 ml) at 0°C. The mixture was warmed to room temperature and stirred for 8 h and then cooled to 0°C, and saturated aqueous $NaHCO_3$ (2.0 ml) was added cautiously. The mixture was extracted with ethyl ether (2 by 5 ml). The organic layer was washed with 1 M HCl and brine, dried, and concentrated. The crude product was purified by flash chromatography (hexane:ethyl acetate, 95:5) affording acetate **7** (0.016 g; 66.7% yield). ^{1}H NMR: δ 0.89 (t, 3H, J = 7.2 Hz), 1.24–1.45 (m, 10H), 1.61 (quint, 2H, J = 6.8 Hz), 2.02 (q, 2H, J = 6.4 Hz), 2.04 (s, 3H), 2.04 (q, 2H, J = 6.0 Hz), 4.04 (t, 2H, J = 6.8 Hz), 5.49–5.62 (m, 2H), 5.95–6.04 (m, 2H). ^{13}C NMR: δ 13.69, 20.98, 22.54, 25.82, 28.55, 29.02, 29.08, 29.28, 32.51, 34.67, 64.60, 130.40, 130.43, 132.22 (2C), 171.22. MS: *m/z* 252 (M^+, 18), 192 (4), 163 (3), 149 (8), 135 (16), 121 (24), 110 (15), 109 (18), 107 (17), 96 (29), 95 (27), 93 (33), 81 (56), 79 (61), 67 (100), 55 (29), 43 (53), 41 (27).

Preparation of (8E,10E)-Tetradecadien-1-yl Formate (8) with Formic Acetic Anhydride. Formic acetic anhydride was freshly prepared by addition of formic acid (96%; 0.10 ml) to acetic anhydride (0.20 ml) at 0°C. When the addition was complete, the mixture was slowly warmed to 50°C, held at this temperature for 20 min and then cooled to room temperature, and used immediately. The mixed anhydride (0.25 ml) was added dropwise to a solution of 8*E*,10*E*-14:OH (0.10 g; 0.48 mmol) and pyridine (0.25 ml) in dry ethyl ether (2.0 ml) at 0°C. The mixture was warmed to room temperature and stirred for 8 h, then cooled to 0°C, and saturated aqueous $NaHCO_3$ (2.0 ml) was added cautiously. The mixture was extracted with ethyl ether (2 by 5 ml). The combined organic layers were washed with 1 M HCl and brine, dried, concentrated, and purified by flash chromatography (hexane:ethyl acetate, 95:5), affording formate **8** (0.098 g; 86.7% yield). ^{1}H NMR: δ 0.89 (t, 3H, J = 7.2 Hz), 1.22–1.45 (m, 10H), 1.65 (quint, 2H, J = 6.8 Hz), 2.03 (q, 2H, J = 6.8 Hz), 2.05 (q, 2H, J = 6.8 Hz), 4.15 (t, 2H, J = 6.8 Hz), 5.49–5.63 (m, 2H), 5.94–6.05 (m, 2H), 8.06 (s, 1H). ^{13}C NMR: δ 13.71, 22.55, 25.73, 28.46, 28.99, 29.01, 29.26, 32.51, 34.68, 64.08, 130.43 (2C), 132.19, 132.28, 161.20. MS: *m/z* 238 (36), 163 (2), 149 (4), 135 (9), 121 (15), 109 (19), 96 (27), 95 (27), 81 (53), 67 (100), 55 (25), 41 (23).

Preparation of (8E,10E)-Tetradecadien-1-yl Formate (8) with Formic Acid. 8*E*,10*E*-14:OH **5** (36 mg) was dissolved in 0.1 ml of 97% formic acid, and the mixture was heated at 60°C for 3 h in a sealed vial. After cooling, the mixture was partitioned between water and hexane. The hexane layer was washed with saturated $NaHCO_3$ and brine, dried, concentrated, and purified as described above, yielding the formate ester **8** completely free of the corresponding acetate.

Field Trials. Four field trials were conducted in a heavily infested organic Ruby Red grapefruit orchard located ≈14 km south of Coachella, Riverside Co., CA (33° 31′38.60″ N, 116° 08′08.78″ W) during June 2002, July 2003, and October 2006. Two additional field trials were conducted during September–November 2006 in a mixed block of 'Lane Late' and 'Powell' navel oranges in the San Joaquin Valley west of Strathmore, Tulare Co., CA (36° 08′0.60″ N, 119° 07′10.19″ W).

Lures consisted of 11-mm gray rubber septa (West Pharmaceutical, Lionville, PA) loaded with heptane solutions of the pheromone components (typically, 0.1 mg of the major component and appropriate amounts of minor components and 5 μg per septum of Topanol CA antioxidant). Unless otherwise stated, compounds were >98% chemically and isomerically pure. Trial 1, conducted during 21–27 June 2002, compared 8*E*,10*E*-14:formate, 8*E*,10*E*-14:OH, and 8*E*,10*E*-14:Ald as single components (100 μg) and in blends (33:100:20, 33:100:0, 0:100:20, and 100:0:60, respectively). Trial 2, conducted during 3–18 July 2003, compared lures with a base blend consisting of 10:80:75:32 μg per septum 8*E*,10*E*-14:Ald, 8*E*,10*E*-14:OH, 8*E*,10*E*-14:formate, and 8*E*,10*E*-14:Ac (94% *EE*), respectively. Trial 3, conducted during 18–28 July 2003, compared lures with a base blend consisting of 10:80:75:32 μg per septum (8*E*,10*E*)-tetradecadienal, (8*E*,10*E*)-tetradecadien-1-ol, (8*E*,10*E*)-tetradecadien-1-yl formate, and (8*E*,10*E*)-tetradecadien-1-yl acetate (>99% *EE*), respectively. Trial 4, conducted during 13–28 September 2006, used lures containing a blend of 8*E*,10*E*-14:Ald, 8*E*,10*E*-14:OH, 8*E*,10*E*-14:formate, and 8*E*,10*E*-14:Ac (8:65:100:67 μg per septum, respectively), alone or in combination with a 1 or 10 μg per septum dose of 8*E*,10*Z*-14:Ac, and single component lures with 100 μg per septum of each of the compounds (excluding the

8*E*,10*Z*-14:Ac). Trial 5, conducted during 12–26 October 2006, tested lures containing single components (100 μg per lure) versus blends of four components (8*E*,10*E*-14:Ald, 8*E*,10*E*-14:OH, 8*E*,10*E*-14:formate, and 8*E*,10*E*-14:Ac, 8:65:100:67 μg per septum, respectively) with and without 1 or 10 μg per septum 8*E*,10*Z*-14:Ac. In trial 6, conducted from 26 October to 16 November 2006, attractiveness of lures treated with 8*E*,10*E*-14:formate lots synthesized by different routes were compared (see above). Single components were tested at the 100 μg per septum dose. Controls were included in all trials and consisted of septa treated only with heptane and stabilizer.

Lures were deployed in green or orange delta sticky traps (Pherocon IIID, Trécé, Inc., Adair, OK) in randomized blocks (replicated five times), which were hung in the outer canopy on the north side of trees, at a height of ≈1.5 m. Trap catches were counted at 2–7-d intervals, with traps being rerandomized at each count for the Coachella trials. Trap count data were summed over the count dates, then transformed (square root $[x + 0.5]$ or $\log_{10}[x + 1]$) and analyzed for treatment and block effects by two-way analysis of variance (ANOVA), followed by a Student–Newman–Keuls means separation test if significant differences were found ($P = 0.05$). Treatments capturing zero insects were not included in the analyses because they violated the assumptions of ANOVA. All statistical analyses were carried out with SigmaStat version 1.0 statistical software (Systat Software, Inc., San Jose, CA).

Results

Identification of Volatiles Collected from Female Moths. GC-EAD analyses of the samples collected by SPME from virgin female moths from early collections from Coachella Valley grapefruit showed at least three relatively large peaks (Fig. 2) that consistently elicited responses from antennae of male moths. The mass spectrum of the first peak (peak 1) gave a molecular ion at *m*/*z* 208 and a base peak at *m*/*z* 67, suggestive of a 14-carbon diunsaturated aldehyde of molecular formula $C_{14}H_{24}O$. That the retention time was significantly longer than that of representative monounsaturated aldehydes, coupled with the relatively large size of the molecular ion peak (17% of base peak), suggested a conjugated dienal. Because 8,10-dienal structures had been reported previously as pheromone components (Svatos et al. 1999) or attractants (Ando et al. 1987) for moths in this subfamily, these seemed the most likely structures for this component. Thus, all four isomers of (8,10)-tetradecadienal were synthesized, and comparison of their mass spectra and GC retention times on polar and nonpolar columns with those of the insect-produced compound determined that the data for the latter compound only matched those of (8*E*,10*E*)-tetradecadienal (8*E*,10*E*-14:Ald).

The second major peak in the SPME extracts (Fig. 2, peak 2) exhibited a significant molecular ion (typical of conjugated alcohols) at *m*/*z* 210, a diagnostic ion at *m*/*z* 192 (M^+-18) from loss of H_2O, and a base peak at *m*/*z* 67. The similarity between the mass spectrum of this compound and the spectrum of 8*E*,10*E*-14:Ald, and the fact that it was 2 mass units larger than 8*E*,10*E*-14:Ald suggested that it was the corresponding alcohol. Comparison of its mass spectrum and chromatographic retention data with those of an authentic standard confirmed the identification as (8*E*,10*E*)-tetradecadien-1-ol (8*E*,10*E*-14:OH). The retention time data of the other three 8,10-isomers did not match those of the insect-produced compound, confirming the stereochemistry of the double bonds as 8*E*,10*E*.

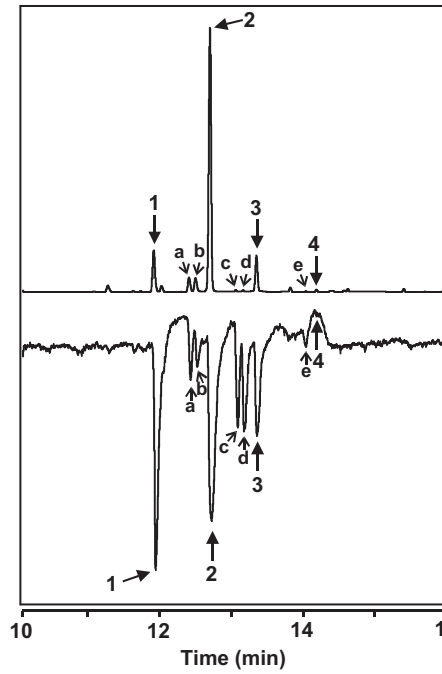

Fig. 2. Coupled GC-EAD traces from an SPME collection of volatiles from three female *M. gulosa* over a single night. Column: DB-5, 30 m by 0.25 mm i.d. by 0.25-μm film, temperature program 100°C/1 min and then 10°C/min to 250°C for 20 min. Peak 1, (8*E*,10*E*)-tetradecadienal; peak 2, (8*E*,10*E*)-tetradecadien-1-ol; peak 3, (8*E*,10*E*)-tetradecadien-1-yl formate; peak 4, (8*E*,10*E*)-tetradecadien-1-yl acetate; peaks a and b, (8*Z*,10*E*)- and (8*E*,10*Z*)-tetradecadien-1-ol, respectively; peaks c and d, (8*Z*,10*E*)- and (8*E*,10*Z*)-tetradecadien-1-yl formate, respectively; and peak e, (8*E*,10*Z*)-tetradecadien-1-yl acetate.

The third GC-EAD peak (Fig. 2, peak 3) exhibited a significant molecular ion at *m*/*z* 238, corresponding to a possible molecular formula of $C_{15}H_{26}O_2$. Its mass spectrum was otherwise quite similar to those of 8*E*,10*E*-14:Ald and 8*E*,10*E*-14:OH, with a base peak at *m*/*z* 67, and significant ions at *m*/*z* 81, 95, and 109. The two oxygen atoms suggested the presence of an ester, with the two most likely structures being the formate ester of 8*E*,10*E*-14:OH or methyl (8*E*,10*E*)-tetradecadienoate. The former structure was deemed more likely due to the lack of both an *m*/*z* 74 fragment, characteristic of McLafferty rearrangement of a methyl ester, and an (M^+-31) ion, from loss of CH_3O from a methyl ester. The two compounds were syn-

thesized, and the mass spectrum and retention data of the (8*E*,10*E*)-tetradecadien-1-yl formate exactly matched those of the insect-produced compound, whereas those of methyl (8*E*,10*E*)-tetradecadienoate were markedly different. In particular, the mass spectrum of methyl (8*E*,10*E*)-tetradecadienoate exhibited a significant fragment at *m*/*z* 74 as expected, and an additional fragment at *m*/*z* 87, from the ion $[CH_2CH_2COOCH_3]^+$. Both of these fragments were absent in the spectrum of peak 3.

A fourth GC peak, which did not seem to elicit a visible response from the antenna (Fig. 2, peak 4) showed a strong molecular ion at *m*/*z* 252 (19%), and a fragment at *m*/*z* 192 (M^+-60), indicative of loss of acetic acid from an acetate ester. The lower mass region of the spectrum was very similar to those of the above-mentioned three compounds, with a base peak at *m*/*z* 67, and other prominent fragments at *m*/*z* 79 (62%), 81 (53%), 95 (28%), and 96 (31%), and a series of ions at *m*/*z* 121, 135, 149, and 163. These data, coupled with its GC retention characteristics, suggested that this compound might be (8*E*,10*E*)-tetradecadien-1-yl acetate, and this was verified with synthetic standards as described above.

For peaks 1, 2, and 4, the position of the diene system in each compound was corroborated by careful examination of the relative ratios of various fragment ions in relation to published mass spectral data (taken under the same conditions of electron impact ionization at 70 eV) on the series of (*E*,*E*)-tetradecadienyl alcohols, aldehydes, and acetates with dienes in the 7,9 to the 11,13 positions (Ando et al. 1988). In particular, for the alcohol and acetate, the fragment at *m*/*z* 96> *m*/*z* 95 was diagnostic for the 8,10-diene structure, whereas for the aldehyde, the base peak at *m*/*z* 67, coupled with the *m*/*z* 109/110 ratio excluded the other possibilities. The stereochemistry of the 8,10-diene function in each structure was further verified by comparison of the retention times of the insect-produced compounds with those of synthetic standards of the four 8,10–14:OH, formate, and acetate isomers on nonpolar (DB-5), midpolarity (DB-17), and polar (DB-WAX and DB-225) GC columns. In all cases, only the (8*E*,10*E*)-isomers matched the insect-produced compounds. In addition, synthetic 8*E*,10*E*-14:Ald, 8*E*,10*E*-14:OH, and 8*E*,10*E*-14:formate all elicited medium to strong responses from male moth antennae in GC-EAD trials.

In addition to the (8*E*,10*E*)-compounds corresponding to the four major peaks seen in SPME extracts from live female moths, chromatograms showed traces of isomers of these components. However, it was unclear whether these trace isomers were indeed produced by the female moths, or whether they were artifacts from the collection or analysis method. In particular, injections of standards of the (8*E*,10*E*)-compounds revealed that the amounts of isomers seen in the resulting GC traces increased with higher injector temperatures (250°C), and when injections were made in splitless mode with the injected compounds remaining in the heated injector for a longer period. For example, an increase in injector temperature from 150 to 250°C resulted in a roughly sevenfold increase in the amount of the 8*E*,10*Z* isomer of the formate when analyzed with the DB-5 column. Higher injector temperatures and splitless injections also resulted in (8*E*,10*E*)-14:OH being detected when 8*E*,10*E*-14:formate was injected (when injector temperatures were increased from 150 to 250°C a roughly eight-fold increase in (8*E*,10*E*)-14:OH was observed on both DB-5 and DB-WAX columns), indicating that small amounts of the formate were hydrolyzed to the corresponding alcohol under these conditions. Because the SPME collections of pheromone had to be injected in splitless mode because of the small amounts of pheromone collected, we cannot be sure that the trace amounts of some isomers and compounds seen in GC analyses were not analytical artifacts. However, the relatively strong antennal response to one particular trace component in the extracts, (8*E*,10*Z*)-14:Ac, suggested that this component might have some role as a semiochemical. This possibility was investigated during field trials (see below).

Comparison of Headspace Collections and Solvent Extractions of Pheromone Glands. Initially, headspace collections were conducted using multiple females from the Coachella valley population of unknown age. These aerations yielded extremely variable ratios of components, with the ratio of the formate to alcohol varying between 12:1 and 1:2. To obtain a better idea of the ratios of the various components, headspace collections were made from single females of known age, by using females reared from pupae from three different locations and hosts (the Riverside squash strain, the Coachella Valley grapefruit strain, and the Parlier squash strain), and changing the SPME collectors daily. Single aerations yielded far more reproducible results, with the formate being consistently the most abundant compound produced by all three populations. When the formate is expressed as a proportion of the combined total of all four components (*E*8,*E*10–14:formate, *E*8,*E*10–14:alcohol, *E*8,*E*10–14:aldehyde, and *E*8,*E*10–14:acetate), it constituted >50% of the blend for all three populations over the first four nights after emergence of the adult female moths. Mean ratios for each population for the first and third nights were (mean ± SD formate:alcohol:aldehyde:acetate): for day 1, 72 ± 10: 18 ± 8:7 ± 6:3 ± 2 for Coachella grapefruit ($n = 6$), 75 ± 16:10 ± 13:4 ± 6:1 ± 1 for the Parlier squash ($n = 6$), and 73 ± 11:22 ± 9:3 ± 2:2 ± 1 for Riverside squash ($n = 5$); for day 3, 54 ± 14:26 ± 9:11 ± 11:9 ± 8 for Coachella grapefruit, 51 ± 10:37 ± 12:10 ± 6:2 ± 1 for Parlier squash, and 60 ± 12:34 ± 10:3 ± 1:3 ± 2 for Riverside squash. In contrast, a solvent extract of 26 ovipositors yielded a ratio of 42:27:3.3:28 formate:alcohol:aldehyde:acetate.

Synthesis of Pheromone Components. The route used to synthesize 8*E*,10*E*-14:OH and the corresponding aldehyde, formate, and acetate is shown in Fig. 1. The key step was the coupling reaction between (2*E*,4*E*)-octadien-1-yl acetate **2,** readily available in one step from the corresponding alcohol **1,** and a

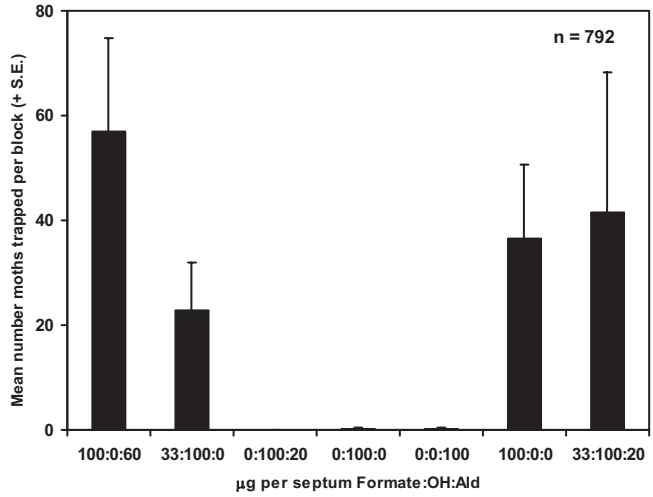

Fig. 3. *M. gulosa* field trial testing (8*E*,10*E*)-tetradecadienal, (8*E*,10*E*)-tetradecadien-1-ol, and (8*E*,10*E*)-tetradecadien-1-yl formate, conducted at the Coachella Valley site 21 June-27, 2002. Treatments capturing more than a single insect were not significantly different (two-way ANOVA: for treatment effect, $F = 1.96$, df = 3, 19, $P = 0.17$; for block effect, $F = 7.16$, df = 4, 19, $P = 0.004$).

Grignard reagent **3,** with dilithium tetrachlorocuprate catalysis. This coupling of allylic acetates with Grignard reagents has been reported to proceed in good yield with high regioselectivity, with retention of the stereochemistry of the double bonds (Fouquet and Schlosser 1974). Thus, commercially available (2*E*,4*E*)-octadien-1-ol **1** was converted to the corresponding acetate **2** (93%) with acetic anhydride in pyridine (Samain et al. 1978). The acetate **2** contained ≈5% of other isomers, which were carried through the synthesis and separated by recrystallization in the final step. Reaction of acetate **2** with the Grignard reagent **3** prepared from (6-bromohexyloxy)-*tert*-butyldimethylsilane in THF at −50°C in the presence of 4 mol % of Li_2CuCl_4 provided the coupling product in good yield. In particular, the regiochemistry of copper-catalyzed reactions between Grignard reagents and allylic substrates is heavily dependent on the reaction conditions, with SN_2 reaction being favored by the formation of the dialkyl- rather than the monoalkylcuprates. Fast addition of the Grignard reagent, low temperature, and low concentration of the catalyst produced these conditions. It is also worth noting that the organocopper intermediate, which was present only in low concentration, reacted faster with the allylic C-O bond than the Grignard reagent reacted with the carbonyl group of the acetate.

The crude (8*E*,10*E*)-tetradecadien-1-yl TBDMS ether **4** then was deprotected with tetrabutylammonium fluoride under the standard conditions (Corey and Venkateswarlu 1972) to give crude 8*E*,10*E*-14:OH **5,** which was purified by recrystallization from hexane at −20°C, yielding 8*E*,10*E*-14:OH **5** in 63% yield over the last two steps. The corresponding 8*E*,10*E*-14:Ald was readily obtained from **5** by oxidation, whereas the corresponding acetate (**7**) and formate (**8**) esters were obtained by reaction of **5** with formic acetic anhydride and acetic anhydride respectively. In the former case, the formate ester product was contaminated with ≈1% of the corresponding acetate ester. Thus, the formate also was synthesized by esterification of the alcohol with formic acid. This method gave the formate ester completely free of the acetate, but with ≈3% isomerization.

Field Trials. The first field trial conducted in a southern California grapefruit orchard concentrated on the three components in extracts that elicited the largest antennal responses in coupled GC-EAD analyses, i.e., the (8*E*,10*E*)-14:Ald, (8*E*,10*E*)-14:OH, and (8*E*,10*E*)-14:formate (Fig. 2, peaks 1, 2, and 3, respectively). The field trial compared the attractiveness of all three components versus binary and single components alone, by using the ratios of the compounds found in multiple female aerations conducted to that date. The results indicated that only blends with the formate as a component were attractive, with no significant differences between any of the blends and the formate alone (Fig. 3).

We subsequently identified (8*E*,10*E*)-14:Ac as a trace component in female aerations (Fig. 2, peak 4) and included it in the field trials despite the lack of strong antennal responses to this compound. In the first trial that included this compound as a lure component (trial 2, Fig. 4), most treatments that contained (8*E*,10*E*)-14:Ac captured significantly fewer moths than the treatments which did not have it. However, the acetate used was not completely isomerically pure (2.6% of the *EZ* isomer and 5.5% of isomers overall). The field trial was repeated using more highly purified material (0.43% *EZ* isomer and 1% isomers overall), with the result that none of the blends differed significantly in attractiveness from the formate alone (Fig. 5), suggesting that one or more isomers of the acetate were antagonistic. To confirm this, we added

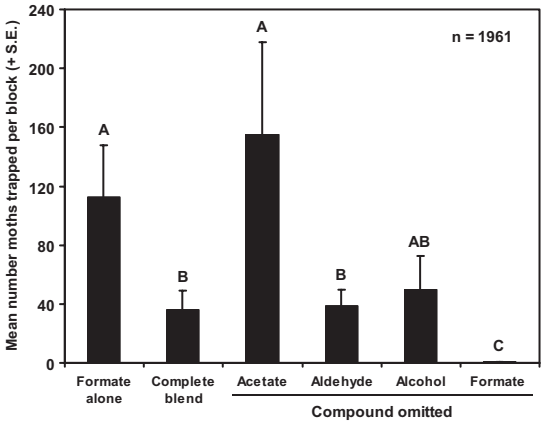

Fig. 4. *M. gulosa* field trial examining the relative attractiveness of a four component blend versus single deletions from that blend and (8*E*,10*E*)-tetradecadien-1-yl formate as a single component. Base blend consisted of 10:80:75:32 μg per septum (8*E*,10*E*)-tetradecadienal, (8*E*,10*E*)-tetradecadien-1-ol, (8*E*,10*E*)-tetradecadien-1-yl formate, and isomerically impure (94% *EE*) (8*E*,10*E*)-tetradecadien-1-yl acetate, respectively. Field trial was conducted at the Coachella Valley site from 3 to 18 July 2003. Treatments with different letters are significantly different (two-way ANOVA followed by Student–Newman–Keuls test, $\alpha = 0.05$: for treatment effect, $F = 4.45$, df = 4, 24, $P = 0.013$; for block effect, $F = 8.03$, df = 4, 24, $P = 0.0009$.

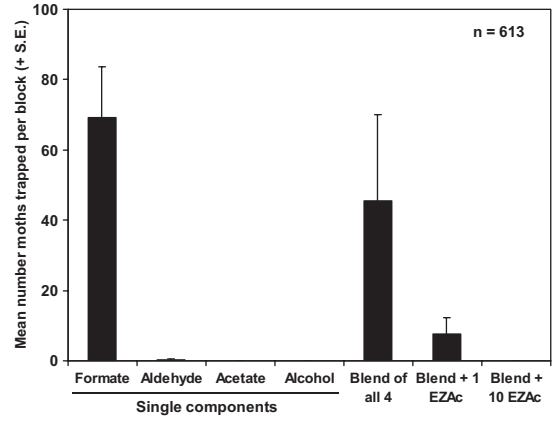

Fig. 6. *M. gulosa* field trial conducted in the San Joaquin Valley, comparing the attractiveness of lures containing a blend of 8*E*,10*E*-14:Ald, 8*E*,10*E*-14:OH, 8*E*,10*E*-14:formate, and 8*E*,10*E*-14:Ac (8:65:100:67 μg per septum, respectively), alone or in combination with a 1 or 10 μg per septum dose of 8*E*,10*Z*-14:Ac, and single component lures with 100 μg per septum of each of the compounds (excluding the 8*E*,10*Z*-14:Ac). Field trial conducted from 14 to 28 September 2006. Bars with different letters are significantly different (two-way ANOVA on $\log_{10}(x + 1)$-transformed data followed by Student–Newman–Keuls test, $\alpha = 0.05$: for treatment effect, $F = 11.48$, df = 3, 19, $P = 0.0008$; for block effect, $F = 3.56$, df = 4, 19, $P = 0.039$.

1% and 10% doses of (8*E*,10*Z*)-14:Ac (as a percentage of the formate) to the formate and tested the blends in both the San Joaquin and Coachella valleys (Figs. 6 and 7, respectively). In both locations, addition of

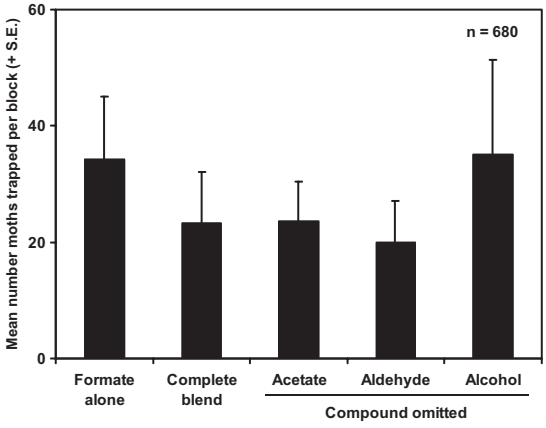

Fig. 5. *M. gulosa* field trial examining the relative attractiveness of highly isomerically pure (99% *EE* isomer) compounds in four component blend versus single deletions from that blend and the formate alone. Base blend consisted of 10:80:75:32 μg per septum (8*E*,10*E*)-tetradecadienal, (8*E*,10*E*)-tetradecadien-1-ol, (8*E*,10*E*)-tetradecadien-1-yl formate, and (8*E*,10*E*)-tetradecadien-1-yl acetate, respectively. Field trial was conducted at the Coachella Valley site from 18 to 28 July 2003. Treatments were not significantly different (two-way ANOVA: for treatment effect, $F = 2.11$, df = 4, 24, $P = 0.13$; for block effect, $F = 34.29$, df = 4, 24, $P < 0.0001$).

(8*E*,10*Z*)-14:Ac reduced or eliminated trap captures entirely. In addition to showing the antagonistic effects of larger amounts of (8*E*,10*Z*)-14:Ac, the field trials again showed that a combination of the four compounds in ratios approximating those found in the headspace collections was no better than the formate alone, and that none of the other compounds alone were attractive (Figs. 6 and 7).

A further field trial in the Coachella Valley revealed that a second batch of the formate (E1043) was not at all attractive when compared with the previous batch (E967) (Fig. 7). The attractive, first batch of 8*E*,10*E*-14:formate had been prepared by esterification of 8*E*,10*E*-14:OH with formic acetic anhydride, and the product was contaminated with ≈1% of the corresponding acetate. In contrast, the E1043 batch was prepared by esterification of the alcohol with formic acid directly, so that the product contained no detectable acetate. Although these data might suggest that 8*E*,10*E*-14:Ac is indeed an important component of the active pheromone at trace levels, addition of the acetate, alcohol, or both failed to render the E1043 batch attractive (Fig. 8), and a 50:50 mix of the two lots was substantially less attractive than the E967 formate batch alone. Although no other obvious differences between the two lots could be determined by GC, GC-MS, or coupled GC-EAD, the data suggest that the E1043 batch contained trace amounts of a very highly antagonistic impurity.

Overall, during the field seasons of 2002–2006, we conducted >20 different field trials in Coachella and San Joaquin Valley citrus orchards and row crops, by

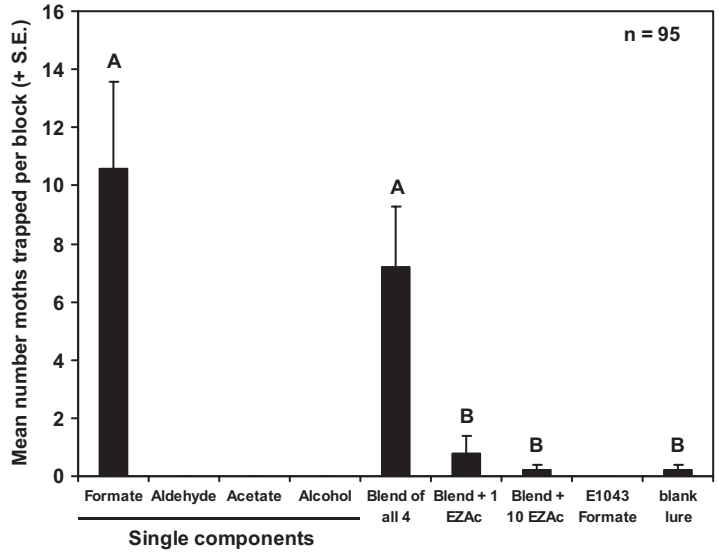

Fig. 7. *M. gulosa* field trial conducted in the Coachella Valley, comparing the attractiveness of lures containing single components to that of a blend of four components with and without 1 or 10 μg per septum 8*E*,10Z-14:Ac. Single components were tested at the 100 μg per septum dose, whereas the ratio in the four-component blend was 8:65:100:67 μg each of 8*E*,10*E*-14:Ald, 8*E*,10*E*-14:OH, 8*E*,10*E*-14:formate, and 8*E*,10*E*-14:Ac, respectively, per septum. Field trial conducted from 12 to 26 October 2006. Bars with different letters are significantly different (two-way ANOVA followed by Student–Newman–Keuls test, $\alpha = 0.05$: for treatment effect, $F = 14.89$, df = 4, 24, $P < 0.0001$; for block effect, $F = 1.02$, df = 4, 24, $P = 0.43$.

using different blends and blend ratios of the four 8*E*,10*E*-formate, alcohol, acetate, and aldehyde components, in the hopes of improving the attractiveness of lures. In no case has any blend been consistently more attractive than the 8*E*,10*E*-formate as a single component.

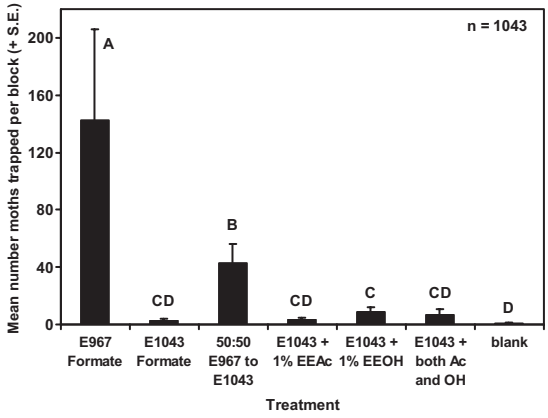

Fig. 8. Field trial conducted in the San Joaquin Valley comparing attractiveness of 8*E*,10*E*-14:formate lots synthesized by different routes. Single components were tested at the 100 μg per septum dose. Field trial conducted from 26 October to 16 November 2006. Bars with different letters are significantly different (two-way ANOVA followed by Student–Newman–Keuls test, $\alpha = 0.05$: for treatment effect, $F = 15.44$, df = 6, 34, $P < 0.0001$; for block effect, $F = 1.17$, df = 4, 34, $P = 0.35$.

Discussion

Trapping of headspace volatiles from live females by dynamic SPME proved to be crucial to the collection of sufficient material for analyses by GC-EAD and GC-MS, with overnight collections from single females providing easily detectable GC peaks. In contrast, the amounts of pheromone components in extracts prepared by solvent extraction of dissected pheromone glands were below the detection limit of the GC-MS, suggesting that the pheromone is biosynthesized and released as needed, rather than being synthesized in advance and then stored until release. However, SPME has the limitation that compounds cannot be recovered from the SPME fibers easily, for example, for derivatization or bioassays. Thus, the identification of the female-produced compounds depended on a combination of mass spectral and retention time matches, coupled with strong biological activity in terms of elicitation of antennal responses in GC-EAD studies, using GC columns of different polarities. The identification of 8*E*,10*E*-14:formate as a pheromone component was further supported by the good attraction of male moths to traps baited with lures containing this compound as a single component or as a component of a blend.

To our knowledge, 8*E*,10*E*-14:OH and 8*E*,10*E*-14:Ac have been identified once before as potential pheromone components for the leafminer *Phyllonorycter emberizaepenella* (Bouché) (Gracillariidae) (Mozûraitis et al. 2002) whereas 8*E*,10*E*-14:Ald and 8*E*,10*E*-14:formate have not been previously reported as lepidopteran pheromone components or attractants. Furthermore,

the latter compound represents the first true example of a formate ester as a component of a lepidopteran pheromone, although several formate compounds have been used as mimics of aldehyde pheromone components (e.g., (7*Z*,9*E*,11)-dodecatrien-1-yl formate as a mimic of (9*Z*,11*E*,13)-tetradecatrienal, a pheromone component of the carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyrallidae) (Todd et al. 1992).

The volatiles collected from 1 to 4 d old unmated female moths consistently contained 8*E*,10*E*-14:Ald, 8*E*,10*E*-14:OH, 8*E*,10*E*-14:formate, and 8*E*,10*E*-14:Ac, the first three of which elicited strong responses from antennae of male moths. Thus, it remains unclear why 8*E*,10*E*-14:formate with a trace of the corresponding acetate (i.e., in a ratio considerably different than that seen in SPME extracts) is as good or better than any blend consisting of the formate with one or more of the other components. Over the space of four years, exhaustive field trials were conducted with various blends and ratios of the four components, but in no case was any blend consistently more attractive than the formate with a trace of acetate. Furthermore, trials were conducted throughout the field season (the moth is multivoltine), in different geographic and climatic regions of California, and in different crops. In all cases, lures prepared from several different batches of 8*E*,10*E*-14:formate prepared by esterification of 8*E*,10*E*-14:OH with formic acetic anhydride (i.e., containing ≈1% of 8*E*,10*E*-14:Ac) were consistently as good or better than 2, 3, or 4-component blends that represented a more complete reconstruction of the blend found in SPME collections of volatiles released by live female moths. As a further complication, trap catches varied with no detectable pattern, with catches often being unexpectedly low in orchards with obvious infestations (as determined from the mines on fruit surfaces or plant stems). Nevertheless, all four of these compounds that were consistently found in SPME extracts must be produced by the moth, because there is no conceivable mechanism by which 8*E*,10*E*-14:formate or any of the other compounds can be interconverted on the SPME fiber or during analysis, as demonstrated by the fact that SPME analyses of pure standards yield only the single peaks of the expected products (plus traces of isomers).

Because of the difficulties in purifying relatively unstable compounds to purities approaching 100%, and further difficulties in analysis of such highly purified compounds with no degradation or isomerization (see above description of small amounts of isomerization during GC analyses), we can only set minimum limits to the purities of the synthetic compounds that were used to formulate lures. Conversely, it is not clear whether the traces of isomers of the four components that were seen in SPME analyses of volatiles collected from female moths were indeed produced by the moths, or were artifacts from the GC analyses.

In summary, our data suggest that the sex pheromone of *M. gulosa* consists of 8*E*,10*E*-14:formate as the major component, possibly with 8*E*,10*E*-14:Ac as a minor component. However, it is possible that one or more of the other compounds found in volatiles collected from virgin female moths by SPME also may be either antagonistic or synergistic. This can only be determined with synthetic compounds of extremely high chemical and isomeric purity, because it is clear that the moth is sensitive to amounts of chemical and isomeric impurities of ≤1% of the major component. Thus, for practical purposes, pheromone lures consisting of 8*E*,10*E*-14:formate with ≈1% of 8*E*,10*E*-14:Ac should be useful for monitoring the presence of the insect and tracking of generations to assist insecticide treatment timing. For example, O'Neal (2007) used the 8*E*,10*E*-14:formate for monitoring populations of *M. gulosa* in walnuts and citrus in the San Joaquin Valley, and was able to use the pheromone to detect the first moth flight and so establish a biofix of early season activity. However, during the rest of the season, the pheromone was variable in its effectiveness in attracting moths. Until the uncertainty as to the true active blend can be resolved, use of pheromone traps for accurately tracking generations and estimating population sizes cannot be recommended.

Acknowledgments

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