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A Symmetrical Diester as the Sex Attractant Pheromone of the North American Click Beetle *Parallelostethus attenuatus* (Say) (Coleoptera: Elateridae)

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Abstract
Hexanoic acid, 1-octanol, 1,8-octanediol, octyl hexanoate, 1,8-octanediol monohexanoate, and 1,8-octanediol dihexanoate were identified in headspace volatiles collected from the crushed abdomen of a female click beetle of the species *Parallelostethus attenuatus* (Say) (Elaterinae, tribe Elaterini). In field trials carried out in Illinois, South Carolina, North Carolina, and Virginia, adult male beetles were strongly attracted to 1,8-octanediol dihexanoate alone. Blends of the dihexanoate with one or more of the other compounds proved to be less attractive than the dihexanoate alone, suggesting that the pheromone of this species may consist of a single compound. The symmetrical diester structure of the pheromone is a novel natural product and appears to be structurally unique among insect pheromones.

Keywords Elateridae · Sex pheromone · Click beetle · 1,8-Octanediol dihexanoate

Introduction

The family Elateridae, commonly known as click beetles because of their "clicking" defensive behaviors, which catapult them into the air, is a highly speciose group in North America, with more than 1000 described species occupying a wide variety of habitat niches (Johnson 2002). The larvae of some species, known as wireworms, are important agricultural pests of root and tuber crops, vegetables, corn, and small grain crops, whereas larvae of other species inhabit rotting woody plants, the plant-soil interface, and forest duff (e.g., Jansson and Seal 1994; Lindroth and Clark 2009). Historically, click beetles caused major crop losses but with the advent and widespread use of broad spectrum and highly toxic insecticides to control other pests, these insects became minor nuisances of crops. Consequently, few detailed studies of their biology, ecology, and life history were carried out. However, the banning of many toxic insecticides and their replacement with less efficacious materials has resulted in a resurgence of wireworms as agricultural pests. Consequently, there is renewed interest in elucidating the biology of click beetles, as well as developing better methods for managing pestiferous species.

Click beetle pheromones have been studied and identified over the last several decades in Europe and, more recently, in Asia. Most of the pheromones or sex attractants of these species are esters of branched chain, unsaturated straight chain, or terpenoid alcohols (reviewed in Tóth 2013). By contrast, the first attractant pheromones for North American elaterids were only identified in 2018, in two species of the genus *Cardiophorus* (Serrano et al. 2018). Since then, pheromones of several additional species have been identified, including agriculturally important species in the genera

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Melanotus (Williams et al. 2019) and *Limonius* (Gries et al. 2021; van Herk et al. 2021). The pheromones of these species are products of biosynthetic pathways different from those of the compounds of the European and Asian species: the *Cardiophorus* compound (methyl [3*R*,6*E*]-2,3-dihydrofarnesoate) is a terpenoid, the straight chain *Melanotus* compound (13-tetradecenyl acetate) probably arises from the fatty acid pathway, and the branched chain *Limonius* compound ([*E*]-5-ethyloct-4-enoic acid) is of uncertain biosynthetic origin. The wide variations in the structures of these compounds, coupled with variation in pheromone chemistry among European and Asian species, suggest that the "chemical space" (i.e., the diversity of chemical compounds utilized as elaterid pheromones) may be large. Thus, the goal of the study reported here was to further elucidate that chemical space, by identifying the pheromone of the North American species *Parallelostethus attenuatus* (Say) (Elaterinae, tribe Elaterini). This black beetle has a reddish-brown prothorax and is the only member of its genus in North America. The larvae inhabit and feed in rotting logs (Blatchley 1910; Kirk 1922; Jewett 1946). It is broadly distributed across North America (Roache 1960), but little else is known about its biology or life history. Here, we report the identification of an unusual symmetrical diester as a female-produced sex attractant pheromone for this beetle, as well as the results of field bioassays of other candidate pheromone compounds in Illinois, South Carolina, North Carolina, and Virginia.

Methods and Materials

Collection and Analysis of Beetle-Produced Compounds

Adults of *P. attenuatus* used for collection of beetle-produced odors and coupled gas chromatography-electroantennogram detection analyses (GC-EAD) were collected in Illinois during August 2019 as apparently random catches by flight-intercept panel traps (see below) during screening trials of compounds known to be sex pheromones of elaterid beetles. In the laboratory, individual beetles were caged separately under ambient room conditions (~12:12 h L:D, ~20 °C) and provided 10% aqueous sucrose solution (glass vials with cotton wicks) for hydration and nutrition. Live beetles were shipped from Illinois to UC Riverside (USDA-APHIS-PPQ permit P526P-17-02384) and held in the UC Riverside quarantine facility. Individual beetles were placed in glass aeration chambers swept with charcoal-purified air, with insect-produced volatiles collected on a small plug of activated charcoal in a glass collection tube attached to the chamber outlet. Tubes were eluted with methylene chloride, and the resulting extracts analyzed by GC-EAD using detached antennae from male beetles mounted between capillary glass electrodes filled with Ringer's solution. Gold wires inside the capillaries provided connections to a custom-built EAD amplifier. The GC and EAD signals were simultaneously recorded with PeakSimple software (SRI Institute, Palo Alto, CA, USA; for details, see Serrano et al. 2018). Extracts were also analyzed by coupled gas chromatography/mass spectrometry (GC/MS) using DB-5 columns (see below).

After several days of headspace collection, females were cold anesthetized, and their abdomens detached and crushed in a 20 ml glass vial. The vial was sealed with aluminum foil, and volatiles were collected from the crushed abdomens for 2 h at room temperature by inserting a polydimethylsiloxane (PDMS) solid phase microextraction fiber through the aluminum foil and into the headspace. The fibers were thermally desorbed by insertion into the injector of an HP 6890N GC (Hewlett-Packard, now Agilent, Santa Clara, CA, USA) held at 250 °C, for 30 s in splitless mode. The desorbed volatiles were analyzed on a DB-5 column (30 m × 0.25 mm ID, 0.5 µm film; J&W Scientific, Folsom, CA, USA), with the oven programmed from 40 °C/1 min, then 10 °C.min^{-1} to 280 °C and held for 10 min. After use, fibers were further cleaned by insertion into a GC injector at 250 °C for 10 min. The GC was interfaced to an HP 5975C mass selective detector in electron impact ionization mode, scanning a mass range from 40 to 350 amu. Compounds were tentatively identified by database matches with the NIST 2014 database, or by interpretation of spectra. Identities were confirmed by matching retention times and mass spectra with those of standards.

Sources of Chemicals

1,8-Octanediol and hexanoic acid were obtained from Aldrich Chemical (Milwaukee, WI, USA), 1-octanol from TCI Americas (Portland, OR, USA), and hexanoyl chloride from Alfa-Aesar (Wardhill, MA, USA).

1,8-Octanediol Monohexanoate 1,8-Octanediol (7.3 g, 50 mmol) was dissolved in ~100 ml refluxing CH_2Cl_2. The mixture was cooled to room temperature, and pyridine (2.0 ml, 25 mmol) and dimethylaminopyridine catalyst (~100 mg) were added, then hexanoyl chloride (3.38 g, 25 mmol) was added by syringe pump over 30 min. The mixture was stirred for 4 h, then quenched by adding 50 ml saturated aqueous NH_4Cl solution and stirring for 30 min. The mixture was then diluted with 200 ml water, and the CH_2Cl_2 layer was removed and concentrated. The residue was taken up in hexane causing excess 1,8-octanediol to precipitate. The hexane solution was decanted, and the residue was rinsed twice more with hexane. The combined hexane layers were washed with 1 M aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over anhydrous Na_2SO_4

and concentrated. The residue was purified by Kugelrohr distillation (oven temperature 110 °C, 0.12 mm Hg), yielding the monoester (3.75 g, 97% pure by GC). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.05 (t, 2H, J = 6.7 Hz), 3.65 (t, 3H, J = 6.6 HZ), 2.30 (t, 2H, J = 7.6 Hz), 1.67–1.52 (m, 6H), 1.4–1.2 (m, 12H) 0.90 (t, 3H, J = 6.9 Hz). ^{13}C NMR (125 MHz, $CDCl_3$); δ 173.99, 64.27, 62.89, 34.28, 32.64, 31.23, 29.20, 29.11, 28.53, 25.77, 25.57, 24.62, 22.24, 13.83 ppm. EIMS, 70 eV, *m/z* (abundance): 214 (1, M^+-30), 201 (1), 188 (1), 155 (3), 117 (98), 110 (34), 99 (100), 95 (16), 85 (23), 82 (59), 81 (33), 71 (42), 69 (59), 68 (42), 67 (45), 55 (54), 43 (46), 41 (35).

1,8-Octanediol Dihexanoate and Octyl Hexanoate 1,8-Octanediol (3.66 g, 25 mmol) was dissolved in 100 ml refluxing CH_2Cl_2, cooled to room temp, and pyridine (4.43 ml, 55 mmol) and dimethylaminopyridine catalyst (~100 mg) added. Hexanoyl chloride (7.7 ml, 55 mmol) was then added by syringe pump over 45 min. The resulting mixture was stirred overnight, followed by the addition of a further 0.44 ml pyridine and 0.77 ml hexanoyl chloride as the esterification was not quite complete. After stirring again overnight, the mixture was quenched with saturated aq. NH_4Cl, then diluted with water. The organic layer was separated and washed with dilute aq. NH_4Cl, then dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by Kugelrohr distillation, taking a forerun (oven temperature up to 110 °C, 0.05 mmHg), followed by distillation of the diester after changing the collection bulbs (8.64 g, quantitative, 98.7% pure by GC, bp ~125–130 °C, 0.05 mm Hg). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.07 (t, 4H, J = 6.7 Hz), 2.30 (t, 4H, J = 7.6 Hz), 1.56–1.7 (m, 8H), 1.24–1.4 (m, 16H), 0.91 (t, 6H, J = 6.9 Hz). ^{13}C NMR (125 MHz, $CDCl_3$); δ 173.94, 64.23, 34.28, 31.24, 29.04, 28.53, 25.77, 24.62, 22.24, 13.84 ppm. EIMS, 70 eV, *m/z* (abundance): 299 (3, M^+-43), 286 (3), 243 (7), 230 (2), 187 (4), 155 (4), 140 (2), 127 (4), 117 (49), 110 (100), 99 (95), 95 (13), 82 (44), 81 (25), 71 (32), 69 (40), 68 (22), 67 (26), 55 (31), 43 (35), 41 (18).

Octyl hexanoate was made in analogous fashion and yield, by substituting 1-octanol for 1,8-octanediol. The crude product was purified by Kugelrohr distillation (bp ~50 °C, 0.1 mmHg).

Field Trials in Illinois

In Illinois, field experiments were conducted in three natural areas: Robert Allerton Park (Piatt Co.; Property of the University of Illinois, http://research.illinois.edu/cna/; 39.996°N, −88.651°W), and two sites in Vermilion County: Forest Glen Preserve (40.0152°N, −87.5677°W; property of the Vermilion County Conservation District; http://www.vccd.org/), and Vermilion River Observatory (40.0655°N, −87.5613°W; property of the University of Illinois). The study sites were mature second-growth or successional forests dominated by hardwood trees, primarily species of oak (*Quercus*), hickory (*Carya*), and maple (*Acer*).

Beetles were caught with flight intercept panel traps (cross-vane black corrugated plastic, Alpha Scents, Portland, OR, USA) with interior surfaces coated with a 10% aqueous solution of the fluoropolymer lubricant Fluon® (Northern Specialty Chemicals, Dudley, MA, USA; Graham et al. 2010) to improve trap efficiency. For field experiments, trap basins were partially filled with saturated aqueous NaCl solution plus a few drops of dish soap to kill and preserve captured beetles. To capture beetles alive for pheromone identification, trap basins were replaced with plastic jars with bottoms removed and covered with aluminum window screen to allow rainwater to drain. Traps were hung (bottoms ~0.5 m above the ground) from inverted L-shaped frames of polyvinyl chloride irrigation pipe. Traps were deployed ~10 m apart in linear transects, one trap per treatment, and one transect per field site. Initially, treatments were assigned randomly to trap position. Traps were serviced every 2–3 d, at which time treatments were rotated one position down the transect to allow for location effects. Lures were replaced at intervals of ~2 wk.

Trap lures consisted of 9 mm grey rubber septa (West Pharmaceutical Services, Lititz, PA, USA) loaded with hexane or acetone solutions of test compounds (see below), unless stated otherwise. Doses used in the various experiments are given below.

Field Trials in South Carolina

In South Carolina, field experiments were conducted at a woodlot at the USDA-ARS U.S. Vegetable Laboratory in Charleston (32.795° N, −80.061° W), which is representative of the Maritime Strand plant community (Porcher and Rayner 2001). In 2020, beetles were captured with black Vernon Pitfall Traps® (VPT; van Herk et al. 2018; Intko Supply Ltd., Chilliwack, BC, Canada) with specimen cups containing 12 ml of a 1:1 mixture of low toxicity propylene glycol antifreeze: tap water as a killing agent and preservative (Williams et al. 2019). A tulip bulb planter was used to dig a hole into which the trap was placed; two heavy duty landscaping pins (25 cm long) were used to anchor each trap into the soil. Lures consisted of grey rubber septa loaded with test solutions, as described below.

Experiments conducted during 2021 used black VPTs hung from fiberglass poles so that lures were ~1 m above the ground. Traps were deployed in linear transects, with one trap per treatment, unless stated otherwise. Treatments were assigned randomly to traps, and positions re-randomized every 2 wk. Traps were serviced and beetles were collected

weekly, except for a 3-wk collection interval from 30 July to 20 August 2021. Lures were grey rubber septa loaded with test attractants (see below).

Field Trials in North Carolina and Virginia

In North Carolina, traps were deployed at the Lake Wheeler Road Field Laboratory, North Carolina State University, Raleigh NC (35.7413000º N, −78.7027579º W). This study consisted of two linear transects of traps (i.e., replicates) established along borders between woodlots and conventionally managed corn fields. The two transects were ~150 m apart. Each transect consisted of six VPTs hung ~1 m above ground on fence posts, spaced 15 m apart. Trap collecting cups were loaded with 12 ml of a 1:1 mixture of propylene glycol-based antifreeze and tap water as a killing agent (Williams et al. 2019). Initial treatment locations within the transect were assigned randomly and treatment locations re-randomized every 2 wk. Grey rubber septum lures were replaced every 4 wk, and trap contents were collected weekly.

In Virginia (Homefield Farm in Whitethorne, 37.202977º N, −80.565366º W), a single transect was established along a fence line separating a stream with forested buffer and organically managed produce and fallow fields. Six VPTs were spaced 20 m apart on the farm side of the fence line. Traps, baited with grey rubber septum lures, were hung ~1 m above the ground by fastening them with duct tape and zip ties to an electric fence post at each trap location. Treatments were assigned randomly, and re-randomized biweekly. Trap catches were tallied weekly, and lures changed every 4 wk.

Field Bioassays of Candidate Pheromone

Screening experiment 1 tested a 1:1:1 blend of three compounds (1,8-octanediol + octanediol monohexanoate + octanediol dihexanoate, 10 mg of each in acetone loaded on a grey rubber septum; control lures were dosed only with solvent) identified from abdomen squashes of female *P. attenuatus*. The lures were included as part of a larger screening trial testing a variety of compounds previously identified as pheromones of European, Asian, and North American elaterid species (Serrano et al. 2018; Tóth 2013; Williams et al. 2019), analogs of those compounds, or candidate pheromones from ongoing research on various species. In Illinois, the experiment was conducted from 28 May to 13 September 2020 at Forest Glen Preserve and from 22 July to 13 September 2020 at Vermilion River Observatory. It was repeated in Illinois at the Allerton Park study site from 3 May to 13 July 2021. The analogous experiment in South Carolina was conducted from 1 April to 30 July 2020.

In 2021, in screening experiment 2, 1,8-octanediol dihexanoate was tested as a single component, among a group of five compounds or blends that were screened as general attractants for click beetle species. Lures were loaded with a hexane solution of 1,8-octanediol dihexanoate (50 µl of 10% solution, 5 mg); controls were treated only with hexane. This experiment was conducted in Illinois from 25 May to 19 August 2021 at Forest Glen, in South Carolina from 3 May to 13 September 2021, in North Carolina from 8 June to 10 August 2021, and in Virginia from 21 May to 12 August 2021.

Targeted field experiment 1 was conducted only in Illinois with treatments that included three individual components identified from the volatiles collected from female *P. attenuatus*, all possible combinations of two of the three components, and all three components, as follows: 1) 1,8-octanediol; 2) 1,8-octanediol monohexanoate; 3) 1,8-octanediol dihexanoate; 4) 1,8-octanediol +1,8-octanediol monohexanoate; 5) 1,8-octanediol +1,8-octanediol dihexanoate; 6) 1,8-octanediol monohexanoate +1,8-octanediol dihexanoate; 7) 1,8-octanediol +1,8-octanediol monohexanoate +1,8-octanediol dihexanoate; 8) solvent control. For this experiment only, emitters consisted of low-density polyethylene sachets (press-seal bags, Bagette model 14,770, 5.1×7.6 cm, 0.05 mm thick, Cousin Corp., Largo, FL, USA) containing a cotton dental roll and loaded with isopropanol solutions of compounds (8 mg/component). The experiment was deployed from 18 June to 22 July 2020 at both Forest Glen Preserve and Vermilion River Observatory.

Targeted experiment 2 tested how attraction of males of *P. attenuatus* to 1,8-octanediol dihexanoate was influenced by other components of the odor collected from females. The treatments were: 1) 1,8-octanediol dihexanoate (5 mg); 2) blend of 1,8-octanediol dihexanoate (5 mg) plus (all 0.5 mg) hexanoic acid, 1-octanol, 1,8-octanediol, octyl hexanoate and 1,8-octanediol monohexanoate; 3) solvent control. Hexane solutions were loaded onto grey rubber septa. In Illinois, the experiment was conducted from 8 July to 13 August 2021 at both Forest Glen and Allerton Park. In South Carolina, the experiment was run from 21 May to 10 September 2021, with three parallel transects of three traps per transect, with grey rubber septum lures as described above. In South Carolina, this experiment was run concurrently with screening experiment 2, with the experiments separated by ~280 m.

Specimens of *Parallelostethus attenuatus* were identified by morphology using taxonomic keys (Dogger 1959; Smith and Enns 1977). Voucher specimens have been deposited with the collection of the Illinois Natural History Survey, Champaign, IL (beetles captured in Illinois), and the National Museum of Natural History through the USDA-ARS Systematic Entomology Laboratory, Washington,

D.C. (beetles captured in South Carolina, North Carolina and Virginia).

Statistical Analyses

Data analyses were limited to treatments relevant to *P. attenuatus* by dropping treatments for other elaterid species, which each caught few, if any, male *P. attenuatus* (data not presented). For each experiment, differences among treatment means of numbers of adult male *P. attenuatus* captured were tested using nonparametric Friedman's test (PROC FREQ, option CMH; SAS Institute 2011), because heteroscedasticity violated conditions of ANOVA (Sokal and Rohlf 1995). Replicates were defined by spatial (block) and temporal (collection date) data. Replicates with the greatest numbers of beetles captured during field experiments in Illinois (i.e., replicates that represented independent responses to bioassay treatments by multiple beetles) were lent greater weight by dropping from the analyses those with fewer than a threshold number of beetles. The threshold numbers were selected, separately for each analysis, to maximize the total number of beetles captured while maintaining sufficient replication for a robust statistical test (at least eight replicates; range of threshold numbers, 0–20 beetles). Assuming the overall Friedman's test was significant, pairs of treatment means were compared with the Ryan-Einot-Gabriel-Welsch multiple range test (REGWQ), controlling the Type I experiment-wise error rate (SAS Institute 2011).

Results

Collection and Analysis of Beetle-Produced Compounds

Analysis of headspace volatiles collected by aeration of four individual live female *P. attenuatus* were barely useful, with only one of the four extracts showing small amounts of hexanoic acid and 1,8-octanediol. In SPME-collected volatiles from crushed abdomens of the same four females, two collections showed small amounts of hexanoic acid, and one of these also contained small amounts of octyl hexanoate. The fourth collection contained several compounds (Fig. 1), including substantial amounts of hexanoic acid and 1,8-octanediol (peaks A and C respectively, Fig. 1), lesser amounts of 1-octanol (peak B), an unknown compound (peak F), and trace amounts of octyl hexanoate (peak D). Peaks A–D were tentatively identified by matches with database spectra. Unknown F was tentatively identified as 1,8-octanediol dihexanoate from its mass spectrum (Fig. 2A), which showed a diagnostic pair of ester fragmentation peaks at *m/z* 99 and 117, assigned as $C_6H_{11}O^+$ and $C_6H_{13}O_2^+$ respectively, and an even mass base peak of

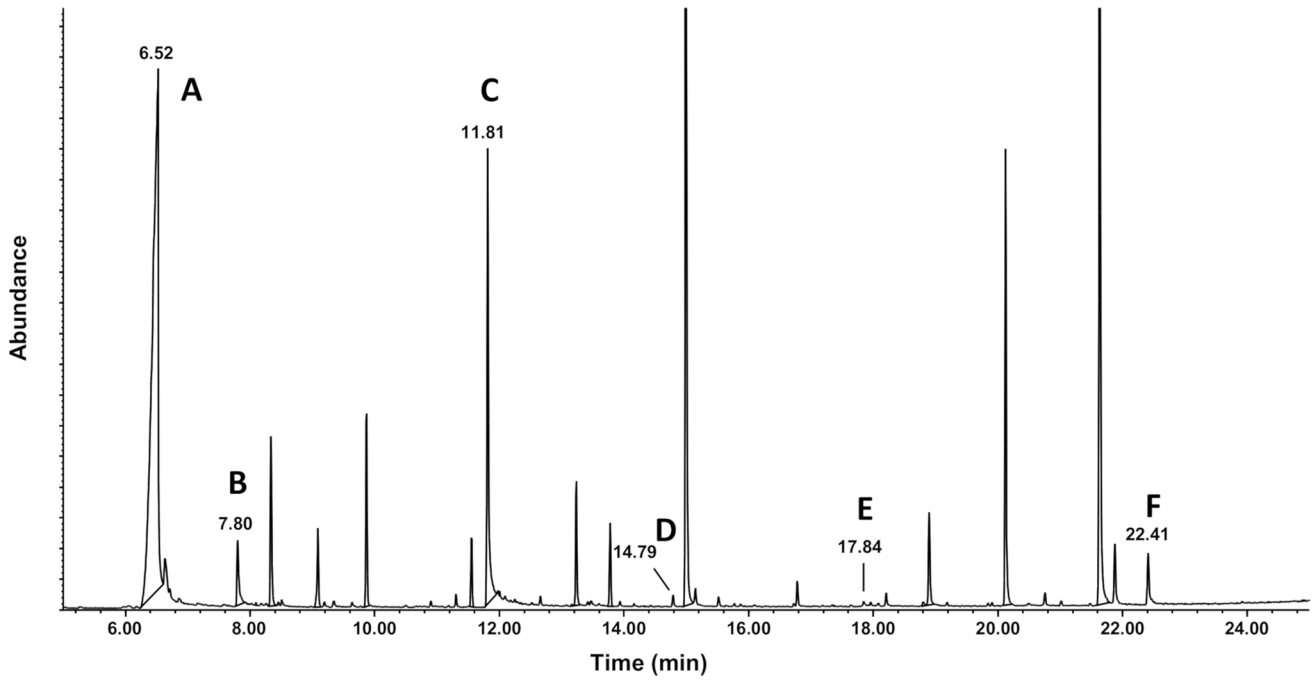

Fig. 1 Total ion chromatogram trace of an SPME collection of headspace volatiles from the squashed abdomen of an adult female *Parallelostethus attenuatus*, showing the major female-specific compounds: **A**) hexanoic acid, **B**) 1-octanol, **C**) 1,8-octanediol, **D**) octyl hexanoate, **E**) 1,8-octanediol monohexanoate, and **F**) 1,8-octanediol dihexanoate. Most other peaks were tentatively identified as common contaminants such as siloxanes from degradation of the SPME fiber, plasticizers, or cuticular hydrocarbons

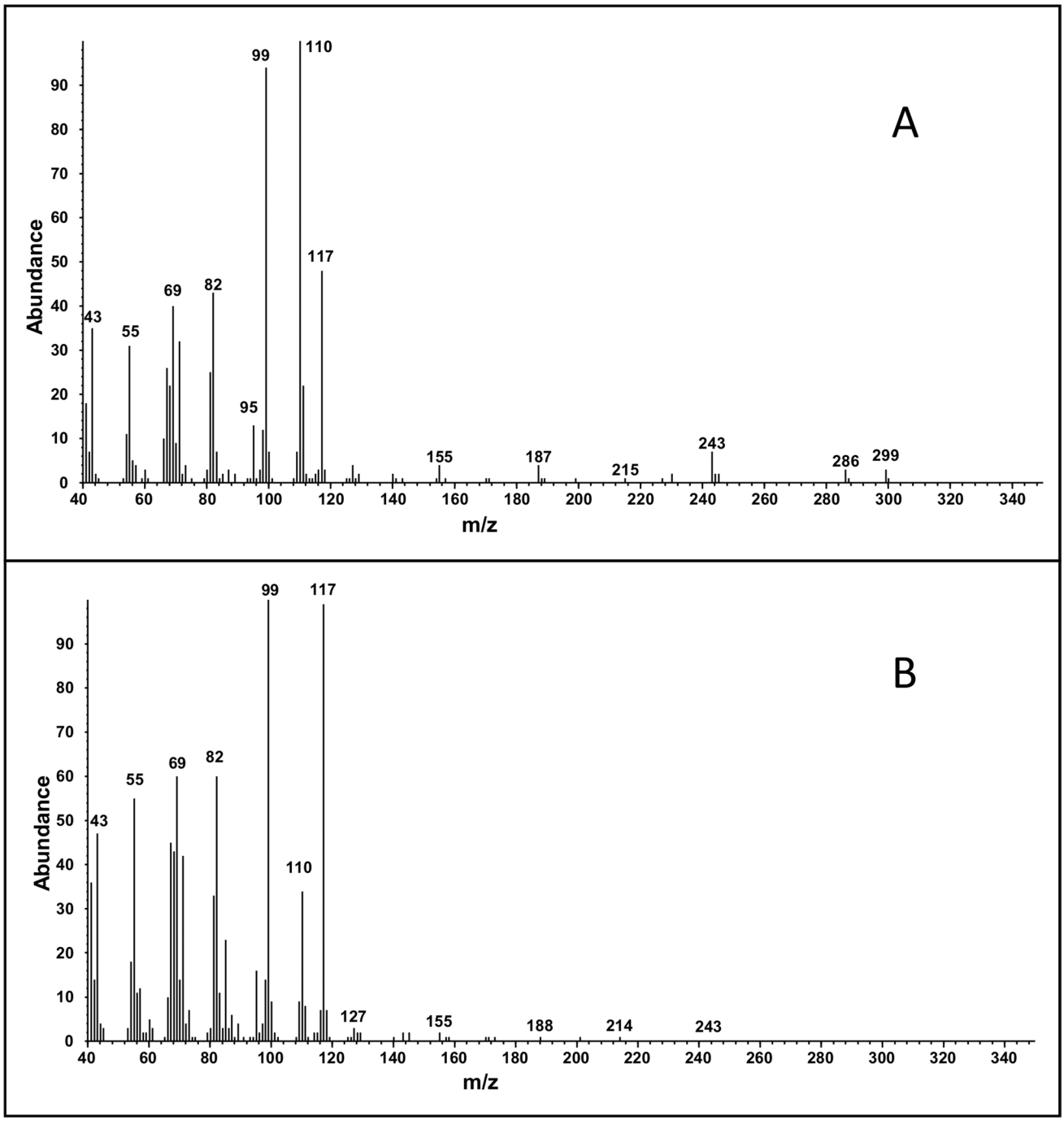

Fig. 2 Electron impact ionization mass spectra (70 eV) of **A**) 1,8-octanediol dihexanoate, and **B**) 1,8-octanediol monohexanoate

m/z 110, assigned as $C_8H_{14}^+$, resulting from loss of both hexanoic acid groups. The molecular ion at *m/z* 342 was not seen, with the highest mass ion being *m/z* 299 from loss of 43 amu (C_3H_7). There was also a small peak at *m/z* 286 (M-56), possibly from McLafferty rearrangement of one of the esters, and a small peak at *m/z* 243 (M-99), possibly from loss of a hexanoyl group. Detailed examination of the chromatogram revealed trace amounts of the corresponding 1,8-octanediol monohexanoate (peak E), the mass spectrum of which (Fig. 2B) also contained the diagnostic ester fragmentation ions at *m/z* 99 and 117, a medium intensity ion at *m/z* 110 from sequential losses of water and hexanoic acid, and a trace ion at *m/z* 243 from loss of a hydrogen from the molecular ion at *m/z* 244. Most of the remaining GC peaks were identified as silicon-containing contaminants from the degradation of the SPME fiber, or other common

contaminants such as phthalate plasticizers, and possible cuticular hydrocarbons. Given that most known elaterid pheromones are esters (Tóth 2013), we focused our attention on these compounds and their precursors for bioassays.

GC-EAD analyses of 1,8-octanediol dihexanoate and monohexanoate standards provided further evidence that one or both compounds were likely pheromone components, with antennae from male beetles responding to both compounds (Fig. 3).

Field Bioassays of Candidate Pheromone

In the first sets of field experiments, the potential pheromone components of *P. attenuatus* were included along with lures testing other possible elaterid pheromone components. Across all experiments, greater numbers of *P. attenuatus* adults were captured in Illinois than in South Carolina, possibly due to differences in local population densities, or efficiency of different trap designs. In the first screening experiment, run in both Illinois and South Carolina, male *P. attenuatus* were captured in significant numbers only in traps baited with the tertiary blend of 1,8-octanediol +1,8-octanediol monohexanoate +1,8-octanediol dihexanoate (henceforth diol, monohexanoate, and dihexanoate). In Illinois in 2020 and 2021, a total of 218 adult males were captured in traps baited with the tertiary blend, compared to only two beetles in control traps (mean catches ± SE: 12.3 ± 2.2 and 0.2 ± 0.1, respectively; $F_{1,26} = 20.3$, $P < 0.001$). In South Carolina, all ten male beetles captured were in traps baited with the tertiary blend, with none in control traps (mean

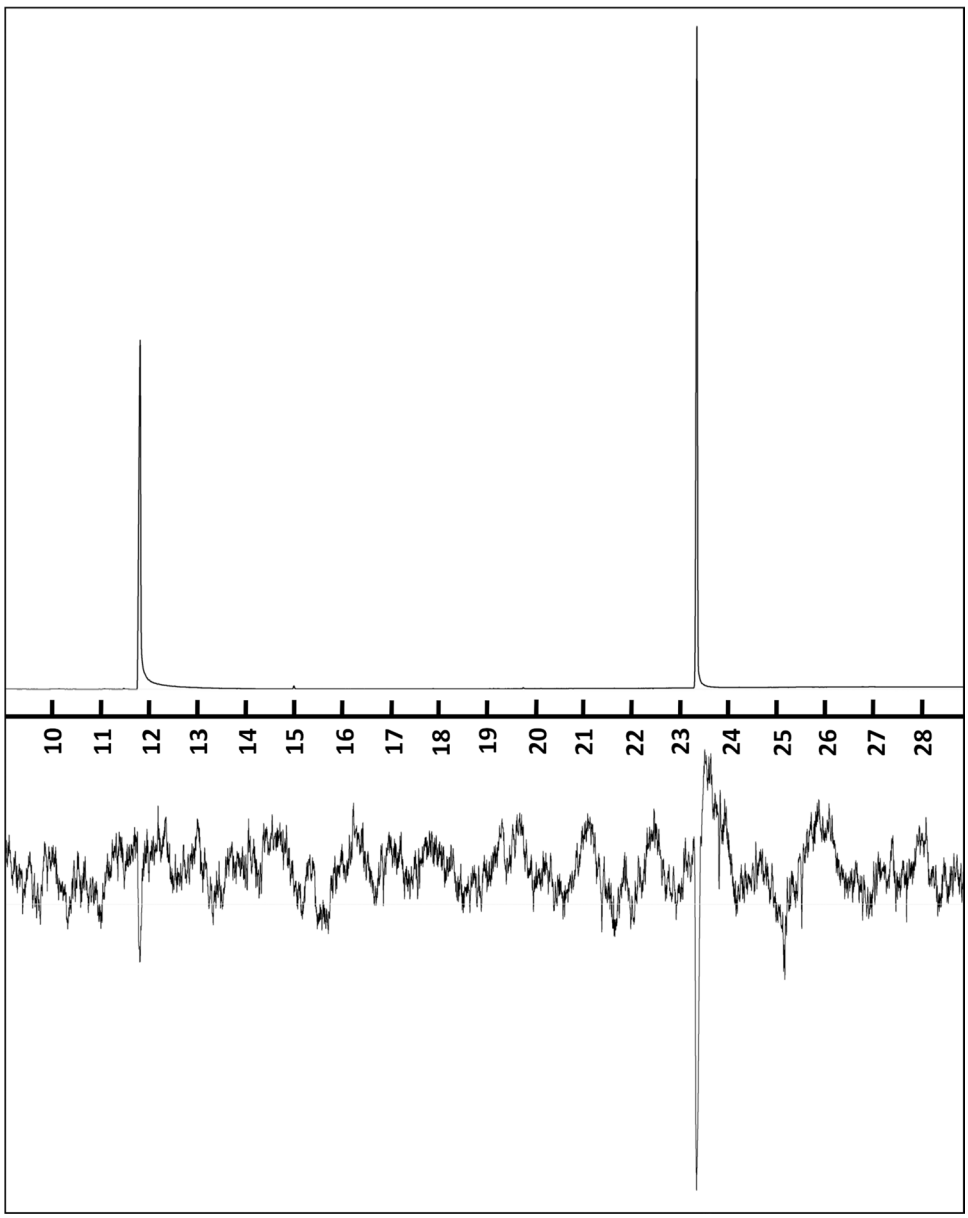

Fig. 3 Representative coupled gas chromatography-electroantennogram detection analysis of 1,8-octanediol monohexanoate (1st peak) and 1,8-octanediol dihexanoate (2nd peak) standards using an antenna from a male *Parallelostethus attenuatus*. Top chromatogram shows flame ionization detection and the bottom shows responses from the antenna

1.7 ± 0.3 and 0; $F_{1,24} = 22.5$, $P < 0.001$). In Illinois, beetles were captured from 22 June to 18 August in 2020 and 14 June to 13 July 2021, and in South Carolina from 28 May to 16 July 2020.

In screening experiment 2 in Illinois, 58 male *P. attenuatus* were captured in traps baited with dihexanoate (mean of 7.3 ± 3.5, different from 0 mean of control; $F_{2,16} = 13.1$, $P = 0.0003$). In South Carolina, all 17 male beetles captured were in traps baited with dihexanoate (mean 1.9 ± 0.26 vs 0 for controls; $F_{1,36} = 34.2$, $P < 0.001$). In Illinois, beetles were captured from 11 June to 26 July 2021, and in South Carolina from 31 May to 16 August 2021.

The dihexanoate was also tested in 2021 in North Carolina and Virginia, as part of ongoing field screening trials of possible click beetle pheromones. In North Carolina, 82 male beetles were caught in baited traps versus none in controls (mean 6.8 ± 2.6 vs 0; $F_{1,24} = 19.8$, $P < 0.0001$). In Virginia, 41 male *P. attenuatus* were caught in dihexanoate-baited traps versus none in controls (mean 5.9 ± 0.86 versus 0; $F_{1,14} = 11.2$, $P = 0.0008$). In North Carolina, beetles were captured from 22 June to 3 August, and in Virginia from 1 July to 12 August.

Subsequent field tests specifically targeted *P. attenuatus*, testing multiple replicates of blends of compounds identified from females. Targeted experiment 1, conducted only in Illinois, tested diol, monohexanoate, and dihexanoate as individual compounds, as all two-component blends, and the three-component blend. A total of 716 adult males of *P. attenuatus* were caught from 22 June to 22 July 2020. Among the individual compounds tested, only treatments containing dihexanoate attracted adult beetles in significant numbers (Fig. 4; $F_{7,111} = 87.7$, $P < 0.001$). Blending the dihexanoate with the monohexanoate resulted in a significant reduction in trap catch, and the diol was an even stronger antagonist. The mean for the tertiary blend was similar to that of the monohexanoate + dihexanoate blend, suggesting the former compound compensated for antagonism by the diol to some extent.

Targeted experiment 2 tested dihexanoate alone versus a blend of dihexanoate with five possible minor components. A total of 452 male *P. attenuatus* were trapped in Illinois, with the dihexanoate-baited trap catching more beetles than the blend of six components (Fig. 5A; $F_{2,27} = 23.8$, $P < 0.001$), the mean of the latter not different from that of the control. The experiment yielded similar results in South Carolina, where 27 males were captured; again, only the mean for dihexanoate was different from that of the control (Fig. 5B; $F_{2,18} = 10.3$, $P = 0.0055$). In Illinois, beetles were captured from 16 July to 17 August 2021 and, in South Carolina, from 4 June to 20 August 2021.

Discussion

The headspace volatiles from a crushed abdomen of female *P. attenuatus* contained six compounds that were identifiable as potential pheromone components, but the field bioassay data suggested that the sex attractant pheromone may only consist of one of them, 1,8-octanediol dihexanoate. Blends of this compound with two other possible components, 1,8-octanediol, and 1,8-octanediol monohexanoate, were less attractive than dihexanoate as a single component, as was a six-component blend of the dihexanoate with hexanoic acid, 1,8-octanediol, octyl hexanoate, 1,8-octanediol monohexanoate, and 1-octanol. Although substantial numbers of beetles were attracted to the dihexanoate, it is possible that one or more of the other components, when presented in the correct ratio, could enhance attraction to this

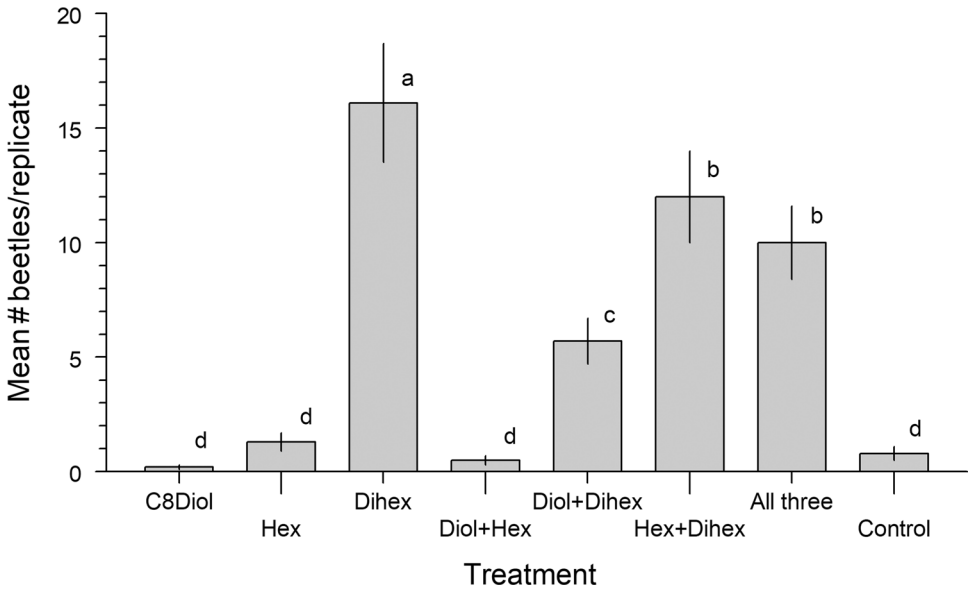

Fig. 4 Captures of adult male *Parallelostethus attenuatus* during targeted experiment 1 in Illinois. Treatments: C8Diol (1,8-octanediol), Hex (1,8-octanediol monohexanoate), Dihex (1,8-octanediol dihexanoate), Diol+Hex (1,8-octanediol +1,8-octanediol monohexanoate), Diol+Dihex (1,8-octanediol +1,8-octanediol dihexanoate), Hex+Dihex (1,8-octanediol monohexanoate +1,8-octanediol dihexanoate), All three (1,8-octanediol +1,8-octanediol monohexanoate +1,8-octanediol dihexanoate), Control. Lures were loaded with 5 mg of each component. Means with different letters are different (REGWQ test, $P < 0.05$)

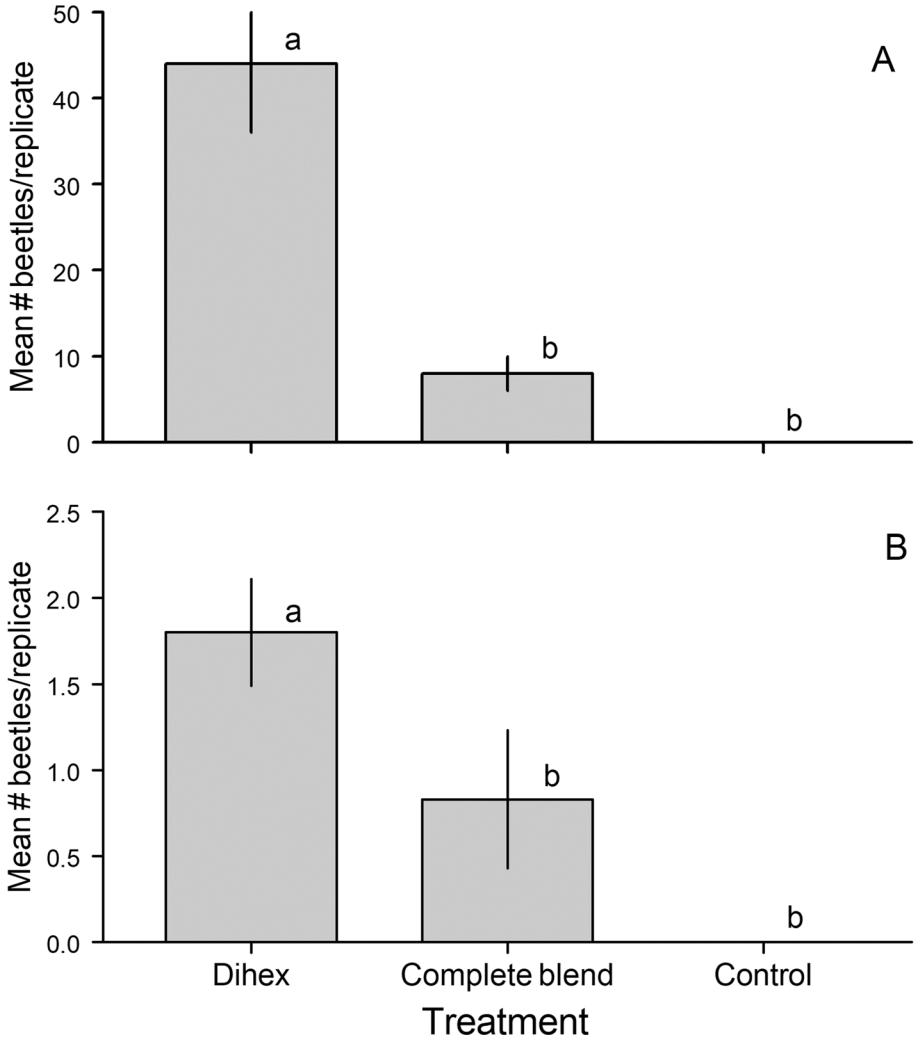

Fig. 5 Captures of adult male *Parallelostethus attenuatus* during targeted experiment 2 in **A**) Illinois, and **B**) South Carolina. Treatments: Dihex (1,8-octanediol dihexanoate), Complete blend (1,8-octanediol dihexanoate + hexanoic acid +1-octanol +1,8-octanediol + octyl hexanoate +1,8-octanediol monohexanoate). Lures contained 5 mg of 1,8-octanediol dihexanoate alone or combined with 0.5 mg each of the other compounds

single component. Unfortunately, we were not able to determine what the natural release ratio might be because analyses of the headspace volatiles collected from live females were indeterminate; the dihexanoate was not detected in any of these extracts. The volatiles detected in abdomen squashes may not reflect the blend that is actually released, either in ratio and/or components, because the volatiles from the squashes may contain pheromone precursors, or even hydrolyzed pheromone components generated by the action of esterases and other enzymes on the pheromone components. It is also unclear why one or more of the other components in the headspace volatiles appeared to inhibit attraction to the diester. In particular, it seems unlikely that the diester could be a pheromone component for related sympatric species, so that minor components might be necessary to produce a species-specific blend, because *P. attenuatus* is the only species in its genus in North America.

To date, a sex attractant pheromone has been identified for only one other species in the tribe Elaterini, to which *P. attenuatus* belongs. Specifically, pheromone gland extracts from females of the European species *Elater ferrugineus* L. contained a blend of four monoesters, 7-methyloctyl 5-methylhexanoate, 7-methyloctyl octanoate, 7-methyloctyl 7-methyloctanoate, and 7-methyloctyl (*Z*)-4-decenoate (Tolasch et al. 2007). The blend was highly attractive to males; analogous to *P. attenuatus*, a subsequent study found that the single component 7-methyloctyl (*Z*)-4-decenoate was both necessary and sufficient for optimal attraction of male *E. ferrugineus* (Svensson et al. 2012). Despite *E. ferrugineus* and *P. attenuatus* being closely related, the structures of their pheromones share few similarities, other than being esters.

Although monoesters are found in numerous insect pheromones, including the pheromones of a number of elaterid species (Tóth 2013) and numerous other insect taxa (El-Sayed 2019), diesters, and particularly symmetrical diesters, appear to be uncommon pheromone constituents. We found only two similar examples: one in the pheromone of the click beetle *Agriotes acuminatus* (Stephens), which produces (*E*)-8-hydroxyneryl dihexanoate

(Tolasch et al. 2010), and the other in the congeneric *A. pilosellus* (Schonherr), which produces (*E*)-8-hydroxygeranyl dibutanoate (Tolasch et al. 2022). Whereas it is known that microorganisms can produce α,ω-diols from substrates such as straight-chain alkanes, fatty acids, or ω-hydroxyacids (e.g., Ahsan et al. 2018), the production of medium length straight-chain diols, such as 1,8-octanediol, has, to our knowledge, not been observed in insects. However, oxidation at the ω or ω-1 position of fatty acids is known in insects, for example, in the biosynthesis of the macrolide pheromones of stored products beetles in the genera *Oryzaephilus* (Silvanidae) and *Cryptolestes* (Laemophloeidae; Vanderwel et al. 1990, 1992). By extension, we speculate that reduction of the carboxyl function in an 8-hydroxyoctanoic acid or a longer chain ω-hydroxylated fatty acid precursor, which is then chain shortened, may provide the 1,8-octanediol moiety for the pheromone of *P. attenuatus*. Although its biosynthetic origin remains to be determined, the identification of 1,8-octanediol dihexanoate as a click beetle pheromone expands the known chemical space of elaterid pheromones, and that of insect pheromones overall.

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Author Contributions JGM, LW III, and LMH secured the funding in support of the work described. JGM identified and synthesized possible pheromone compounds and contributed to writing the manuscript. LW III, ASH, and TPK carried out field trials and contributed sections of the manuscript. JMS and SH prepared and carried out analyses of pheromone extracts. ACG assisted with field trials in Illinois. LMH organized the field work in Illinois, carried out the data analyses, and contributed to writing the manuscript. All authors assisted in editing and polishing the manuscript.

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Declarations

Competing Interests The authors declare that they have no competing interests.

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