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Interspecific Cross-Attraction between the South American Cerambycid Beetles *Cotyclytus curvatus* and *Megacyllene acuta* is Averted by Minor Pheromone Components

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Abstract

During field screening trials conducted in Brazil in 2015, adults of both sexes of the cerambycid beetles *Cotyclytus curvatus* (Germar) and *Megacyllene acuta* (Germar) (subfamily Cerambycinae, tribe Clytini) were significantly attracted to racemic 3-hydroxyhexan-2-one and racemic 2-methylbutan-1-ol, chemicals which previously have been identified as male-produced aggregation-sex pheromones of a number of cerambycid species endemic to other continents. Subsequent analyses of samples of beetle-produced volatiles revealed that males of *C. curvatus* sex-specifically produce only (*R*)-3-hydroxyhexan-2-one, whereas males of *M. acuta* produce the same compound along with lesser amounts of (2*S*,3*S*)-2,3-hexanediol and (*S*)-2-methylbutan-1-ol. Follow-up field trials showed that both sexes of both species were attracted to synthetic reconstructions of their respective pheromones, confirming that males produce aggregation-sex pheromones. The minor pheromone components of *M. acuta*, (*S*)-2-methylbutan-1-ol and (2*S*,3*S*)-2,3-hexanediol, synergized attraction of that species, but antagonized attraction of *C. curvatus* to (*R*)-3-hydroxyhexan-2-one. Beetles of other cerambycine species also were attracted in significant numbers, including *Chrysoprasis linearis* Bates, *Cotyclytus dorsalis* (Laporte & Gory), and *Megacyllene falsa* (Chevrolat). Our results provide further evidence that 3-hydroxyhexan-2-one is a major component of attractant pheromones of numerous cerambycine species world-wide. Our results also highlight our increasing understanding of the crucial role of minor pheromone components in imparting species specificity to cerambycid pheromone blends, as is known to occur in numerous species in other insect families.

Keywords Coleoptera · Longhorned beetle · Reproductive isolation · Mate location · Semiochemical · Pheromone chemistry

Introduction

Research over the last decade has revealed considerable parsimony within the large beetle family Cerambycidae with regard to attractant pheromones, with many closely-related species being shown to share pheromone structures, or even produce pheromones of apparently identical composition (reviewed by Millar and Hanks 2017). For example, many species in the subfamily Cerambycinae have been documented to use pheromones composed of 6, 8, or 10 carbon chains with ketone or hydroxyl groups on the second and third carbons (reviewed by Hanks and Millar 2016). These 3-hydroxyalkan-2-ones and 2,3-alkanediols appear to represent conserved structural motifs among cerambycine species endemic to all continents except Antarctica (Hanks and Millar 2016). For all known cases within the subfamily Cerambycinae, the compounds serve as aggregation-sex pheromones (sensu Cardé 2014), produced by males, but attractive to both sexes. As a consequence of this sharing of pheromone motifs, a single pheromone compound may attract beetles of multiple species simultaneously (e.g., Hanks et al. 2012; Millar et al. 2017; Sweeney et al. 2014; Wickham et al. 2014).

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Many species in the cerambycine tribe Clytini, including representatives of several genera native to North America, Europe, Asia, and Australia, are known to be attracted by 3-hydroxyalkan-2-ones, and in many cases the attractive compounds have been confirmed to be pheromone components (Hanks and Millar 2016). Recently, (*R*)-3-hydroxyhexan-2-one also was identified as an important or even sole component of pheromones produced by males of several South American clytine species (Silva et al. 2017).

We report here the identification and field bioassays of the male-produced aggregation-sex pheromones of two additional clytine species, *Cotyclytus curvatus* (Germar) and *Megacyllene acuta* (Germar). Both species are endemic to South America, where they are broadly distributed (Monné 2017). The adults are diurnal, and at least the adults of *M. acuta* are known to visit flowers and feed on rotting fruit (Martins and Galileo 2011). The larvae of both species are polyphagous, with host plants including more than 50 species of angiosperms in 21 families, primarily Anacardiaceae, Fabaceae, and Mimosaceae (Monné 2017). Their larvae bore within branches and stems of freshly cut or dying trees (Martins and Galileo 2011), and can cause economic damage to fruit trees such as apple, pear, quince (Rosaceae), avocado (Lauraceae), and fig (Moraceae; Martins and Galileo 2011). Moreover, larvae of *M. acuta* are known to be readily transported by international commerce, for example in shipments of timber from Brazil to Europe (Duffy 1953), and thus may pose a threat as exotic and invasive species. The two species were targeted for pheromone identification after being caught in screening trials of known cerambycid pheromones. In particular, beetles of both sexes of *C. curvatus* and *M. acuta* were significantly attracted to racemic 3-hydroxyhexan-2-one as a single component, or blended with racemic 2-methylbutan-1-ol (WDS, unpub. data).

We provide evidence that males of *C. curvatus* produce only (*R*)-3-hydroxyhexan-2-one, that males of *M. acuta* produce the same compound along with lesser amounts of (2*S*,3*S*)-2,3-hexanediol and (*S*)-2-methylbutan-1-ol, and that adults of both species were optimally attracted by synthetic reconstructions of their respective pheromones. Furthermore, during field trials, adults of several other cerambycine species were attracted in significant numbers, including *Megacyllene falsa* (Chevrolat), *Cotyclytus dorsalis* (Laporte & Gory), and *Chrysoprasis linearis* Bates, suggesting that their pheromones may also contain 3-hydroxyhexan-2-one as a major component.

Methods and Materials

Sources of Chemicals Racemic 3-hydroxyhexan-2-one was purchased from Bedoukian Research Inc. (Danbury, CT, USA), and 1-hexanol, racemic 2-methylbutan-1-ol, and (*S*)-2-methylbutan-1-ol were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). (*R*)-3-Hydroxyhexan-2-one was synthesized as described in Lacey et al. (2007), and (2*S*,3*S*)-2,3-hexanediol was made as described in Lacey et al. (2004).

Field Bioassay 1 Attraction of *C. curvatus* and *M. acuta* to synthetic chemicals representative of their suspected pheromones was first assessed with a preliminary field bioassay of racemic 3-hydroxyhexan-2-one and racemic 2-methylbutan-1-ol conducted in a remnant of the Atlantic Rain Forest on the campus of the University of São Paulo, Piracicaba, SP Brazil (−22.712 lat., −47.628 long.). Beetles were captured with custom-built panel traps (black corrugated plastic; see Silva et al. 2016a,b) with internal surfaces coated with a 50% aqueous emulsion of Fluon® (Insect-a-Slip, BioQuip Products Inc., Rancho Dominguez, CA, USA). Traps were fitted with 5-l plastic jars containing ~100 ml of saturated aqueous sodium chloride and a few drops of dish detergent to kill and preserve captured beetles. Traps were deployed ~2 m above the ground on inverted L-shaped frames of PVC pipe (2 cm i.d.) mounted on steel rods (1-m long) driven halfway into the ground. Lures were transparent plastic sachets (polyethylene resealable, 5 × 7.5 cm, 50 μm wall thickness; Bagettes® model 14770, Cousin Corp., Largo, FL, USA) containing a cotton dental roll, and were loaded with 50 mg of the racemic chemicals (i.e., 25 mg per enantiomer) in 1 ml of isopropanol (Dinâmica Química Contemporânea Ltda., Diadema, SP, Brazil). Control lures were loaded with 1 ml of neat isopropanol. Treatments were: 1) racemic 3-hydroxyhexan-2-one; 2) racemic 2-methylbutan-1-ol; 3) a 1:1 blend of racemic 3-hydroxyhexan-2-one and 2-methylbutan-1-ol; and 4) the solvent control. Four traps were deployed ~15 m apart in each of four blocks, with the blocks separated by at least 30 m. Test lures were assigned randomly to traps within blocks on the day of setup, and traps were serviced every 2–3 d, at which time treatments were rotated within blocks to control for positional effects. The experiment was conducted from 23 September to 28 October 2015. Dead adults of the two species that were collected from traps were sexed by examining them for male-specific pores on the prothorax (Online Supplement, Figs. S1-S3).

Collection and Identification of Beetle-Produced Volatiles from *C. curvatus* and *M. acuta* Live beetles for collection of headspace volatiles were procured during Field Bioassay 1 with a separate set of three traps baited with the same chemical treatments as described above, but excluding the solvent control. Trap jars for those traps were modified to capture beetles alive by omitting the killing solution and by drilling 2-mm holes in the bottom of jars to allow rainwater to drain (Silva et al. 2016a,b). Traps were checked for beetles daily, which yielded ~20 adult males and females of *C. curvatus* and *M. acuta*.

Live adults of each species were sexed by caging arbitrarily selected pairs of beetles together and monitoring them for mating behavior (i.e., males mount females). Males and females were kept separately in 0.5-l plastic jars under ambient laboratory conditions (23 ± 2 °C, 60 ± 10% RH, ~5000 lx from fluorescent lights, and 12:12 h L:D) for 48 h prior to collection of headspace volatiles. Beetles were provided with a 10% aqueous solution of sucrose for nutrition, dispensed from cotton-stoppered vials. Beetles were aerated in groups of two to five conspecific individuals of the same sex, in 0.5-l cylindrical glass chambers that also contained four of the sucrose solution vials. Individual beetles were aerated as many as three times. Headspace odors were trapped on 150 mg of 80/100 mesh HayeSep® Q adsorbent (Supelco, Bellefonte, PA, USA) in a glass pipette (8.5 cm long × 0.5 cm i.d.) with the adsorbent held in place with glass wool plugs. Collectors were attached to the outlets of chambers with a screw cap with a Teflon ferrule. Charcoal-filtered air was passed through the apparatus at ~300 ml/min. Volatiles were collected continuously for 24–48 h under the environmental conditions described above. Volatiles were collected simultaneously from chambers containing only the sucrose solution vials as controls for system contaminants. Trapped volatiles were recovered by eluting the collectors with 4 × 0.25-ml methylene chloride into 2-ml silanized glass vials, which were stored at −30 °C until analysis.

The resulting extracts initially were analyzed in Brazil, to confirm that they contained beetle-produced volatiles, using a GC-2010 gas chromatograph (Shimadzu Corp., Kyoto, Japan) with flame ionization detection, and fitted with an HP5-MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film; Agilent Scientific, Santa Clara, CA, USA). Injections of 1 μl were made in splitless mode (split valve opened after 1 min) with an injector temperature of 250 °C and He carrier gas at a linear velocity of 25 cm/s. The GC oven was programmed from 35 °C for 1 min, increased to 40 °C at 2 °C/min (hold 1 min), and then increased to 250 °C at 10 °C/min (hold 10 min). Extracts that contained sex-specific peaks subsequently were sent to the University of California, Riverside, where compounds were tentatively identified with an Agilent 7820A GC coupled to a 5977E mass selective detector, using an DB5-MS column (same dimensions as above), in splitless mode with He carrier gas (linear velocity 37 cm/s), and a temperature program of 40 °C/1 min, 10 °C/min to 280 °C (hold 10 min). Retention times and mass spectra of compounds in the extracts were compared with those of authentic standards to confirm identifications. Retention indices were calculated relative to linear alkanes (Table 1). Representative mass spectra are shown in Figs. S4-S7 in the Online Supplement.

The absolute configurations of the insect-produced compounds were determined by analysis of extracts on a chiral stationary phase Cyclodex B GC column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA), with an injector temp of 150 °C to minimize isomerization of the thermally unstable hydroxyketones. Injections were made in split mode at 25 psi head-pressure, and the oven was programmed from 50 °C/1 min, 3 °C/min to 220 °C, hold 20 min. Retention indices calculated relative to linear alkanes are listed in Table 1.

Table 1 Retention indices (RI) of the stereoisomers of 2-methylbutan-1-ol, 3-hydroxyhexan-2-one, and 2,3-hexanediols on a DB-5MS column and a chiral stationary phase Cyclodex B column, calculated relative to linear alkane standards. Chromatographic conditions are listed in the Methods and Materials

Compound	RI on DB-5MS	RI on Cyclodex B
(*R*)-2-methylbutan-1-ol	737	939
(*S*)-2-methylbutan-1-ol	737	941
(*R*)-3-hydroxyhexan-2-one	896	1082
(*S*)-3-hydroxyhexan-2-one	896	1108
(2*S*,3*S*)-2,3-hexanediol	976	1260
(2*R*,3*R*)-2,3-hexanediol	976	1270
(2*R*,3*S*)-2,3-hexanediol	980	1287
(2*S*,3*R*)-2,3-hexanediol	980	1292

Field Bioassay 2 The biological activities of the reconstructions of the respective pheromones of *C. curvatus* and *M. acuta* from synthesized compounds were field tested in the same study area as described for Field bioassay 1, and using the same methods, from 16 July to 22 September 2016. This bioassay included traps baited with (*R*)-3-hydroxyhexan-2-one and racemic 3-hydroxyhexan-2-one, to test for antagonism of attraction by the non-natural (*S*)-enantiomer for the two target species. Because 1-hexanol was present only in trace amounts, if at all, in the volatiles from males of *M. acuta* (see Results), it was not included in the test treatments.

Pheromone lures, prepared as described above, were loaded with 25 mg of (*R*)-3-hydroxyhexan-2-one, or 50 mg each of the racemic compounds (individually or blended), in 1 ml of isopropanol. Treatments were as follows: 1) (*R*)-3-hydroxyhexan-2-one; 2) racemic 3-hydroxyhexan-2-one; 3) racemic *syn*-2,3-hexanediol [(2*R**,3*R**)-2,3-hexanediol]; 4) racemic 2-methylbutan-1-ol; 5) the binary blend of racemic 3-hydroxyhexan-2-one and *syn*-2,3-hexanediol; 6) the binary blend of racemic 3-hydroxyhexan-2-one and 2-methylbutan-1-ol; 7) the ternary blend of racemic 3-hydroxyhexan-2-one, racemic *syn*-2,3-hexanediol, and racemic 2-methylbutan-1-ol; and 8) solvent control. Traps were deployed ~15 m apart in four blocks of eight traps (i.e., one trap per treatment), with the blocks separated by ~30 m. Traps were serviced at intervals of 5–7 d, at which time treatments were rotated within blocks, and pheromone lures were replaced every 2 wk.

Statistical Analysis Differences between treatment means, blocked by location and date, were tested separately for species that were represented by at least 20 specimens per field bioassay using the nonparametric Friedman's Test (PROC FREQ; SAS Institute 2011) because data violated assumptions of ANOVA (Sokal and Rohlf 1995). Replicates were defined by block and collection date. Replicates in which no specimens of the species in question had been caught in any trap (e.g., due to inclement weather) were dropped from analyses. In recognition of the multiple statistical tests of treatment effects, significance levels were adjusted to $\alpha = 0.010$ for Field Bioassay 1 with four treatments (N = five analyses; see Results) and $\alpha = 0.017$ for Field Bioassay 2 with eight treatments (N = three analyses) according to the Bonferroni procedure (Quinn and Keough 2002). Assuming a significant overall Friedman's test, pairs of treatment means were compared using the REGWQ multiple range test, which controls the Type I experimentwise error rate (SAS Institute 2011). For the best represented species in each bioassay, the sex ratio of captured beetles was compared to a nominal proportion of 0.5 with 95% Clopper-Pearson exact confidence intervals at 5% probability (Newcombe 1998).

Results

Field Bioassay 1 A total of 358 cerambycid beetles, representing 28 species in three subfamilies, were caught during the bioassay, including 38 and 33 specimens of the target species *C. curvatus* and *M. acuta*, respectively (Table 2). Adults of *C. curvatus* were significantly attracted only by 3-hydroxyhexan-2-one, and attraction apparently was strongly antagonized by 2-methylbutan-1-ol (Fig. 1a; means significantly different, Friedman's $Q_{3,108} = 83.8$, $P < 0.001$). Adults of *M. acuta* showed a different response, being attracted only by the blend of 3-hydroxyhexan-2-one and 2-methylbutan-1-ol, and not by either compound alone (Fig. 1b; $Q_{3,92} = 68.9$, $P < 0.001$).

Among the remaining species, the cerambycines *C. linearis*, *C. dorsalis*, and *M. falsa* were captured in numbers sufficient for statistical analysis. Adults of *C. linearis* were significantly attracted only by racemic 3-hydroxyhexan-2-one (Fig. 2a; $Q_{3,72} = 65.6$, $P < 0.001$), indicating that the 2-methylbutan-1-ol strongly inhibited attraction to 3-hydroxyhexan-2-one. In contrast, adults of *C. dorsalis* and *M. falsa* were attracted only by the blend of racemic 3-hydroxyhexan-2-one and 2-methylbutan-1-ol, indicating strong synergism between the two compounds (Fig. 2b and c; $Q_{3,68} = 65.7$ and $Q_{3,168} = 155.9$, respectively; $P < 0.001$ for both).

Collection and Identification of Beetle-Produced Volatiles from *C. curvatus* and *M. acuta* Analyses of extracts of headspace volatiles from males of *C. curvatus* and *M. acuta* revealed the presence of sex-specific compounds that were absent in equivalent extracts of females and system controls (Fig. 3). Compounds were tentatively identified by matching their mass spectra with spectra in the NIS mass spectral database (2-methylbutan-1-ol, 2,3-hexanediol, and 1-hexanol), or by matching them with literature spectra (3-hydroxyhexan-2-one; Fettköther et al. 1995). Identifications were then confirmed by matching retention indices (Table 1) and mass spectra with those of authentic standards (Figs. S4-S7 in the Online Supplement). Finally, the enantiomer of each chiral insect-produced compound was determined by analyses of extracts, alone and spiked with authentic standards, on a chiral stationary phase Cyclodex B column. The retention indices of the various compounds and isomers on the Cyclodex B column are listed in Table 1, and the chromatograms are shown in Figs. S8-S11 in the Online Supplement.

Extracts from males of *C. curvatus* showed essentially a single peak, identified as (*R*)-3-hydroxyhexan-2-one (peak 3 in Fig. 3). Volatiles from males of *M. acuta* were dominated by the same compound, with lesser amounts of (*S*)-2-methylbutan-1-ol (peak 1 in Fig. 3) and (2*S*,3*S*)-2,3-hexanediol (peak 4), as well as traces of 1-hexanol (peak 2). The average proportions (± SD) of peaks 1, 2, and 4 (relative to peak 3) in eight aeration extracts of male *M. acuta* were 23.8 ± 25.0, 1.0 ± 0.5, and 2.7 ± 1.4, respectively.

Field Bioassay 2 During this bioassay, traps captured a total of 501 beetles of 11 species, with three species being caught in significant numbers (Table 2; 99 adults of *C. curvatus,* 257 adults of *M. acuta*, and 116 adults of *M. falsa*). The responses of *C. curvatus* and *M. acuta* to the experimental treatments were consistent with the results from Field Bioassay 1, as well as with the compounds released by their males. Thus, adults of *C. curvatus* were attracted by both racemic 3-hydroxyhexan-2-one and its (*R*)-enantiomer, but the latter was slightly but significantly a stronger attractant, indicating some measure of inhibition by the (*S*)-enantiomer (Fig. 4a; means significantly different, $Q_{7,280} = 118.4$, $P < 0.001$). Attraction to the hydroxyketone again was antagonized by 2-methylbutan-1-ol, and even more strongly by the *syn*-(2,3)-hexanediol.

For *M. acuta*, as in Field Bioassay 1, neither 3-hydroxyhexan-2-one nor 2-methylbutan-1-ol were attractive alone, but the blend was clearly attractive (Fig. 4b; $Q_{7,360} = 235.5$, $P < 0.001$). Attraction was further increased by addition of *syn*-2,3-hexanediol to the blend, indicating that all three chemicals are components of the natural pheromone.

Megacyllene falsa was the only other species collected in significant numbers in Field Bioassay 2, with both sexes being attracted only by the blend of 3-hydroxyhexan-2-one and 2-methylbutan-1-ol (Fig. 4c; $Q_{7,296} = 197.2$, $P < 0.001$). Attraction of this species also appeared to be antagonized by addition of *syn*-2,3-hexanediol to the blend. Two of the species caught in significant numbers during Field Bioassay 1

Table 2 Taxonomy and total numbers of adult cerambycid beetles captured during two field bioassays conducted in Brazil: 1) during 2015, and 2) during 2016. The two target species are indicated by bold font. Question marks indicate that sex could not be determined

Taxon	Field bioassay 1			Field bioassay 2		
	♂	♀	Total	♂	♀	Total
Cerambycinae						
Achrysonini						
Achryson surinamum (L.)	9	8	17			
Clytini						
***Cotyclytus curvatus* (Germar)**	**17**	**21**	**38**	**35**	**64**	**99**
Cotyclytus dorsalis (Laporte & Gory)	7	17	24			
***Megacyllene acuta* (Germar)**	**21**	**12**	**33**	**88**	**169**	**257**
Megacyllene falsa (Chevrolat)	68	93	161	53	63	116
Compsocerini						
Aglaoschema ventrale (Germar)	0	2	2			
Compsocerus violaceus (White)	1	2	3			
Elaphidiini						
Ambonus distinctus (Newman)	1	0	1	1	0	1
Ambonus interrogationis (Blanchard)	2	3	5			
Centrocerum variatum (Newman)				?	?	3
Mallocera glauca Audinet-Serville	?	?	1			
Protosphaerion variabile Gounelle	2	2	4			
Heteropsini						
Chrysoprasis aurigena (Germar)	0	3	3			
Chrysoprasis linearis Bates	14	15	29			
Mallosoma zonatum (Sahlberg)	3	7	10			
Hexoplonini						
Gnomidolon elegantulum Lameere	?	?	1			
Neoibidionini						
Tropidion investitum (Martins)	?	?	2			
Trachyderini						
Chydarteres dimidiatus dimidiatus (F.)	0	2	2			
Trachyderes succinctus duponti Aurivillius	0	2	2			
Lamiinae						
Acanthocinini						
Eutrypanus dorsalis (Germar)	?	?	1	?	?	1
Oedopeza umbrosa (Germar)	?	?	2			
Acanthoderini						
Macropophora accentifer (Olivier)	0	1	1	1	4	5
Psapharochrus cylindricus (Bates)	?	?	3	?	?	11
Psapharochrus jaspideus (Germar)	2	4	6	0	2	2
Colobotheini						
Colobothea rubroornata Zajciw	?	?	2			
Compsosomatini						
Aerenea posticalis Thomson				?	?	3
Aerenea quadriplagiata (Boheman)	?	?	1	?	?	1
Onciderini						
Hesycha inermicollis (Breuning)	?	?	1			
Plerodia syrinx (Bates)	?	?	2			
Prioninae						
Macrotomini						
Mallodon spinibarbis (L.)	0	1	1			
Total:			358			501

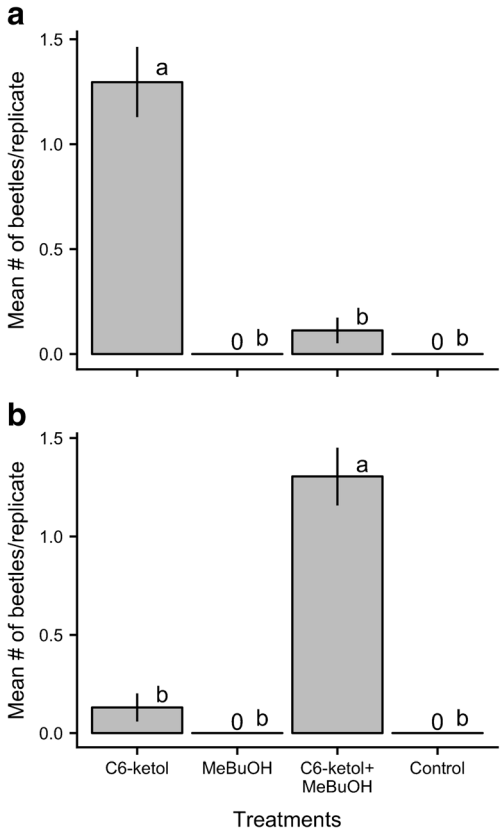

Fig. 1 Mean (± SE) number of adults of both sexes of **a**) *Cotyclytus curvatus* and **b**) *Megacyllene acuta* captured during Field Bioassay 1. Compounds: C6-ketol = racemic 3-hydroxyhexan-2-one; MeBuOH = racemic 2-methylbutan-1-ol, and Control = solvent control (isopropanol). Means within species with different letters are significantly different (REGWQ test, $P < 0.05$)

(*C. linearis* and *C. dorsalis*) were not caught the following year in Field Bioassay 2, most likely due to the differences in the time periods that the two bioassays were deployed (23 September-28 October, and 16 July to 22 September, respectively).

In both field bioassays, the sex ratios of beetles within the most attractive treatments, for all the species for which sex could be reliably determined, were not significantly different from 50:50 (Clopper-Pearson $P > 0.05$), with the exception of a slight female bias for *M. acuta* in Field Bioassay 2 (66% females; 95% Clopper-Pearson exact confidence interval: 0.56–0.73, $P < 0.001$).

Discussion

Attraction of both sexes of the target species *C. curvatus* and *M. acuta* to traps baited with reconstructed blends of the compounds produced by their conspecific males confirmed that males of both species produce aggregation-sex pheromones, as has been shown for many species in the cerambycid subfamilies Cerambycinae, Lamiine, and Spondylidinae (Millar

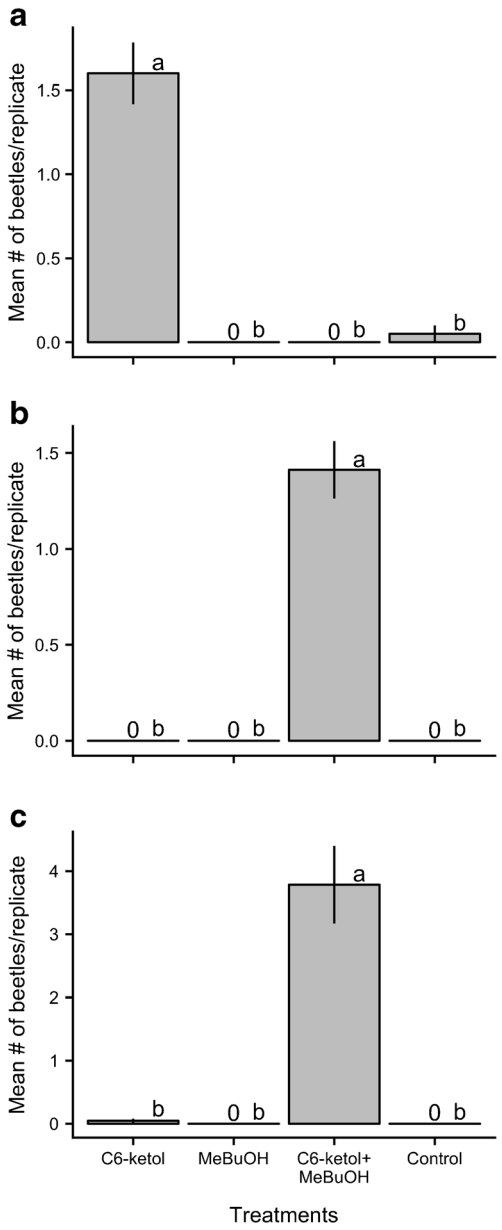

Fig. 2 Mean (± SE) number of adults of both sexes of **a**) *Chrysoprasis linearis*, **b**) *Cotyclytus dorsalis*, and **c**) *Megacyllene falsa* captured during Field Bioassay 1. Compounds: C6-ketol = racemic 3-hydroxyhexan-2-one; MeBuOH = racemic 2-methylbutan-1-ol, and Control = solvent control (isopropanol). Means within species with different letters are significantly different (REGWQ test, $P < 0.05$)

and Hanks 2017). Demonstrated production of (*R*)-3-hydroxyhexan-2-one by males of *C. curvatus* and *M. acuta,* and attraction of a number of related species to lures containing this compound (*C. linearis*, *C. dorsalis*, and *M. falsa*) provides further evidence of the ubiquity of this chemical as a pheromone component of cerambycine beetles (see Hanks and Millar 2016).

The field bioassays also highlighted the importance of minor pheromone components in averting cross attraction among sympatric species of cerambycids, as shown previously in several

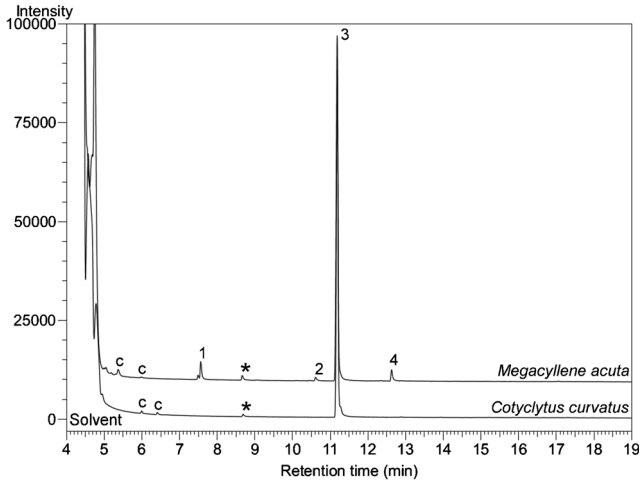

Fig. 3 Representative gas chromatograms of extracts of volatiles produced by adult males of *Cotyclytus curvatus* and *Megacyllene acuta*. Male-specific compounds are indicated by numbers as follows: 1 = (*S*)-2-methylbutan-1-ol, 2 = 1-hexanol, 3 = (*R*)-3-hydroxyhexan-2-one, 4 = (2*S*,3*S*)-2,3-hexanediol. Asterisks indicate analytical artifacts and "c" indicates contaminants from the aeration system

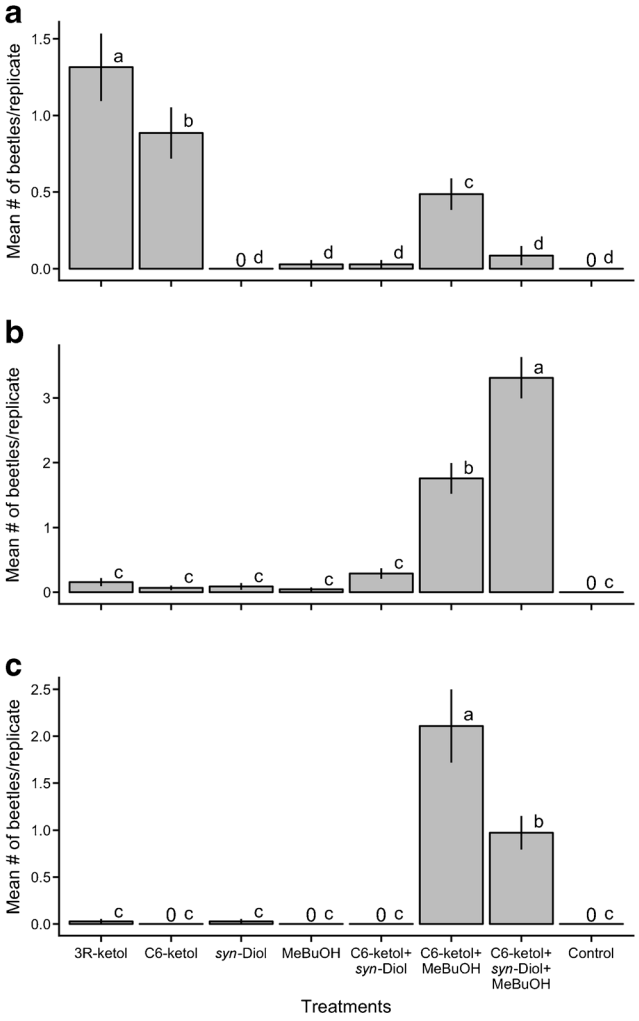

Fig. 4 Mean (± SE) number of adults of both sexes of **a**) *Cotyclytus curvatus,* **b**) *Megacyllene acuta*, and **c**) *Megacyllene falsa* captured during Field Bioassay 2. Compounds: 3R–ketol = (*R*)-3-hydroxyhexan-2-one, C6-ketol = racemic 3-hydroxyhexan-2-one, *syn*-Diol = *syn*-2,3-hexanediol, MeBuOH = racemic 2-methylbutan-1-ol, and Control = solvent control (isopropanol). Means within species with different letters are significantly different (REGWQ test, $P < 0.05$)

other instances (e.g., Mitchell et al. 2015; Narai et al. 2015; Silva et al. 2017). Specifically, the minor components (*S*)-2-methylbutan-1-ol and (2*S*,3*S*)-2,3-hexanediol in the pheromone of *M. acuta* appeared to be strong synergists, and thus this species would be unlikely to respond to any species that produces only (*R*)-3-hydroxyhexan-2-one, such as *C. curvatus*. In turn, attraction of *C. curvatus* to (*R*)-3-hydroxyhexan-2-one present in the pheromone of *M. acuta* would likely be prevented by both the (*S*)-2-methylbutan-1-ol and the (2*S*,3*S*)-2,3-hexanediol components in the pheromone of the latter species.

We have not yet tested the responses of *M. acuta* to individual enantiomers of 2-methylbutan-1-ol or *syn*-2,3-hexanediol. However, the fact that racemic 2-methylbutan-1-ol acts as a strong synergist of the 3-hydroxyhexan-2-one, and that racemic *syn*-2,3-hexanediol further increases that attraction, suggests that the "non-natural" enantiomers present in the racemates of both of these compounds are unlikely to be strongly inhibitory. It is also noteworthy that *M. acuta* produces (*S*)-2-methylbutan-1-ol, analogous to its North American congener *Megacyllene caryae* (Gahan) (Mitchell et al. 2012). In contrast, all other cerambycid species known to have 2-methylbutan-1-ol as a pheromone component produce the (*R*)-enantiomer (listed in Hanks and Millar 2016). Thus, production of the less common (*S*)-enantiomer by *M. acuta* may play a role in its reproductive isolation from related sympatric species.

Even though the pheromone blends of the other species caught in significant numbers remain to be elucidated, the bioassay results provide persuasive evidence for synergistic and inhibitory actions among blend components for those species as well. For example, in Field Bioassay 1, *C. dorsalis* and *M. falsa* were only attracted by the blend of 3-hydroxyhexan-2-one and 2-methylbutan-1-ol, with no sign of attraction to either compound alone. Conversely, in the same bioassay, *C. linearis* was only attracted to 3-hydroxyhexan-2-one as a single component; adding 2-methylbutan-1-ol to the 3-hydroxyhexan-2-one completely abolished attraction, indicating strong inhibition by at least one of the enantiomers of 2-methylbutan-1-ol. However, because racemic 2-methylbutan-1-ol was used in these bioassays, and not the individual enantiomers, we cannot rule out the possibility that one enantiomer of 2-methylbutan-1-ol is strongly inhibitory to *C. linearis*, while the other may act additively or synergistically with the 3-hydroxyhexan-2-one.

For *M. falsa*, these results were reproduced in Field Bioassay 2, with the blend of 3-hydroxyhexan-2-one and 2-methylbutan-1-ol being required to achieve any degree of attraction. Furthermore, attraction to that blend was diminished

by addition of the *syn*-2,3-hexanediol, a minor component in the blend of its sympatric congener *M. acuta*. Overall, the bioassay results suggest that chemical mechanisms for averting cross attraction, based on minor components of pheromone blends, are operative for the species discussed here, because they overlap broadly in seasonal phenology and diel flight activity (WDS, pers. obs.). Further work to identify the compounds actually emitted by *C. linearis*, *C. dorsalis*, and *M. falsa* should help to verify the complex interplay of attraction and inhibition suggested by the bioassays described here.

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References

Cardé RT (2014) Defining attraction and aggregation pheromones: teleological versus functional perspectives. J Chem Ecol 40:519–520. https://doi.org/10.1007/s10886-014-0465-6

Duffy EAJ (1953) A monograph of the immature stages of British and imported timber beetles (Cerambycidae). Brit. Mus. (Nat. Hist.), London

Fettköther R, Dettner K, Schröder F, Meyer H, Francke W, Noldt U (1995) The male pheromone of the old house borer *Hylotrupes bajulus* (L.) (Coleoptera: Cerambycidae): identification and female response. Experientia 51:270–277. https://doi.org/10.1007/BF01931111

Hanks LM, Millar JG (2016) Sex and aggregation pheromones of cerambycid beetles: basic science and practical applications. J Chem Ecol 42:631–654. https://doi.org/10.1007/s10886-016-0733-8

Hanks LM, Millar JG, Mongold-Diers JA, Wong JCH, Meier LR, Reagel PF, Mitchell RF (2012) Using blends of cerambycid beetle pheromones and host plant volatiles to simultaneously attract a diversity of cerambycid species. Can J For Res 42:1050–1059. https://doi.org/10.1139/x2012-062

Lacey ES, Ginzel MD, Millar JG, Hanks LM (2004) Male-produced aggregation pheromone of the cerambycid beetle *Neoclytus acuminatus acuminatus*. J Chem Ecol 30:1493–1507. https://doi.org/10.1023/B:JOEC.0000042064.25363.42

Lacey ES, Moreira JA, Millar JG, Ray AM, Hanks LM (2007) Male-produced aggregation pheromone of the cerambycid beetle *Neoclytus mucronatus mucronatus*. Entomol Exp Appl 122:171–179. https://doi.org/10.1111/j.1570-7458.2006.00508.x

Martins UR, Galileo MHM (2011) Tribo Clytini Mulsant, 1839. In: Martins UR, Galileo MHM (eds) Cerambycidae Sul-Americanos (Coleoptera): taxonomia, vol 12. Sociedade Brasileira de Entomologia, São Paulo, pp 8–264 (In Portuguese)

Millar JG, Hanks LM (2017) Chemical ecology of cerambycids. In: Wang Q (ed) Cerambycidae of the world: biology and pest management. CRC press. Taylor & Francis, Boca Raton, pp 167–202

Millar JG, Mitchell RF, Mongold-Diers JA, Zou Y, Bográn CE, Fierke MK, Ginzel MD, Johnson CW, Meeker JR, Poland TM, Ragenovich I, Hanks LM (2017) Identifying possible pheromones of cerambycid beetles by field testing known pheromone components in four widely separated regions of the United States. J Econ Entomol. https://doi.org/10.1093/jee/tox312

Miller DR, Crowe CM, Mayo PD, Reid LS, Silk PJ, Sweeney JD (2017) Interactions between ethanol, *syn*-2,3-hexanediol, 3-hydroxyhexan-2-one, and 3-hydroxyoctan-2-one lures on trap catches of hardwood longhorn beetles in southeastern United States. J Econ Entomol 110:2119–2128. https://doi.org/10.1093/jee/tox188

Mitchell RF, Hughes DT, Luetje CW, Millar JG, Soriano-Agaton F, Hanks LM, Robertson HM (2012) Sequencing and characterizing odorant receptors of the cerambycid beetle *Megacyllene caryae*. Insect Biochem Mol Biol 42:499–505. https://doi.org/10.1016/j.ibmb.2012.03.007

Mitchell RF, Reagel PF, Wong JCH, Meier LR, Silva WD, Mongold-Diers J, Millar JG, Hanks LM (2015) Cerambycid beetle species with similar pheromones are segregated by phenology and minor pheromone components. J Chem Ecol 41:431–440. https://doi.org/10.1007/s10886-015-0571-0

Monné MA (2017) Catalogue of the Cerambycidae (Coleoptera) of the neotropical region. Part I: subfamily Cerambycinae http://cerambyxcat.com/Parte1_Cerambycinae.pdf. Accessed 10 Oct 2017

Narai Y, Zou Y, Nakamuta K, Mongold-Diers JA, Hanks LM, Millar JG (2015) Candidate aggregation pheromones of two potentially invasive Asian cerambycid species in the genus *Xylotrechus*. J Econ Entomol 108:1444–1446. https://doi.org/10.1093/jee/tov087

Newcombe RG (1998) Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med 17:857–872. https://doi.org/10.1002/(SICI)10970258(19980430)17:8<857::AID-SIM777>3.0.CO;2-E

Quinn GP, Keough MJ (2002) Experimental design and data analysis for biologists. Cambridge University Press, Cambridge

SAS Institute (2011) SAS/STAT 9.3 user's guide. SAS Institute Inc., Cary

Silva WD, Millar JG, Hanks LM, Bento JMS (2016a) 10-Methyldodecanal, a novel attractant pheromone produced by males of the south American cerambycid beetle *Eburodacrys vittata*. PLoS One 11:e0160727. https://doi.org/10.1371/journal.pone.0160727

Silva WD, Millar JG, Hanks LM, Bento JMS (2016b) (6*E*,8*Z*)-6,8-Pentadecadienal, a novel attractant pheromone produced by males of the cerambycid beetles *Chlorida festiva* and *Chlorida costata*. J Chem Ecol 42:1082–1085. https://doi.org/10.1007/s10886-016-0742-7

Silva WD, Zou Y, Bento JMS, Hanks LM, Millar JG (2017) Aggregation-sex pheromones and likely pheromones of 11 south American cerambycid beetles, and partitioning of pheromone channels. Front Ecol Evol 5:101. https://doi.org/10.3389/fevo.2017.00101

Sokal RR, Rohlf FJ (1995) Biometry, 3rd edn. W. H., Freeman, New York

Sweeney JD, Silk PJ, Grebennikov V (2014) Efficacy of semiochemical-baited traps for detection of longhorn beetles (Coleoptera: Cerambycidae) in the Russian far east. Eur J Entomol 111:397–406. https://doi.org/10.14411/eje.2014.049

Wickham JD, Harrison RD, Lu W, Guo Z, Millar JG, Hanks LM, Chen Y (2014) Generic lures attract cerambycid beetles in a tropical montane rain forest in southern China. J Econ Entomol 107:259–267. https://doi.org/10.1603/EC13333