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ORIGINAL PAPER

Pheromone identification by proxy: identification of aggregation-sex pheromones of North American cerambycid beetles as a strategy to identify pheromones of invasive Asian congeners

Jocelyn G. Millar[1] · Austin B. Richards[2] · Sean Halloran[1] · Yunfan Zou[1] · Elizabeth A. Boyd[3] · Kristin N. Quigley[3] · Lawrence M. Hanks[4]

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Abstract

Research over the past decade has shown that attractant pheromones used by cerambycid beetles are often highly conserved, with the same compound being used as a pheromone component by multiple related species, even among species on different continents which have been separated for millions of years. We describe how this conservation of structures can be exploited to identify possible pheromone components for Asian target species with a high risk of invading North America. Thus, collection and analysis of volatiles from five North American species, *Semanotus amethystinus* (LeConte), *Semanotus ligneus* (F.), *Semanotus litigiosus* (Casey), *Callidium antennatum hesperum* Casey, and *Callidium pseudotsugae* Fisher, showed that males of the two *Callidium* species sex-specifically produced 3-hydroxyhexan-2-one, a previously known cerambycid pheromone component, along with a novel natural product, 1-(1*H*-pyrrol-2-yl)-1,2-propanedione (henceforth pyrrole). In contrast, males of the three *Semanotus* species produced only the pyrrole. In field bioassays, both sexes of *C. antennatum hesperum* were significantly attracted to the blend of the two compounds, and *S. amethystinus* were equally attracted to the pyrrole alone, or the blend of the two compounds. Even before completing field bioassays with these species in California, field testing of the two compounds in Japan and China had revealed that several related target species, including the invasive *Callidiellum rufipenne* (Motschulsky), *Callidiellum villosulum* (Fairmaire), and *Semanotus bifasciatus* (Motschulsky), were attracted to one or both compounds (data reported elsewhere), providing proof of concept of the "pheromone identification by proxy" strategy.

Keywords 1-(1*H*-pyrrol-2-yl)-1,2-propanedione · 3-Hydroxyhexan-2-one · Biosynthetic parsimony · Monitoring

Key messages

- Because pheromones are often conserved among related cerambycids, identifying pheromones for one species can enable identification of pheromones for related taxa, even native to different continents.
- This strategy can be exploited to identify pheromones for invasive species which may be difficult or expensive to study in their native countries, or in quarantine.
- We demonstrate proof of concept by identifying pheromones for North American cerambycids in the genera *Semanotus* and *Callidium*, which enabled identification of pheromones for related invasive Asian species.

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✉ Jocelyn G. Millar
millar@ucr.edu

[1] Department of Entomology, University of California, 900 University Ave, Riverside, CA 92521, USA

[2] Aquatic Bioassessment Laboratory, California State University, Chico, CA 95929, USA

[3] College of Agriculture, California State University, Chico, 400 West First Street, Chico, CA 95929, USA

[4] Department of Entomology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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Introduction

Cerambycid beetles have considerable potential to become invasive species because their long-lived wood-boring larvae are often moved to new countries and continents by international commerce, hidden in finished wooden products, or wooden packing cases and dunnage (Eyre and Haack 2017). As such, Asian species such as the Asian longhorned beetle *Anoplophora glabripennis* (Motschulsky) (Haack et al. 2010), *Callidiellum rufipenne* (Motschulsky) (Zou et al. 2016), and *Trichoferus campestris* (Faldermann) (Bullas-Appleton et al. 2014) have recently invaded and become established in North America and Europe. Other Asian species with major potential to invade, such as *Aromia bungii* (Faldermann), *Callidiellum villosulum* (Fairmaire), and *Anoplophora chinensis* (Forster), have not yet become established in North America (Eyre and Haack 2017).

The US Department of Agriculture's Animal and Plant Health Inspection Service (USDA-APHIS) has assembled a list of Asian species that are considered likely to invade and become significant pests in North America due to the availability of susceptible tree species (USDA-APHIS 2017). Consequently, APHIS is supporting proactive efforts to identify semiochemical attractants for invasive species on their target list, to strengthen surveillance efforts to detect new invasions as soon as possible, and for use in eradication efforts should a new species become established. However, identification of pheromones or related semiochemical attractants for species which are not yet present in continental North America presents substantial logistical problems. For example, pheromones may be identified by collaborating with research groups in a target insect's region of origin, but such efforts may be hampered by lack of appropriate equipment or expertise. Alternatively, targeted species can be imported, maintained, and studied in a quarantine facility in North America, but such efforts are extraordinarily costly and labor-intensive, and may require both export and import permits.

Because of these drawbacks, we explored a new strategy, "pheromone identification by proxy," hoping to exploit the biosynthetic parsimony of cerambycid pheromones. Specifically, pheromone structures are often highly conserved among congeneric species, and even to tribal or higher taxonomic levels (Hanks and Millar 2016). For example, (*R*)-3-hydroxyhexan-2-one is a known or suspected pheromone component for numerous species within at least 25 genera in 10 tribes in the cerambycid subfamily Cerambycinae, and 2-(undecyloxy)ethanol (monochamol) is a known or suspected pheromone for species in five genera in three tribes in the subfamily Lamiinae (reviewed in Hanks and Millar 2016). Furthermore, some pheromone structures appear to have been conserved among related species that have been geographically separated for millions of years. For example, (*R*)-3-hydroxyhexan-2-one has been shown to be a pheromone or likely pheromone for cerambycid species native to all continents except Antarctica (Hanks and Millar 2016).

Thus, we reasoned that if we could identify pheromones from comparatively readily accessible North American cerambycid species, there would be a reasonable chance that these compounds or very similar structures would also be pheromone components of their close Asian relatives, such as species in the same genera or possibly tribe. The North American taxa that we targeted included several species in the genera *Semanotus* and *Callidium*, as proxies for Asian target species in the genera *Semanotus* and *Callidiellum*, such as *S. bifasciatus* (Motschulsky), *C. rufipenne*, and *C. villosulum*.

We report here the identification and bioassay of pheromones or likely pheromones for five North American species in the subfamily Cerambycinae, tribe Callidiini, including *Callidium antennatum hesperum* Casey and *Callidium pseudotsugae* Fisher, and *Semanotus amethystinus* (LeConte), *Semanotus ligneus* (F.), and *Semanotus litigiosus* (Casey). The pheromones consist of 1-(1*H*-pyrrol-2-yl)-1,2-propanedione (henceforth pyrrole), alone or in combination with (*R*)-3-hydroxyhexan-2-one. The identification of these compounds subsequently enabled the identification of pheromones or likely pheromones for the target Asian species *C. rufipenne* (described in Zou et al. 2016) and *C. villosulum* (described in Wickham et al. 2016), both of which use blends of pyrrole + 3-hydroxyhexan-2-one as their pheromones, and *S. bifasciatus*, for which the pyrrole alone was identified as an attractant (Wickham et al. 2016).

Materials and methods

Sources of chemicals

Racemic 3-hydroxyhexan-2-one (henceforth 3-ketol) was purchased from Bedoukian Research (Danbury, CT, USA), and the pyrrole was synthesized as described by Zou et al. (2016). Standards of other minor components found in some of the extracts of *Semanotus* species were obtained from the following sources: benzyl alcohol, 2-heptanone, 2-octanone, 2,3-butanediol from Sigma-Aldrich (Milwaukee, WI, USA); benzaldehyde and 1-hexanol from Alfa-Aesar (Ward Hill, MA, USA); acetoin from TCI America (Portland, OR, USA).

Sources of insects for collection of insect-produced volatiles

Live specimens of the target species for this study were collected from various sites in northern California, either

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directly in the field or reared out of infested branches of host trees. *Callidium pseudotsugae* and *S. litigiosus* were both reared from branches of white fir (*Abies concolor* [Gordon]), *C. a. hesperum* was reared from Douglas fir (*Pseudotsugae menziesii* [Mirb.] Franco), and *S. amethystinus* was reared from incense cedar (*Calocedrus decurrens* Torr.). All specimens of *S. ligneus* were hand-collected from standing *C. decurrens* and several specimens of *S. litigiosus* were hand-collected from *A. concolor*. Rearing chambers consisted primarily of 19-l plastic buckets or similar containers with mesh-covered holes in the lids for ventilation. Each container contained branches cut from a single host tree at a single collection site, to retain the host-to-beetle association. Containers were checked daily or more frequently for freshly emerged beetles. The beetles were removed from the containers before they could mate and placed singly into 9- or 25-dram plastic tubes (Bioquip, Rancho Dominguez CA, USA) for transport. It is unknown whether the field-caught beetles had mated prior to capture.

Beetles were shipped to UC Riverside by overnight courier. Individual beetles were placed in modified, ~ 0.5-l wide-mouth screw-cap canning jars (one per jar), with a vial of 10% sugar water provided for nutrition. The metal lids of the jars were replaced with Teflon circles fitted with Swagelok bulkhead unions (Swagelok, Solon OH, USA) to connect inlet and outlet tubes. The jars were flushed with charcoal-filtered air (500 ml/min), and odors released by the beetles were trapped on activated charcoal collectors made from ~ 10-cm-long glass tubes (0.5 cm ID) with a 1-cm-long bed of activated charcoal (50–200 mesh; Fisher Scientific, Pittsburgh, PA, USA) held in place by glass wool plugs. A collector was attached to the outlet of each aeration chamber via one of the two bulkhead unions. Collectors were changed every 24 h, with the trapped volatiles being recovered by elution of the collectors with methylene chloride (0.5 ml).

In addition, for *S. litigiosus*, beetles that had been reared out of naturally infested conifer logs were aerated in groups of 20 beetles of each sex. The group of males was aerated twice for 24 h, and the group of females was aerated three times for 24 h.

Adult *C. a. hesperum* caught in pheromone traps in the San Bernardino National Forest during bioassays in spring 2017 were transported in coolers to UC Riverside. The beetles were sexed and placed into 15-cm-diameter plastic rearing cages along with sugar water and a wet cotton ball. Within 24 h of collection, males and females were separately placed into ~ 0.5-l canning jars, modified as described above, for aerations. *Callidium a. hesperum* were either aerated individually or in groups of 3 or 6, by pumping charcoal-filtered air into the jars and out through an activated charcoal collector (250 ml/min). Trapped volatiles were eluted as described above. A total of three 24-h aerations of females and nine 24-h aerations of males were carried out.

Identification of compounds from headspace volatiles

Extracts of volatiles were analyzed by coupled gas chromatography–mass spectrometry (GC–MS) in splitless mode, using an Agilent 7820A GC interfaced to a 5977E mass selective detector (Agilent Technologies, Santa Clara, CA, USA). The GC was equipped with an HP-5 column (30 m × 0.25 mm i.d. × 0.25 µm film; Agilent Technologies), with helium carrier gas (37 cm/s linear velocity) and a temperature program of 40 °C/1 min, 10 °C/min to 280 °C, hold for 10 min). High-resolution mass spectra were taken on a Waters GCT GC–MS instrument (Waters Corp., Milford, MA, USA).

Extracts from the two *Callidium* species that contained 3-hydroxyhexan-2-one (see Results) also were analyzed on a chiral stationary phase Cyclodex B column (30 m × 0.25 mm ID, 0.25 µm film; J&W Scientific, Folsom, CA, USA) in split mode, programmed from 50 ℃/1 min, 3 ℃ per min to 220 ℃. The injector temperature was set at 125 ℃ to minimize thermal isomerization of the 3-hydroxyhexan-2-one to the regioisomeric 2-hydroxyhexan-3-one (Sakai et al. 1984).

To isolate sufficient pyrrole for nuclear magnetic resonance (NMR) analysis, two aeration extracts from male *C. a. hesperum* and an aeration extract from five male *C. pseudotsugae* were combined and concentrated to ~ 20 µl under a gentle stream of nitrogen. Two aliquots of 10 µl of the concentrated extract then were fractionated by preparative GC (2 m × 2 mm ID glass column, 10% SP-1000 on 80/100 mesh Supelcoport, head pressure 240 kPa). The split ratio between the GC's flame ionization detector and the heated collection port (200 ℃) was ~ 28:1, and the oven was programed from 50 ℃/1 min, 10 ℃/min to 200 ℃, hold 30 min). Fractions were collected in glass capillary tubes chilled with dry ice. After warming to room temperature, the tubes containing the desired compound were rinsed into a conical-bottomed vial with ~ 25 µl of deuterated methylene chloride, and an aliquot of the resulting yellow solution was transferred into a microbore NMR tube. Proton NMR spectra were recorded on a Bruker Avance 600 instrument at 600 MHz (Bruker Biospin, Fremont, CA, USA).

Bioassays of synthesized compounds

Field bioassays in the Sierra Nevada mountains of northern California were carried out from April 19 to September 4, 2016. Three sites were chosen based on the presence of host trees for the target beetle species. The first site was located on ABR's property in Magalia (Butte Co.) [39°50′19″N 121°37′01″W, 730 m]. This site was dominated by black oak (*Quercus kelloggii* Newb.), with incense cedar, ponderosa pine, and Douglas fir also present. The second site was located in Forest Ranch (Butte Co.), off highway

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CA-32 [39°52′42″N 121°40′34″W, 720 m]. Incense cedar and ponderosa pine were abundant, but black oak was also present. The final site was a large property near Childs Meadows (Tehama Co.), off highway CA-36 [40°20′42″N 121°29′06″W, 1490 m]. White fir, incense cedar, ponderosa pine, and lodgepole pine (*Pinus contorta* Douglas) were all present.

Field bioassays tested the attraction of the various species to the pyrrole alone, racemic 3-ketol alone, and the pyrrole blended with racemic 3-ketol, versus a solvent control. The racemic 3-ketol (50 mg/ml) and pyrrole (25 mg/ml) were diluted in isopropanol. Solvent controls consisted of isopropanol only. Each treatment was randomly assigned and deployed singly in a black cross-vane intercept trap (Alpha Scents, Portland, OR, USA) coated with fluon® (Bioquip, Rancho Dominguez, CA, USA). Once a treatment was assigned to a trap, no other treatment was used with that trap, to avoid contamination. The Magalia and Forest Ranch sites each consisted of one set of the four treatments (= 1 block). Two blocks of traps, eight traps total, were deployed at the Childs Meadows site. All traps within a block were hung at least 20 m apart. The two blocks at the Childs Meadows site were separated by 90 m. To prevent damage from bears, all traps were hung roughly 3–5 m above the ground. Lures were prepared by pipetting 1 ml of a test solution into a 5 × 7.5 cm low-density polyethylene resealable bag (2 mil wall thickness; Fisher Scientific, Pittsburgh, PA, USA). Loaded bags were then hung from a wire hook in the center of the trap vanes. A fluon-coated bucket was placed below the cross-vanes to serve as a collection container for beetles captured in the traps. The buckets contained propylene glycol as a field preservative. Beetles and other captured insects were removed from the traps ~ weekly (shortest interval = 6 days, longest interval = 9 days), and lures were replaced biweekly. Captured beetles were transferred into 70% ethanol to preserve them.

Field bioassays in the San Bernardino National Forest in southern California were carried out from March 15 to May 11, 2017. Two sites were chosen, both located approximately 5–8 km north of Crestline, CA (San Bernardino Co.), USA, along highway CA-138 [34°15′55.3″N 117°17′30.0″W]. Both sites contained a mixture of tree species, with the majority being ponderosa pine along with white fir and incense cedar. At each site, four intercept traps coated with fluon® were hung from each tree species at a height of ~ 2 m, for a total of 12 traps per site. For each tree species the traps were placed in a straight transect roughly 15 m apart. Beetles were collected in fluon®-coated jars hung below the cross-vanes. Beetles were live trapped so that they could be used for collection of volatiles and/or electrophysiological recordings.

For each of the three tree species, treatments consisted of a solvent blank, racemic 3-ketol alone, the pyrrole alone, or a mixture of both racemic 3-ketol and the pyrrole. The racemic 3-ketol (50 mg/ml) and pyrrole (25 mg/ml) were diluted in ethanol. Solvent controls consisted of ethanol only. Lures were prepared by placing 1 ml of test solutions into 5 × 7.5 cm plastic bags as described above. Lures were replaced biweekly, and traps were checked multiple times each week. Within each tree species transect, each of the four treatments was assigned randomly, and after each count the traps were rotated to the next tree along the transect. Captured beetles were counted and sexed by gently pressing on the abdomen to extrude the genitalia.

Statistical analyses

For each bioassay, differences between treatment means, blocked by site and date, were tested separately for each species using the nonparametric Friedman's test (PROC FREQ, option CMH; SAS Institute 2011) because data violated homoscedasticity assumptions of ANOVA (Sokal and Rohlf 1995). Thus, replicates were defined by study site and collection date. Data for the three experiments in northern California were combined, because the methods were identical. Assuming a significant overall Friedman's test, pairs of treatment means were compared with the REGWQ means separation test, which controls for maximum experimentwise error rates (PROC GLM, SAS Institute 2011). Replicates in which no specimens of the species in question were caught, for example due to inclement weather, were excluded from analyses.

Results

Identification of compounds from headspace volatiles (Table 1)

Analysis of extracts from beetles of the *Callidium* and *Semanotus* species revealed that extracts from males of the *Callidium* species contained essentially two compounds, one of which was also present in the *Semanotus* extracts. The earlier eluting compound in the *Callidium* extracts was readily identified from its mass spectrum as the well-known cerambycid pheromone component 3-ketol, and the identification was confirmed by matching its mass spectrum and retention time on two GC columns (HP-5 and Cyclodex B) with that of an authentic standard. Analysis on the latter column determined that the insects produced only (*R*)-3-hydroxyhexan-2-one. The ratio of the 3-ketol to the other compound in the extracts (mean ± SD) was 1.3 ± 0.32 for *C. pseudotsugae* ($N = 7$) and 1.02 ± 0.24 ($N = 6$) for *C. a. hesperum*.

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Table 1 Compounds detected in aeration extracts from beetles of five species in the subfamily Cerambycinae native to California, USA. Compounds: Pyrrole = 1-(1*H*-pyrrol-2-yl)-1,2-propanedione, 3*R*-ketol = (*R*)-3-hydroxyhexan-2-one

Species	# of aerations/(# with compounds)	Producing sex	Pyrrole	3*R*-ketol	Other compounds
S. amethystinus	2 (2)	Male	Yes	No	–
S. ligneus	12 (4)	Unknown	Yes	No	Acetoin, 1-hexanol, 2,3-butanediol
S. litigiosus	8 (4)	Male[a]	Yes	No	Acetoin, benzyl alcohol, benzaldehyde, 1-hexanol, 2-heptanone, 2-octanone
C. a. hesperum	9 (6)	Male	Yes	Yes	–
C. pseudotsugae	8 (8)	Male	Yes	Yes	–

[a]Determined from comparisons of two aerations of 20 adult males and three aerations of 20 adult females that were reared from logs in the laboratory

The second compound, present in extracts from all five species, had a relatively simple mass spectrum consisting primarily of three ions, including a likely molecular ion at *m/z* 137 (30% of base peak), a base peak at *m/z* 94 (100%), and an ion at *m/z* 66 (43%). The closest match in the NIST mass spectral database was to 1-(1*H*-pyrrol-2-yl)-ethanone, the mass spectrum of which also had a base peak at *m/z* 94 and an abundant ion at *m/z* 66 (50%), and a strong molecular ion at *m/z* 109, 28 mass units less than the unknown pyrrole. These data, coupled with the fact that the *m/z* 94 ion in the spectrum of 1-(1*H*-pyrrol-2-yl)-ethanone was likely due to the pyrrole ring with a carbonyl attached, suggested that the unknown could be either the analog, 1-(1*H*-pyrrol-2-yl)-butanone, or 1-(1*H*-pyrrol-2-yl)-1,2-propanedione. The high-resolution mass spectrum gave a molecular weight of 137.0476, corresponding to a molecular formula of $C_7H_7NO_2$ (calc. 137.0471), supporting the latter structure.

To provide a sample sufficient for NMR analysis, the extracts from male *C. pseudotsugae* and male *C. a. hesperum* that contained the largest amounts of the unknown were combined and the compound was purified by preparative GC, producing an estimated several tens of micrograms as yellow crystals on the inside of the collection tube. The proton NMR of the isolated compound confirmed the 1-(1*H*-pyrrol-2-yl)-1,2-propanedione structure, with a broad singlet at 9.93 for the proton on the pyrrole nitrogen, three individual protons at 7.30 (br s), 7.19 (br s), and 6.36 ppm (m) corresponding to the three sp^2 protons on the pyrrole ring, and a 3-proton methyl singlet at 2.46 ppm, shifted downfield by the vicinal carbonyl group. The structure was confirmed by synthesis (described in Zou et al. 2016).

The extracts from the two *Callidium* species contained essentially only the 3-ketol and pyrrole compounds, whereas the extracts from the *Semanotus* species contained several additional compounds (Table 1), in amounts that varied among aerations.

Bioassays of synthesized compounds

In a bioassay conducted in northern California, adults of *S. amethystinus* were significantly attracted to the pyrrole alone and the blend of the pyrrole with racemic 3-ketol, but not to 3-ketol alone (Fig. 1; means significantly different; Friedman's $Q_{3,112} = 46.4$, $P < 0.0001$). These results suggested that the pyrrole is the major if not sole component of the pheromone of this species and that attraction was not inhibited by the presence of the 3-ketol. In addition, 15 *C. pseudotsugae* were caught during this bioassay, with all 15 specimens being captured in two traps baited with the blend of 3-ketol and pyrrole. Despite all specimens being attracted to one treatment, the results for this species should be treated with caution because 14 of the 15 beetles captured were caught in one trap on a single date.

In a bioassay targeting *C. a. hesperum*, conducted in the San Bernardino mountains of southern California with the same test treatments, adults were only significantly attracted to the blend of 3-ketol + pyrrole (Fig. 2a; $Q_{3,308} = 200.0$, $P < 0.0001$). Substantial numbers of *Neoclytus conjunctus* (LeConte) also were caught in this trial, with similar numbers in traps baited with the 3-ketol alone and the blend of 3-ketol + pyrrole (Fig. 2b; $Q_{3,144} = 71.3$, $P < 0.0001$).

Despite the fact that the pyrrole was the major component of the volatiles collected from males of *S. litigiosus* and *S. ligneus*, only five specimens of *S. litigiosus* were caught during the field trials in southern California, with no discernable pattern to the catches, and no *S. ligneus* at all were caught.

Discussion

We have presented data showing that the pyrrole is a pheromone component for at least two North American cerambycid species in the genera *Semanotus* and *Callidium*, and a likely pheromone component for two additional *Semanotus*

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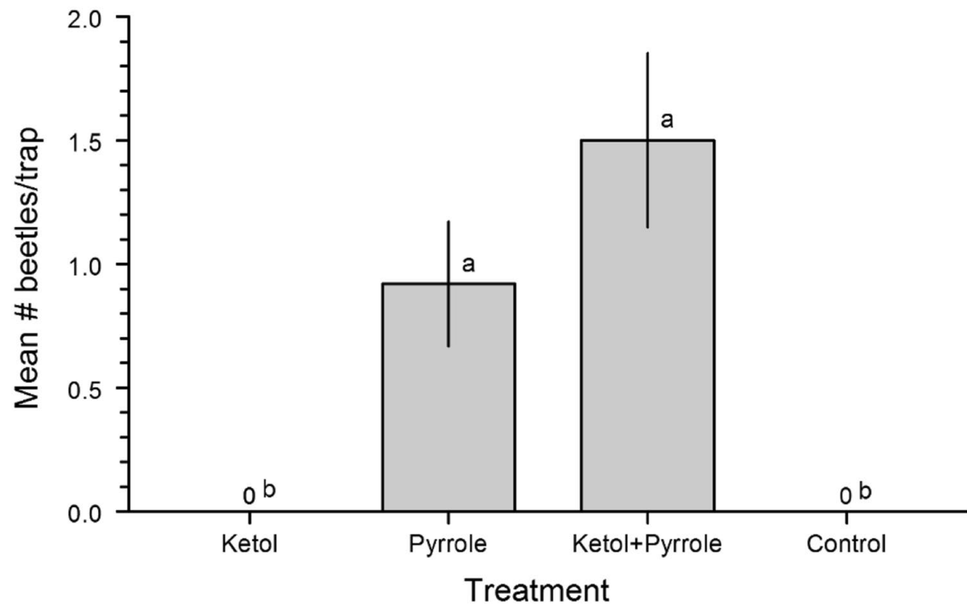

Fig. 1 Mean (± SE) number of adults of *Semanotus amethystinus* captured per replicate during a field bioassay of synthesized candidate pheromones in the Sierra Nevada mountains of northern California. Data were from 24 replicates, from June 7 to August 27, 2016. Compounds: Ketol = racemic 3-hydroxyhexan-2-one, Pyrrole = 1-(1*H*-pyrrol-2-yl)-1,2-propanedione, Ketol + Pyrrole = blend of the two compounds, Control = solvent control (isopropanol). Means with different letters are significantly different (REGWQ means separation test, $P < 0.05$)

and one additional *Callidium* species, based on the fact that it is present in volatiles produced sex-specifically by males of the latter three species. For *S. amethystinus*, the pyrrole was attractive as a single component, whereas for *C. a. hesperum*, the optimal attractant consisted of a blend of the pyrrole with the previously known 3-ketol, with neither compound being attractive alone. The pheromone of the other species caught in substantial numbers during these trials, *N. conjunctus*, had been previously identified as (*R*)-3-hydroxyhexan-2-one (Ray et al. 2015), so its attraction to the 3-ketol lures was expected. However, it was useful to know that attraction of this species to the ketol was not antagonized by the presence of the pyrrole. Thus, other mechanisms, such as differences in diel activity patterns between *N. conjunctus* and sympatric species such as *Callidium* species which also produce 3-ketol as part of their pheromone, are likely operative to minimize unproductive cross-attraction to heterospecifics. Such diel partitioning is known to occur for other sympatric cerambycid species which share pheromone components (e.g., Mitchell et al. 2015).

A total of five *S. litigiosus* were caught in the field trials in southern California, indicating that the beetles were indeed present at the sites where and when the trials were deployed. There are at least two possible reasons for the low trap catches of this species. First, as shown in Table 1, aeration extracts from *S. litigiosus* males contained a number of other compounds besides the pyrrole, and any one or some combination of those compounds may be crucial additional pheromone components for this species. For example, 2-nonanone has been shown to be a crucial minor component in the pheromone blend of the cerambycine species *Cyrtophorus verrucosus* (Olivier) (Mitchell et al. 2015). Second, it is well established that the pheromones of many cerambycid species (Hanks and Millar 2016), and particularly those that infest conifers (Collignon et al. 2016), are strongly synergized by host plant volatiles. In addition, for *S. ligneus*, for which no specimens were caught in bioassays in southern California, this species may not have been present at the sites and/or during the time periods that the field experiments were deployed. To date, we have not been able to conduct further field trials to test these possibilities due to the limited amount of pyrrole which we have been able to synthesize.

Due to the timing of the identification and synthesis of the pyrrole in relation to the field seasons of the North American species, the logistics of deploying bioassays, and the priorities of the agency funding the research (USDA-APHIS), the first trials of the pyrrole as a possible cerambycid pheromone component were actually carried out in Japan and China rather than in North America. Both trials resulted in immediate successes. In Japan, the target Asian species *C. rufipenne* was strongly attracted to a blend of pyrrole + 3-ketol, and these data were corroborated by subsequent field trials in eastern North America (previously reported in Zou et al. 2016), where this species has invaded and become established. In China, *C. villosulum* was similarly attracted to a blend of pyrrole + 3-ketol, whereas *S. bifasciatus* was attracted to the pyrrole as a single component (previously reported in Wickham et al. 2016). Furthermore, two additional Asian species were attracted in the same trial, *Xylotrechus buqueti* (Castelnau & Gory) (tribe Clytini) specifically to a blend of pyrrole with 3-ketol, and *Allotreus asiaticus* (Schwarzer) (tribe Phoracanthini) specifically to the pyrrole alone. It is particularly noteworthy that the latter two species are not only in different genera than the other species, but in different tribes within the Cerambycinae, demonstrating

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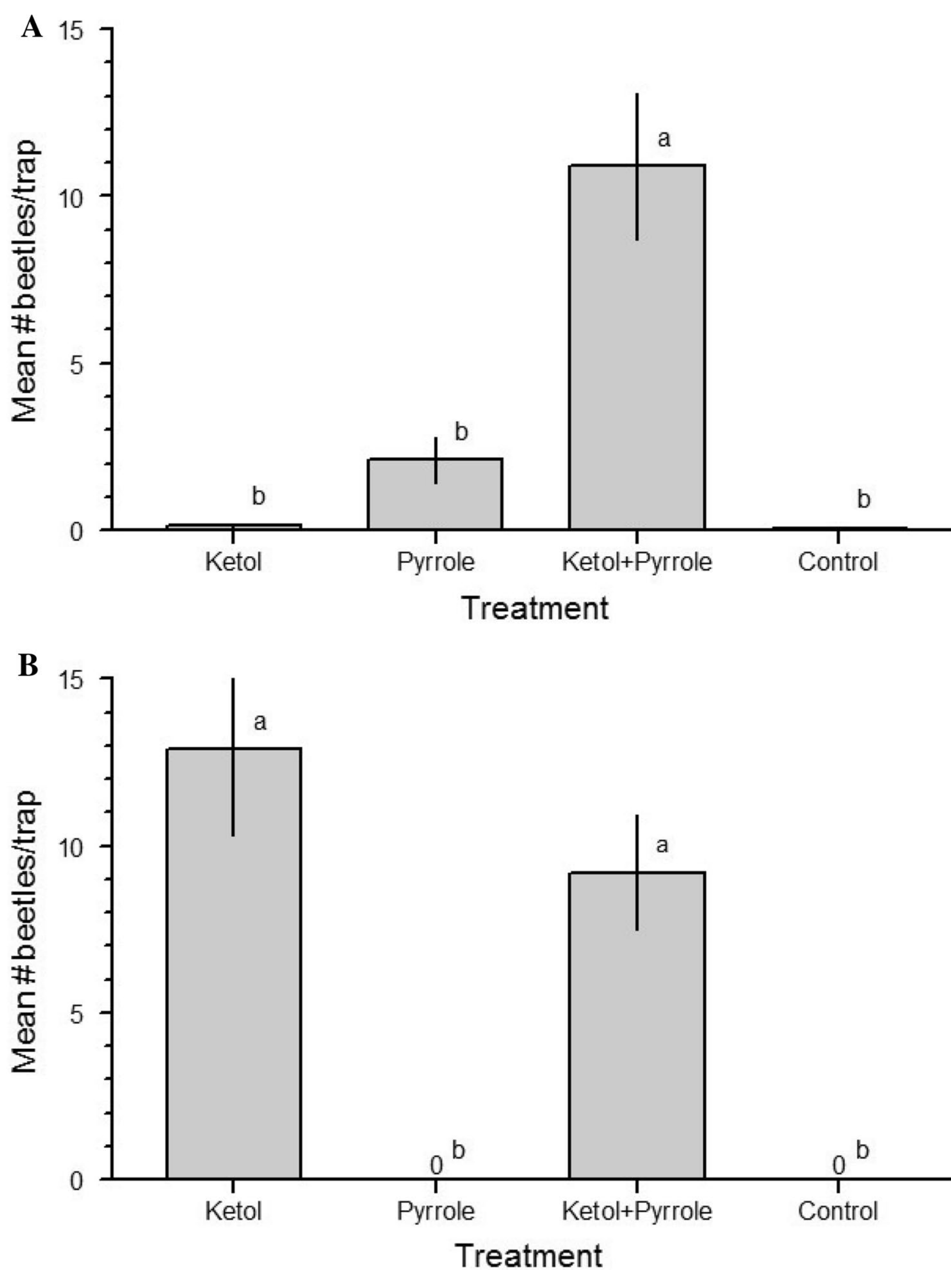

Fig. 2 Mean (± SE) number of adults of **a** *Callidium antennatum hesperum* and **b** *Neoclytus conjunctus* captured during a field bioassay of synthesized candidate pheromones in the San Bernardino mountains of southern California. For *C. a. hesperum*, data were from 36 replicates, from March 15 to May 11, 2017. For *N. conjunctus*, data were from 77 replicates, from March 8 to May 11, 2017. Compounds: Ketol = racemic 3-hydroxyhexan-2-one, Pyrrole = 1-(1*H*-pyrrol-2-yl)-1,2-propanedione, Ketol + Pyrrole = blend of the two compounds, Control = solvent control (ethanol). Means within species with different letters are significantly different (REGWQ means separation test, $P < 0.05$)

how widely conserved these structures are within related taxonomic groups. In fact, the pyrrole has now been found as a pheromone component in another North American species, *Dryobius sexnotatus* Linsley (tribe Dryobiini; Diesel et al. 2017), as well as in South American species in the genus *Ambonus* (tribe Elaphidiini; Silva et al. 2017). Thus, to date, the pyrrole has been shown to be a pheromone component for species on three continents in at least five different tribes suggesting that, similar to the 3-ketol, it represents a highly conserved pheromone structure within the Cerambycinae.

In sum, the results described above provide strong support for the "pheromone identification by proxy" strategy as a means of developing good leads for the identification of pheromones of exotic species which may not be easily accessible for research. Since the inception of this project, we have found at least one additional successful example of this strategy, in the identification of 3-methylthiopropanol as a pheromone component of several South American cerambycids (Silva et al. 2017). With this background information, we then recently found 3-methylthiopropanol, and a related analog, in volatiles produced by two North American species (JGM, unpub. data). The two compounds will be tested with these and other North American species during the upcoming field season.

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Author contributions

JGM organized the study, isolated and identified the pyrrole, and wrote the first draft of the manuscript. ABR collected the study species and assisted with northern California field trials. SH organized and carried out the field trials in the San Bernardino mountains, CA, USA. EAB funded KNQ, assisted with acquisition of field sites and, with KNQ and ABR, assisted with organization, deployment, and data collection for northern California field trials. YZ synthesized the pyrrole. LMH designed and executed the statistical analyses and, with JGM, obtained the grants that funded the project. All authors read, reviewed, and contributed to the final manuscript draft.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The laboratory research was conducted at the University of California, Riverside, and field bioassays were conducted at field sites in the Sierra Nevada mountains near Chico, California, and in the San Bernardino mountains of southern California. All methods met the ethical requirements of the respective universities and followed guidelines of the Committee of Publication Ethics.

Research involving human participants and/or animals This article does not involve any studies with human participants or animals.

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