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SHORT COMMUNICATION

Blends of (*R*)-3-hydroxyhexan-2-one and alkan-2-ones identified as potential pheromones produced by three species of cerambycid beetles

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Abstract We present data indicating that three species of cerambycid beetles (subfamily Cerambycinae) produce the common cerambycine pheromone component (*R*)-3-hydroxyhexan-2-one as well as an alkan-2-one component, a possible new motif for cerambycid pheromone components. GC/MS analyses of headspace volatiles produced by male beetles indicated that *Cyrtophorus verrucosus* (Olivier) produced (*R*)-3-hydroxyhexan-2-one but also nonan-2-one at ~18 % of the hydroxyketone component, whereas *Orwellion gibbulum arizonense* (Casey) and *Parelaphidion aspersum* (Haldeman) produced decan-2-one at ~40 and 7 % of the amount of the hydroxyketone, respectively. In field bioassays, adult *C. verrucosus* were attracted by (*R*)-3-hydroxyhexan-2-one alone, but attraction was significantly enhanced by nonan-2-one. This effect was lost if the quantity of nonan-2-one exceeded 100 % of the hydroxyketone, suggesting that beetles could discern ratios of the two chemicals and were most strongly attracted to those approximating the blend produced by males. We suggest that nonan-2-one plays a role in the species specificity of the pheromone signal of *C. verrucosus*, and that decan-2-one plays a similar role in the semiochemical communication of *O. g. arizonense* and *P. aspersum*.

Keywords Sex pheromone · Reproductive isolation · Longhorned beetle · Nonan-2-one · Decan-2-one

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Introduction

Pheromone chemistry appears to be remarkably conserved among closely related and even quite distantly related species of cerambycid beetles, to the extent that some species produce a single chemical that potentially could attract many other species (e.g., Barbour et al. 2011; Hanks et al. 2012; Hanks and Millar 2012; Macias-Samano et al. 2012). For example, (*R*)-3-hydroxyhexan-2-one (hereafter "3*R*-ketone") appears to be the sole or primary component of aggregation pheromones for many sympatric and synchronic species in the subfamily Cerambycinae, with the result that traps baited with the synthetic compound capture multiple species (e.g., Hanks and Millar 2012; Hanks et al. 2007, 2012; Lacey et al. 2007, 2009; Wong et al. 2012). Species may avoid cross attraction by differential responses to certain host plant volatiles in combination with the pheromone, or by differing in seasonal or circadian activity periods (e.g., Hanks et al. 2012). Species specificity of pheromone signals also might be maintained by minor pheromone components. For example, males of the cerambycine *Phymatodes lengi* Joutel produce 3*R*-ketone, but neither sex is attracted to the synthetic compound unless it is released along with a second component, (*R*)-2-methylbutan-1-ol (Hanks et al. 2012). Similarly, the cerambycids *Xylotrechus colonus* (F.) and *Sarosesthes*

fulminans (F.) produce 3*R*-ketone and are attracted to traps baited with the synthetic compound, but species specificity may be imparted by the different isomers of 2,3-hexanediol that males produce in lesser quantities (Lacey et al. 2009; Hanks and Millar 2012).

Here, we summarize research on the pheromones of three North American cerambycine species: *Cyrtophorus verrucosus* (Olivier) (Tribe Anaglyptini), *Orwellion gibbulum arizonense* (Casey), and *Parelaphidion aspersum* (Haldeman) (both Elaphidiini) (taxonomy per Monné and Bezark 2012). The pheromone chemistry of these beetles has not previously been described, although *C. verrucosus* is attracted to traps baited with 3*R*-ketone (Hanks and Millar 2012). We provide evidence that males of all three species produce 3*R*-ketone as a likely pheromone component, but additionally produce nonan-2-one or decan-2-one as minor components of their pheromone blends.

Materials and methods

Source of synthetic pheromones

3*R*-ketone and (*S*)-3-hydroxyhexan-2-one ("3*S*-ketone") were synthesized as described in Lacey et al. (2007), racemic 3*RS*-ketone was synthesized as described in Imrei et al. (2013), and nonan-2-one (99 % purity) and decan-2-one (>99 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and TCI America (Portland, OR, USA), respectively.

Pheromone identification

Pheromone-baited black cross-vane panel traps (Alpha-Scents, Portland, OR, USA) (for details, see Graham et al. 2010) were used to obtain live males and females of the three species for collection and analysis of insect-produced volatiles. Pheromone lures consisting of polyethylene sachets (Bagettes™ model 14770, 5.1 × 7.6 cm; Cousin Corp., Largo, FL, USA) loaded with 50 mg 3*RS*-ketone in 1 ml ethanol were suspended in the center of each trap. Adult *P. aspersum* were trapped during August 2008 at the municipal Landscape Recycling Center of Urbana, IL (Champaign Co.; 40°7′19.23″N, 88°10′44.79″W), a recycling facility for woody plant material. Adult *C. verrucosus* were trapped at Forest Glen Preserve, IL (Vermilion Co., IL; 40°0′51.97″N, 87°34′0.74″W) during May 2009. A single male *O. g. arizonense* was trapped during June 2009 in a private lumber yard south of Stephenville, TX (Erath Co., 32°9′25.77″N, 98°11′45.65″W), which contained stacked logs of many species of native trees.

Specimens of *P. aspersum* and *O. g. arizonense* were sexed by the relative length of the antennae (Linsley 1963; both under former genus name *Elaphidionoides*). Male and female *C. verrucosus* are very similar in morphology, so sexes were confirmed by briefly holding pairs of beetles in cages and observing mating behavior (i.e., males mounting females). Females and males of the three species were housed separately in the laboratory in cylinders of aluminum window screen (9 cm diameter, ~10 cm height) under laboratory conditions (12:12 h L:D, ~20 °C) and provided with 10 % aqueous sucrose solution dispensed from 8 ml vials plugged with cotton rolls. Beetles were allowed at least 3 days to acclimate prior to being used for headspace collections.

Volatiles produced by beetles were collected using the Mason-style canning jar chambers described in Ray et al. (2011), but collectors contained ~150 mg HayeSepQ (Sigma-Aldrich) and were connected to the aeration chamber and the vacuum source with Teflon® tubing (~8 cm long, 9.5 mm diameter). Headspace volatiles were collected for 24 h under natural light (aeration chambers positioned near closed windows; ~14:10 h L:D, ~20 °C). Insects usually were sampled individually, or in groups of two to three of the same sex to increase the chances of collecting pheromones. Control aerations without insects were run simultaneously to check for system contaminants. Collectors were extracted with 1.5 ml of dichloromethane into silanized glass vials that were stored at −20 °C. After each collection session, beetles were returned to cages for at least 24 h before being reused. Voucher specimens have been deposited in the collection of the Illinois Natural History Survey, Champaign, IL, USA.

Extracts were analyzed by coupled gas chromatography–mass spectrometry (GC/MS) with an HP 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) fitted with an AT-5 ms column (30 m × 0.25 mm i.d., 0.25 μm film; Alltech Associates, Inc., Deerfield, IL, USA) and coupled to an HP 5973 mass selective detector. Injector temperature was 250 °C, and oven temperature was programmed from 40 °C for 1 min, 10 °C/min to 210 °C, and held 3 min. Sex-specific peaks were tentatively identified by matching their mass spectra to the GC/MS database (National Institute of Standards and Technology, Gaithersburg, MD), and confirmed by comparing spectra and retention times with those of authentic standards.

The absolute configuration of the 3-hydroxyhexan-2-one produced by male beetles was determined with an HP 5890 GC fitted with a Cyclodex B column (30 m × 0.25 mm i.d., 0.25 μm film; Agilent Technologies, Inc., Santa Clara, CA, USA). Injector temperature was 110 °C, and oven temperature was held at 50 °C for 1 min, increased at 5 °C/min to 130 °C, and held 10 min. 3*R*- and 3*S*-ketone were resolved to baseline (retention times 15.04 and 15.78 min, respectively) and the identities of the

enantiomers were confirmed by co-injection of 3*S*-ketone with insect-produced samples.

Field bioassays of synthetic compounds

Field bioassays were conducted to evaluate the effect of nonan-2-one as a possible pheromone component for *C. verrucosus*. The surfaces and collecting buckets of the flight intercept traps (see above methods) were coated with Fluon® PTFE dispersion (AGC Chemicals Americas, Inc., Exton, PA) to enhance trap efficiency (Graham et al. 2010). Lures were as described above, except that synthetic pheromones were diluted in 1 ml isopropanol instead of ethanol, with controls being 1 ml of isopropanol. Traps were positioned 10 m apart in linear transects, and treatments were assigned randomly to traps on the first day, with one trap per treatment and one complete set of treatments per study site. Beetles were collected from traps at intervals of 1–3 days, at which time treatments were rotated within the transect to control for location effects. Lures were replaced as needed, usually every 5–7 days.

The biological activities of male-produced compounds of *C. verrucosus* were confirmed by testing synthetic blends in two independent field bioassays. Experiment 1, conducted at Forest Glen Preserve, tested the effect of nonan-2-one on activity of 3*R*-ketone, and whether the unnatural 3*S*-ketone enantiomer affected attraction to 3*R*-ketone. The experimental treatments consisted of various combinations of 3*R*-ketone (25 mg per lure), 3*RS*-ketone (racemic; 50 mg), and nonan-2-one (5 mg per lure), that simulated the relative amount of nonan-2-one in aeration extracts of males (~20 % of the amount of 3*R*-ketone), dissolved in 1 ml isopropanol, as follows: (1) 3*R*-ketone alone, (2) 3*RS*-ketone alone, (3) nonan-2-one alone, (4) 3*R*-ketone + nonan-2-one, (5) 3*RS*-ketone + nonan-2-one, and (6) isopropanol control. The experiment was conducted during 29th April to 1st June 2011 (average low temperature 11.3 °C, average high temperature 23.1 °C, total precipitation 11 cm; Weather Underground, Inc., Ann Arbor, MI, USA).

Experiment 2 tested the relationship between the relative amount of nonan-2-one and the degree to which it enhanced attraction to 3*R*-ketone, and was conducted at Allerton Park near Monticello, IL (Piatt County; 39°59′11.01″ N, 88°39′3.75″ W), a 600 ha riparian forest. The five treatments represented varying doses of nonan-2-one relative to the amount of 3*R*-ketone (i.e., 25 mg 3*R*-ketone in the 50 mg per lure dose of 3*RS* ketone that was used), including 0, 10, 20, 100, 500, and 1,000 % nonan-2-one (i.e., 0, 2.5, 5, 25, 125, and 250 mg). 3*RS*-ketone was used because it was more economical and more readily available than the chiral material, and because Experiment 1 confirmed that the presence of the (*S*)-enantiomer did not influence attraction of *C. verrucosus* to 3*R*-ketone (see Results). Experiment 2 was conducted during 2nd May to 31st May 2011 (average low temperature 11.5 °C, average high temperature 23.2 °C, total precipitation 10.6 cm).

We conducted similar bioassays to evaluate the effect of decan-2-one on the behavior of *P. aspersum* and *O. gibbulum arizonense*, but too few beetles were captured at the IL and TX study sites to provide meaningful results.

Statistical analysis

Differences between treatment means were tested by fitting the data to a negative binomial distribution with a log-link function (PROC GENMOD; SAS Institute 2010) and using a Type 3 analysis (TYPE3 option) to generate a likelihood ratio statistic (calculated as a Chi-square) that tested for an overall treatment effect. Date-site combinations that had fewer than three specimens of *C. verrucosus* were dropped from the analysis so as to exclude periods of low beetle activity (leaving ten replicates per experiment). For both experiments, a priori contrasts (CONTRAST option) were used to compare pairs of treatment means and test specific hypotheses. In Experiment 1, we tested the hypotheses that nonan-2-one and 3*R*-ketone were separately attractive to adult *C. verrucosus* (Contrasts 1 and 2 in Table 1), that the ketone + nonan-2-one was significantly attractive (Contrast 3), and that nonan-2-one enhanced attraction to the ketone (Contrast 4). The remaining contrasts (5 and 6) tested for interference by 3S-ketone in the racemic blend. In Experiment 2, we used five contrasts to test whether *C. verrucosus* could discriminate among different blends: pure 3*RS*-ketone treatment (i.e., ketone +0 % nonan-2-one) was compared with each of the additional treatments that included nonan-2-one (Table 1). Because contrasts were non-orthogonal, *P* values were estimated with a Šidák correction that controlled family-wise error to $\alpha = 0.05$, based on the total number of contrasts in each experiment (Abdi and Williams 2010).

The effect of nonan-2-one was also tested for two non-target species in the Cerambycinae that had been captured during Experiment 1: *Anelaphus pumilus* (Newman) (tribe Elaphidiini) and *Phymatodes aereus* (Newman) (Callidiini). Because these species were represented by fewer specimens, treatments were combined to test hypotheses, as follows: the total number of specimens was pooled for traps baited with both 3*R*-ketone and nonan-2-one (i.e., the 3*R*-ketone + nonan-2-one and 3*RS*-ketone + nonan-2-one treatments) and traps baited with lures without nonan-2-one (the 3*R*-ketone and 3*RS*-ketone treatments). Deviations from the expected 1:1 ratio of beetles between the two treatments (under the null hypothesis) were tested with Chi-square analyses.

Table 1 A priori linear contrasts of treatment pairs for field bioassays of *C. verrucosus* during Experiments 1 and 2 (means presented in Figs. 1 and 2)

Contrast	χ^2	*P*
Experiment 1		
1. nonan-2-one versus control	1.12	0.29
2. 3*R*-ketone versus control	8.4	0.004
3. (3*R*-ketone + 20 % nonan-2-one) versus control	16.3	<0.001
4. 3*R*-ketone versus (3*R*-ketone + 20 % nonan-2-one)	4.18	0.13
5. 3*R*-ketone versus 3*RS*-ketone	0.08	0.78
6. (3*R*-ketone + 20 % nonan-2-one) versus (3*RS*-ketone + 20 % nonan-2-one)	0.11	0.74
Experiment 2		
1. 3*RS*-ketone versus (3*RS*-ketone + 10 % nonan-2-one)	7.85	0.005
2. 3*RS*-ketone versus (3*RS*-ketone + 20 % nonan-2-one)	6.41	0.01
3. 3*RS*-ketone versus (3*RS*-ketone + 100 % nonan-2-one)	7.85	0.005
4. 3*RS*-ketone versus (3*RS*-ketone + 500 % nonan-2-one)	1.89	0.17
5. 3*RS*-ketone versus (3*RS*-ketone + 1000 % nonan-2-one)	2.85	0.09

Percentages in Experiment 2 refer to the amount of nonan-2-one expressed as a percentage of the 25 mg of 3*R*-ketone in the lure

$df = 1$ for all contrasts

3R-ketone (*R*)-3-hydroxyhexan-2-one, *3RS-ketone* racemic 3-hydroxyhexan-2-one

Target significance levels were $P = 0.008$ in Experiment 1 and $P = 0.01$ in Experiment 2, based on a Šidák correction for the number of comparisons

Results

Pheromone identification

Extracts of headspace volatiles from male *C. verrucosus*, *O. g. arizonense,* and *P. aspersum* all contained two peaks (Fig. 1) that were absent in control aerations and in headspace extracts from female *C. verrucosus* and *P. aspersum* (no female *O. g. arizonense* were available for analysis). Mass spectra and retention times of the early-eluting components matched those of 3-hydroxyhexan-2-one for all three species, consistent with the collection of these species by traps baited with the same chemical (Hanks and Millar 2012, unpub. data). Co-injection on the Cyclodex B column with a pure standard of 3*S*-ketone resulted in a novel peak, proving that all three species produced the 3*R*-enantiomer. Extracts of volatiles from *C. verrucosus* also contained nonan-2-one, whereas those of both *O. g. arizonense* and *P. aspersum* contained decan-2-one. The alkan-2-ones were tentatively identified by interpretation of their mass spectra and matching with database spectra, and the identifications were confirmed by matching their retention times and mass spectra with those of standards. Trace amounts of 2,3-hexanedione were also detected in extracts from male beetles, but similar amounts of this chemical have been detected in synthetic standards of 3*RS*-ketone analyzed under the same conditions (unpub. data), suggesting it is a degradation product or analytical artifact (see Fettköther et al. 1995).

For each species, the amount of the alkan-2-one component was less than half that of 3*R*-ketone. Three different aeration extracts from male *C. verrucosus* contained nonan-2-one in quantities of 18.8, 16.8, and 17.4 % (mean ± SD: 17.7 ± 0.59 %) relative to the 3*R*-ketone. The amount of decan-2-one (relative to 3*R*-ketone) was 39.4 % in an aeration extract from the one male *O. g. arizonense* and 6.6 % in an aeration extract from two male *P. aspersum*.

Field bioassays of synthetic compounds

A total of 105 and 174 adult *C. verrucosus* were captured by panel traps during Experiments 1 and 2. In Experiment 1, traps baited with nonan-2-one alone were not significantly more attractive to beetles than control traps (Figs. 2, 3; Contrast 1 in Table 1). The 3*R*-ketone and 3*R*-ketone + nonan-2-one treatments were significantly more attractive than controls (Contrasts 2 and 3; overall likelihood ratio $\chi^2 = 21.7$, $df = 5$, $P = 0.0006$), but not significantly different from one another (Table 1, Contrast 4), suggesting that nonan-2-one did not enhance attraction to the ketone. The remaining two contrasts (5 and 6) confirmed that 3*R*-ketone was similar in activity to 3*RS*-ketone (Table 1), demonstrating that the 3*S* enantiomer did not influence attraction of *C. verrucosus*.

During Experiment 1, 239 adult *A. pumilus* and 69 *P. aereus* were captured, all in traps baited with lures that

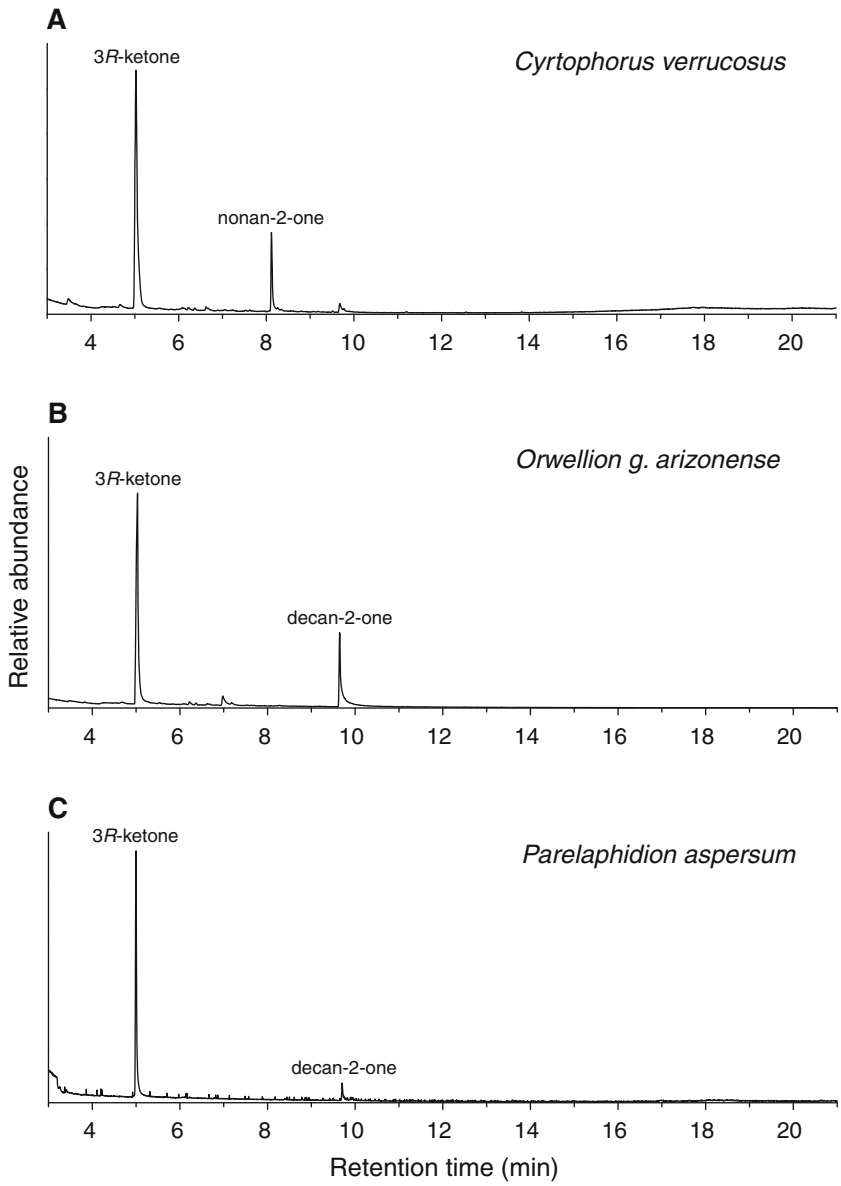

Fig. 1 Representative total ion chromatograms of headspace extracts from male **a** *Cyrtophorus verrucosus*, **b** *Orwellion gibbulum arizonense*, and **c** *Parelaphidion aspersum*. AT-5 ms column, 40 °C/1 min, then 10 °C/min to 210 °C/1 min. *Note* contaminant peak eluting at 9.75 min in the extract of *C. verrucosus* is not decan-2-one, despite apparent overlap in retention times

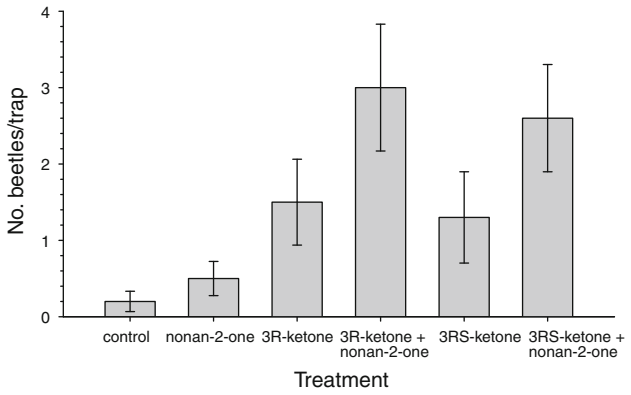

Fig. 2 Mean (±SE) number of *Cyrtophorus verrucosus* captured during Experiment 1 by traps baited with pheromone components. Compound abbreviations as in Table 1

contained 3*R*-ketone as a single component or a blend component. The presence of nonan-2-one did not affect attraction of these species (97 and 116 *A. pumilus*, and 39 and 30 *P. aereus* in the treatments with nonan-2-one versus treatments lacking nonan-2-one; $\chi^2 = 1.69$, $P = 0.19$, and $\chi^2 = 1.17$, $P = 0.28$, respectively).

Even though nonan-2-one had not appeared to enhance attraction of *C. verrucosus* in Experiment 1, it clearly did so in Experiment 2 (Fig. 3; overall likelihood ratio $\chi^2 = 11.02$, $df = 5$, $P = 0.05$). Traps baited with the 3*RS*-ketone blended with 10, 20, and 100 % of the amount of nonan-2-one captured more than three times as many beetles as traps baited with 3*RS*-ketone alone (0 % nonan-2-one, Fig. 3; Table 1, Contrast 1–3). However, greater amounts of nonan-2-one (500 and 1,000 %, Fig. 3) did not

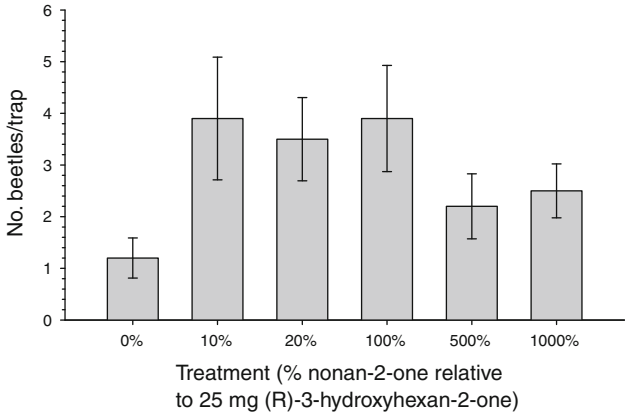

Fig. 3 Mean (±SE) number of *Cyrtophorus verrucosus* captured during Experiment 2 by traps baited with 50 mg 3*RS*-ketone combined with varying amounts of nonan-2-one. The amount of nonan-2-one is expressed as a percentage of the 25 mg of 3*R*-ketone in the lure

significantly enhance the attractiveness of the ketone (Table 1, Contrasts 4 and 5).

Discussion

The results reported above add three new species to the growing list of cerambycids that produce 3*R*-ketone, and also provide the first indication that alkan-2-ones might serve as pheromone components for cerambycid beetles. The fact that these chemicals are produced sex-specifically by males of three cerambycine species in two tribes (Anaglyptini and Elaphidiini) suggests that they may be common in other cerambycid species for which 3*R*-ketone is the primary pheromone component. Both nonan-2-one and decan-2-one are pheromone components for beetles in other families (Bartelt et al. 2009; Francke et al. 1979), and the former is a pheromone component of other types of insects, especially the Trichoptera (Bergmann 2002; Löfstedt et al. 1994). Nonan-2-one is also a very common plant volatile, associated with more than 200 species, as are other alkan-2-ones (El-Sayed 2012).

In Experiment 2, *Cyrtophorus verrucosus* was more attracted to a blend of its two pheromone components than to the major component 3*R*-ketone, suggesting that the addition of nonan-2-one enhances or clarifies this chemical signal. The most attractive ratios approximated the blend collected by aerations of adult males, but this must be taken with some caution because the blend ratio loaded onto the lures may have differed from the actual release rates due to differences in the vapor pressures of the components. Furthermore, *C. verrucosus* was significantly attracted to 3*R*-ketone alone, suggesting that nonan-2-one may not be critical for mate location. Recent studies have suggested that such minor components may also play a role in maintaining species specificity of the pheromone signals of cerambycids (Hanks and Millar 2012). It is possible that nonan-2-one may act as a deterrent for other cerambycids that would be attracted to 3*R*-ketone, although at least two sympatric species, *A. pumilus* and *P. aereus*, were seemingly unaffected by the presence of nonan-2-one in this study. Ongoing research aims to further elucidate the relationships among these and other species that respond to 3*R*-ketone, and that co-occur with *C. verrucosus*, *O. g. arizonense*, and *P. aspersum*.

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