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Cerambycid Beetle Species with Similar Pheromones are Segregated by Phenology and Minor Pheromone Components

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Abstract Recent research has shown that volatile sex and aggregation-sex pheromones of many species of cerambycid beetles are highly conserved, with sympatric and synchronic species that are closely related (i.e., congeners), and even more distantly related (different subfamilies), using the same or similar pheromones. Here, we investigated mechanisms by which cross attraction is averted among seven cerambycid species that are native to eastern North America and active as adults in spring: *Anelaphus pumilus* (Newman), *Cyrtophorus verrucosus* (Olivier), *Euderces pini* (Olivier), *Neoclytus caprea* (Say), and the congeners *Phymatodes aereus* (Newman), *P. amoenus* (Say), and *P. varius* (F.). Males of these species produce (*R*)-3-hydroxyhexan-2-one as their dominant or sole pheromone component. Our field bioassays support the hypothesis that cross attraction between species is averted or at least minimized by differences among species in seasonal phenology and circadian flight periods of adults, and/or by minor pheromone components that act as synergists for conspecifics and antagonists for heterospecifics.

Keywords Reproductive isolation · Sex pheromone · Aggregation pheromone · Cerambycidae · Longhorned beetle · *Anelaphus pumilus* · *Cyrtophorus verrucosus* · *Euderces pini* · *Neoclytus caprea* · *Phymatodes aereus* · *Phymatodes amoenus* · *Phymatodes varius*

Introduction

Insects produce a variety of signals that serve to expedite location of mates, with many species relying on volatile pheromones that advertise the availability of receptive mates over relatively long distances to bring the sexes together (Francke and Dettner 2005). Species specificity in pheromone chemistry is considered necessary because shared pheromones would diminish signal clarity and interfere with mate location (Cardé and Minks 1995; Linn and Roelofs 1995). Thus, the pheromones of closely-related and sympatric species may differ in the ratios or subsets of components, and include components that antagonize interspecific attraction (Baker 2008; Leal 1996; Löfstedt et al. 1991).

Recent research on species of wood-boring beetles in the large family Cerambycidae has revealed a seemingly maladaptive congruency in the pheromone chemistry of sympatric species (Barbour et al. 2011; Hanks and Millar 2013; Mitchell et al. 2011, 2013; Sweeney et al. 2010; Wickham et al. 2014). In fact, pheromone chemistry among species native to North America, Europe, and Asia appears to be highly conserved, with a few basic chemical structures ("motifs") accounting for pheromones of dozens, if not hundreds of species. Male-produced

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pheromones that attract both sexes include 3-hydroxyalkan-2-ones and related 2,3-alkanediols from many species in the subfamily Cerambycinae (Hanks et al. 2014; Mitchell et al. 2013; Wickham et al. 2014), 2-(undecyloxy)ethanol ("monochamol") from species in the Lamiinae (Allison et al. 2012; Fierke et al. 2012; Pajares et al. 2010; Teale et al. 2011; Wickham et al. 2014), and (*E*)-6,10-dimethyl-5,9-undecadien-2-ol ("fuscumol") and its acetate from species in the Lamiinae and Spondylidinae (Fonseca et al. 2010; Liendo et al. 2005; Mitchell et al. 2011; Sweeney et al. 2010; Vidal et al. 2010). On the other hand, it appears that females of species in the Prioninae produce sex pheromones that attract only males, including 2,3-alkanediols and 3,5-dimethyldodecanoic acid ("prionic acid"; Barbour et al. 2011; Ray et al. 2012; Wickham et al. 2014).

Here, we summarize research that investigated mechanisms by which cross attraction is averted among seven cerambycid species that share similar pheromone chemistry, yet are sympatric throughout much of eastern North America. Males of several species in the subfamily Cerambycinae that are active in spring produce (*R*)-3-hydroxyhexan-2-one as their dominant or sole pheromone component (Mitchell et al. 2013). This chemical is among the most common pheromone components reported to date for cerambycids of the northern hemisphere (Hanks and Millar 2013; Imrei et al. 2013; Wickham et al. 2014). Our field studies tested the hypotheses that cross attraction among these species is averted or minimized by differences in seasonal phenology of adults, their circadian flight periods, and/or by minor pheromone components that act as synergists for conspecifics and/or antagonists for heterospecifics.

Methods and Materials

Study Species Our study system consisted of seven primary species representing five tribes of the Cerambycinae (Table 1; Lingafelter 2007), including *Anelaphus pumilus* (Newman), *Cyrtophorus verrucosus* (Olivier), *Euderces pini* (Olivier), *Neoclytus caprea* (Say), and the congeners *Phymatodes aereus* (Newman), *P. amoenus* (Say), and *P. varius* (F.). These species were chosen because they are active as adults in spring (determined from previous research; Hanks et al. 2014), and use (*R*)-3-hydroxyhexan-2-one ("3*R*-ketone") as their dominant or sole pheromone component, or were presumed to do so based on attraction to racemic 3-hydroxyhexan-2-one in field bioassays ("3-ketone"; Table 1). In addition, we knew from previous experience that adults of these species could be trapped in reasonable numbers in the area of our studies (east-central Illinois). We also included in this study another four species that fly later in the year, but may overlap in activity period with some of the primary species, and that also use 3*R*-ketone as their sole or dominant pheromone component. These later species were *Neoclytus mucronatus mucronatus* (F.), *Phymatodes lengi* Joutel, *Sarosesthes fulminans* (F.), and *Xylotrechus colonus* (F.). All eleven of the study species are native to North America (Linsley 1963, 1964). Larvae of *A. pumilus*, *C. verrucosus*, *E. pini*, *P. aereus*, and *X. colonus* are polyphagous, feeding within the woody tissues of many species of hardwoods, especially species of *Quercus*, *Carya*, and *Castanea* that also are larval hosts of *N. caprea*, *N. m. mucronatus*, *P. varius*, and *S. fulminans* (Linsley 1963, 1964; Yanega 1996). Larvae of *P. amoenus* develop in dead grape vines (*Vitis* species), whereas hosts of *P. lengi* are not known (Lingafelter 2007).

Study Sites The research was conducted at the following sites in east-central Illinois: Allerton Park (Piatt Co., 600 ha, mixed hardwoods; 39°59′11.01″N, 88°39′3.75″W), Forest Glen Preserve (Vermilion Co., 728 ha, beech-maple and oak-hickory forest; 40° 1′18.43″N, 87° 33′59.58″W), Nettie Hart Memorial Woods (Champaign Co., 16 ha, second-growth upland and mesic woods; 40° 13′42.40″N, 88° 21′24.70″W), Trelease Woods (Champaign Co., 29 ha, virgin deciduous upland forest; 40° 8′5.54″N, 88° 8′34.06″W), and a residential neighborhood in Urbana, Illinois (Champaign Co., mature deciduous and coniferous ornamentals; 40°5′49.30″N, 88°12′11.33″W).

Identification of Pheromones Pheromones had already been identified for five of the species in previous studies, and those of the remaining species are reported here (Table 1). Beetles were captured for identification of pheromones using cross-vane panel traps (Alpha Scents, Portland, OR, USA) with surfaces coated with Fluon® PTFE dispersion (Northern Specialty Chemicals, Dudley, MA, USA) to improve capture efficiency (Graham et al. 2010). Traps were modified for catching live beetles by replacing the standard collecting bucket with a ~1 l clear plastic jar. Trap lures consisted of polyethylene sachets (Bagettes™ model 14770, 5.1 cm × 7.6 cm × 50 μ thick; Cousin Corp., Largo, FL, USA) containing synthetic pheromone diluted to 1 ml in ethanol (during 2008–2010) or isopropanol (2010–2013). Trap lures were loaded with 50 mg of racemic 3-ketone, or blends of 50 mg 3-ketone with 50 mg racemic 2-methylbutan-1-ol to target *Phymatodes* species (see below). Lures were replaced at intervals of 7–10 d.

Captured beetles of most species were sexed by the relative lengths of their antennae and morphology of the terminal antennomere (Linsley 1963, 1964), or by caging beetles together and observing mating behavior (i.e., males mount females). The sexes subsequently were housed separately in screen cages under laboratory conditions (~12:12 h L:D, ~20 °C) and provided 10 % sucrose solution as nourishment (Mitchell et al. 2013). We collected volatiles emitted by beetles by placing 1–4 individuals of the same species and sex in Mason-style canning jar chambers (~0.5 l). Air was drawn into the chamber at 1 l/min through a charcoal filter, and headspace was drawn

Table 1 Compounds detected in aeration extracts from males of seven species in the subfamily Cerambycinae that were the primary study species, and four later-season species that overlapped with the primary species in activity period

Category	Tribe	Species	Compounds (% of blend)	Reference
Primary	Elaphidiini	*Anelaphus pumilus*	3*R*-ketone (100 %)	Present paper
	Tillomorphini	*Euderces pini*	3*R*-ketone (100 %)	Present paper
	Clytini	*Neoclytus caprea*	3*R*-ketone (100 %)	A. M. Ray, pers. comm.
	Callidiini	*Phymatodes aereus*	3*R*-, 3*S*-ketone (90, 10 %)	Present paper
	Callidiini	*Phymatodes amoenus*	3*R*-ketone, (*R*)-2-methylbutan-1-ol (75, 25 %)	Present paper
	Callidiini	*Phymatodes varius*	3*R*-ketone, (*R*)-2-methylbutan-1-ol (96, 4 %)	Present paper
	Anaglyptini	*Cyrtophorus verrucosus*	3*R*-ketone, nonan-2-one (83, 17 %)	Mitchell et al. 2013
Later season	Clytini	*Neoclytus m. mucronatus*	3*R*-ketone (100 %)	Lacey et al. 2007
	Callidiini	*Phymatodes lengi*	3*R*-ketone, (*R*)-2-methylbutan-1-ol (94, 6 %)	Present paper
	Clytini	*Sarosesthes fulminans*	3*R*-ketone, (2*S,3R*)-2,3-hexanediol (83, 17 %)	Lacey et al. 2009; Hanks and Millar 2013
	Clytini	*Xylotrechus colonus*	3*R*-ketone (70 %), 3*S*-ketone (10 %), (2*S,3S*)-, (2*R,3R*)-2,3-hexanediol (17 %, 3 %)	Lacey et al. 2009

Species are ordered by increasing complexity of the pheromone blend within each category. Chemical abbreviations: 3*R*-ketone = (*R*)-3-hydroxyhexan-2-one, 3*S*-ketone = (*S*)-3-hydroxyhexan-2-one

out through collectors containing 150 mg of the absorbent polymer HayeSep® Q (Sigma-Aldrich, St. Louis, MO, USA; for details, see Mitchell et al. 2013). Headspace volatiles were collected for 24 h under laboratory conditions with aeration chambers near a north-facing window that provided natural photoperiod (~14:10 h L:D, ~20 °C). Aerations of empty chambers 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 until analyzed.

Aeration 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. Injections were made in splitless mode with an injector temperature of 250 °C, and an oven temperature program of 40 °C for 1 min, increased at 10 °C/min to 210 °C, and held for 3 min. Extracts were analyzed separately to detect 2-methylbutan-1-ol with an oven temperature program of 30 °C for 5 min, increased at 10 °C/min to 210 °C, and held 3 min. Sex-specific peaks were identified by comparing spectra and retention times with those of authentic standards.

The absolute configurations of 3-ketone enantiomers were 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 set at 110 °C to minimize isomerization of the 3-ketone (Millar et al. 2009), with an oven temperature program of 50 °C for 1 min, increased at 5 °C/min to 130 °C, and held for 10 min (retention times: 3*R*-ketone, 10.56 min; 3*S*-ketone, 11.10 min). Enantiomers of 2-methylbutan-1-ol were resolved with an oven temperature program of 40 °C for 10 min, increased at 5 °C/min to 200 °C ([*R*]-2-methylbutan-1-ol, 13.74 min; [*S*]-2-methylbutan-1-ol, 13.87 min). Identities were confirmed by co-injection of extracts with authentic standards.

Standards of some chemicals were synthesized as described in earlier publications, including 3*R*- and 3*S*-ketone (Lacey et al. 2007), racemic 3-ketone (Imrei et al. 2013), and (*R*)-2-methylbutan-1-ol (Mitchell et al. 2012). Nonan-2-one and racemic 2-methylbutan-1-ol were purchased from commercial sources (Sigma-Aldrich), as was (*S*)-2-methylbutan-1-ol (TCI America, Portland, OR, USA).

Segregation by Seasonal Phenology Flight phenology of the primary seven study species was characterized using data from field bioassays that tested attraction of the local community of cerambycid species to synthetic pheromones, blends of pheromones, host plant volatiles, and blends of pheromones with plant volatiles (Hanks et al. 2014). Those bioassays were consistent in their methods (using the same types of traps and lures), but varied in their objectives. Data for the present study were from field bioassays conducted at the five study sites during 15 March – 9 June 2012 and 3 April – 27 June 2013. We supplemented this analysis with data from dedicated experiments conducted in 2012 that measured the phenology of cerambycid species in eastern Illinois (Hanks et al. 2014).

Seasonal flight periods of species were characterized by grouping captured specimens into deciles based on date of collection. Species whose flight periods did not overlap, or overlapped by only 1 decile, (i.e., ≤ 10 % overlap in flight period) were considered to be segregated in seasonal phenology.

Segregation by Circadian Flight Period Segregation of species by circadian flight period was assessed only for the primary seven study species, excluding *N. caprea* because it overlapped little if at all in seasonal flight period with the remaining species (see Results). The same panel traps were used, but with the collecting basins replaced with mechanisms that rotated eight trap jars at programmable intervals ("timer traps"; model #2850, BioQuip Products, Rancho Dominguez, CA, USA). Trap lures were loaded with 25 mg per enantiomer of synthetic pheromones in 1 ml isopropanol, with blends of chemicals approximating the ratios produced by study species (see Table 1). In 2012, pairs of timer traps were used, one baited with 50 mg of racemic 3-ketone and the other with 3-ketone blended with 50 mg of racemic 2-methybutan-1-ol. In 2013, the same two treatments were used, but with an additional two timer traps, one baited with 3-ketone blended with nonan-2-one (25 mg), and one with the full blend of 3-ketone, 2-methybutan-1-ol, and nonan-2-one so as to maximize the number of target species that would be attracted. Treatments were assigned randomly to traps at the Allerton Park and Forest Glen Preserve study sites throughout flight seasons of the target species (19 April–17 May 2012, 16 May–15 June 2013; traps stopped during periods of rain).

During 2012, timer traps were programmed to rotate jars at six 2-h intervals, beginning at 10:00 and ending at 22:00, followed by two 6-h intervals (22:00–4:00 and 4:00–10:00), the latter intervals confirming that no species were active later at night or early in the morning. During 2013, pairs of traps were used to capture beetles during 1-h periods throughout the flight period of the target species (10:00–24:00), as follows: 1) one trap was set to capture only diurnal beetles, with one jar rotating per hour for 7 h (first jar starting at 10:00, seventh jar ending at 17:00), and the remaining jar removed so that beetles trapped during the layover period could escape (and so be available for capture by the second trap); 2) the second trap was set to capture only crepuscular and nocturnal species, with one jar accepting beetles per hour for 7 h (first jar starting at 17:00, seventh jar ending at 24:00), and the remaining jar again removed to allow escape of beetles captured during the layover period (and available for capture by the first trap). Thus, the pairs of traps could be run continuously, with each jar receiving trapped beetles during the same 1-h period every day, and beetles trapped during every hour of the activity period.

Circadian flight periods of species were characterized by pooling data from both years into 2-h blocks, and flight periods of different species were compared by overlap of quartiles (small sample size for some species precluded the use of deciles). Species were considered temporally segregated if their flight periods overlapped by less than 1 quartile. We also tested for segregation of species by comparing "peak" circadian period of flight (i.e., testing for differences in the distribution of specimens across circadian periods) using the Wilcoxon rank-sum test (PROC NPAR1WAY; SAS Institute 2011), with family-wise error controlled to $P<0.05$ using the false discovery rate (Benjamini and Hochberg 1995).

Segregation by Pheromone Chemistry We used field bioassays to assess the role of the minor pheromone components nonan-2-one and 2-methylbutan-1-ol in mediating attraction of beetles to 3*R*-ketone. These tests targeted the primary seven study species, but again excluded *N. caprea* due to its isolated flight season. The same panel traps were used, but with the standard collecting buckets partly filled with ~0.25 l of saturated brine to kill trapped beetles, and to prevent captured males from emitting pheromone. Trap lures were loaded with 25 mg per enantiomer of synthetic pheromone in 1 ml isopropanol, and treatments were as follows: 1) 3-ketone (simulating pheromones of *A. pumilus*, *E. pini*, and *P. aereus*; Table 1), 2) 3-ketone + 2-methylbutan-1-ol (simulating pheromones of *P. amoenus* and *P. varius*), 3) 3-ketone + nonan-2-one (simulating pheromone of *C. verrucosus*), and 4) solvent control (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 block. The experiment was run as a single block at Allerton Park, Forest Glen Preserve, and Nettie Hart Memorial Woods during 22 March – 14 May 2012. The experiment was repeated during 15 May – 14 June 2013 as one block at Forest Glen Preserve and two blocks (separated by >500 m) at Allerton Park. Specimens were collected from traps every 1–4 d, at which time treatments were moved round one position within transects to control for location effects.

Bioassay data for each species were combined across years, and treatment effects on numbers of beetles captured were tested with the nonparametric Friedman's test (PROC FREQ, option CMH2; SAS Institute 2011) because the data violated assumptions of ANOVA (Sokal and Rohlf 1995). Replicates consisted of a single collection date at a single study site, and replicates with no specimens caught in any trap were dropped from analyses. Pairs of treatment means were compared using multiple Wilcoxon rank-sum tests, and family-wise error controlled with the false discovery rate.

Results

Identification of Pheromones Extracts of headspace volatiles from males of all 11 species contained a prominent peak of 3-ketone that was absent in negative controls and headspace samples of females (Supplementary Online Resource 1), as

had already been reported for five species (Table 1). Extracts from most species contained only 3*R*-ketone (Table 1), but the 3*S* enantiomer was a minor component in extracts from *P. aereus* and *X. colonus*. Extracts from *P. amoenus*, *P. lengi*, and *P. varius* contained the minor component (*R*)-2-methylbutan-1-ol. Additional minor components had previously been reported from three other species: nonan-2-one from *C. verrucosus*, and stereoisomers of 2,3-hexanediol from *S. fulminans* and *X. colonus* (Table 1). Structures of compounds produced by the seven primary study species are detailed in Fig. 1.

Segregation by Seasonal Phenology We captured 4648 specimens of the 11 cerambycid species, and whereas adults of all the species were active earlier in 2012 than in 2013, the progression of flight periods was similar across years (Fig. 2). Earlier emergence in 2012 probably was due to an unusually warm spring (average temperatures in March of 2012 and 2013: 13.3 and 1.6 °C, respectively; Weather Underground, Inc., Ann Arbor, MI, USA). *Neoclytus caprea* appeared earliest, and was isolated from the six other primary study species for nearly its entire flight period (segregation by seasonality indicated by "S" in Fig. 3). The remaining primary species subsequently emerged in near synchrony and overlapped broadly in flight season. Adults of *S. fulminans*, *P. lengi*, *X. colonus*, and *N. m. mucronatus* emerged after the primary study species, overlapping to some degree with the tail ends of flight periods of *A. pumilus* and *C. verrucosus* (Fig. 2; summarized in Fig. 3).

Segregation by Circadian Flight Period Among the primary study species that overlapped in seasonal activity period (i.e., all but *N. caprea*), adults were active for discrete periods of the day, with clear differences among some species (Fig. 4). Adult *E. pini*, *P. amoenus*, and *C. verrucosus* were active during afternoons (14:00–18:00 h), and did not overlap significantly with the nocturnal *P. aereus* and *A. pumilus* (most specimens captured between 20:00 and 22:00 h; segregation by circadian flight period indicated by "C" in Fig. 3). Adults of *P. varius* differed significantly from both the afternoon and nocturnal groups in terms of peak flight period (Fig. 4), with 70 % being captured between 18:00–20:00 h, when few adults of any other species were active.

Segregation by Pheromone Chemistry Pheromone chemistry apparently serves to prevent cross attraction among the primary study species that overlap in flight period. For example, attraction of adult *E. pini* to 3-ketone was strongly antagonized by both nonan-2-one or 2-methybutan-1-ol (Fig. 5), so that this species would not be attracted to the 3*R*-ketone in the pheromone blends of the synchronous species *C. verrucosus* and *P. amoenus,* which contain these minor components, respectively (segregation by pheromone chemistry indicated by "P" in Fig. 3). On the other hand, adult *C. verrucosus* and *P. amoenus* were most strongly attracted by the blends of 3-ketone and their minor components (Fig. 5), and so would be unlikely to be attracted to pheromones of one another, and to the pure 3*R*-ketone produced by *E. pini* (Fig. 3). Thus, the segregating effects of the pheromone minor components are complementary such that not only does the absence of the minor component in the pheromone of *E. pini* inhibit attraction of *C. verrucosus* to *E. pini*, but its presence in the pheromone of *C. verrucosus* also inhibits attraction of *E. pini* to *C. verrucosus* (Fig. 3). The unique quality of the pheromone blend of *C. verrucosus* should also reduce cross attraction to the 3*R*-ketone in pheromones of the later season species with which it slightly overlaps. The results suggest that minor pheromone components may offer an additional level of protection from cross attraction for many species that are segregated primarily by phenology (Fig. 3).

Discussion

This study supports the hypotheses that temporal segregation and pheromone chemistry together serve to avert or minimize cross attraction between sympatric cerambycid species that share one or more pheromone components. The two mechanisms may complement one another, with pheromone chemistry offering an additional barrier to cross attraction between species that may partially overlap in flight phenology. Segregation of species by phenology and/or pheromone chemistry has been

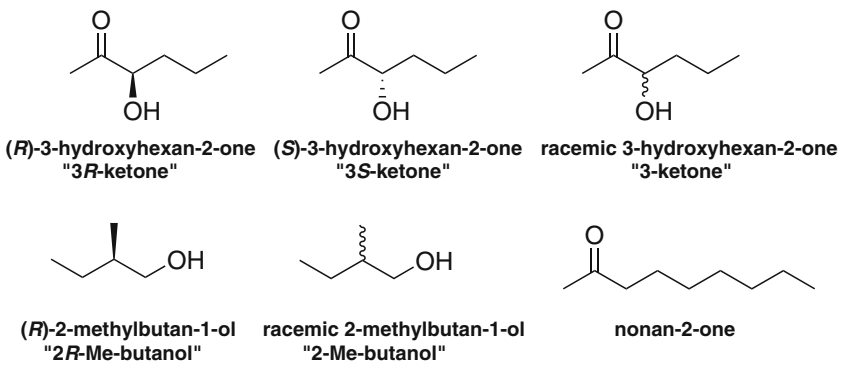

Fig. 1 Structures of pheromones produced by males of the seven primary study species

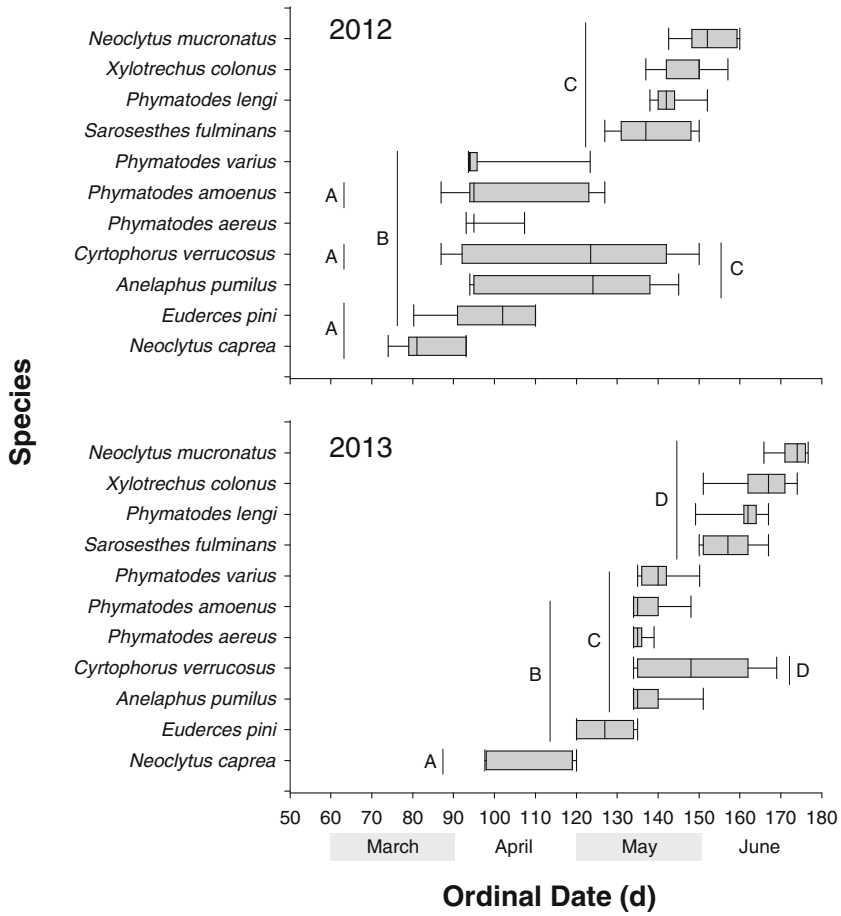

Fig. 2 Seasonal phenology of cerambycid species as estimated by the number captured by pheromone-baited panel traps during 2012 and 2013, ordered in reverse by onset of flight period. Sample sizes for *N. m. mucronatus, X. colonus, P. lengi, S. fulminans, P. varius, P. amoenus, P. aereus, C. verrucosus, A. pumilus, E. pini,* and *N. caprea* were 31, 587, 389, 15, 11, 52, 65, 214, 369, 6, and 47 in 2012, and 8, 891, 141, 18, 33, 422, 332, 253, 701, 41, and 22 in 2013, respectively (*gray box* includes 50 % of the data points, median indicated by the *vertical line*, whiskers indicate the 90th and 10th percentiles; *letters* indicate groups of species that were considered synchronous i.e., overlap in >10 % of data points between each species in the group)

reported previously for other insects, but rarely for complexes of species such as described here (Ishikawa et al. 1999; Löfstedt et al. 1991; Mazor and Dunkelblum 2005; McElfresh et al. 2001; Müller and Eggert 1987).

Our findings suggest that the pheromone systems of cerambycid communities are tightly integrated and a product of strong selective forces against cross attraction, and/or favoring mate location, that drive temporal segregation (over the season, or over the course of the day) and divergence in pheromone chemistry. It follows that sympatric species that do not overlap in flight phenology would be free to use the same or very similar pheromones. As examples, we offer the study species whose pheromones appear to consist solely of 3*R*-ketone, and which are segregated by seasonal flight period (*N. caprea*, *E. pini, N. m. mucronatus*) or by circadian flight period (*E. pini*, *A. pumilus*). Similarly, species whose

	Primary study species							Later-season species			
	N. caprea	*E. pini*	*A. pumilus*	*C. verrucosus*	*P. aereus*	*P. amoenus*	*P. varius*	*Sarosesthes fulminans*	*Phymatodes lengi*	*Xylotrechus colonus*	*Neoclytus mucronatus*
Neoclytus caprea								S	S	S	S
Euderces pini	S							S	S	S	S
Anelaphus pumilus	S	C						S?	S?	S?	S?
Cyrtophorus verrucosus	S	P	C					*	*	*	*
Phymatodes aereus	S	C	?	C				S	S	S	S
Phymatodes amoenus	S	P	C	P	C,P			S	S	S	S
Phymatodes varius	S	C,P	C	C,P	C,P	C		S	S	S	S

Fig. 3 Mechanisms of isolation among cerambycid species that share the pheromone component (*R*)-3-hydroxyhexan-2-one, including the seven primary study species and four later-season species. Species are ordered by approximate onset of flight period within these categories (mechanism by which species are mutually segregated is indicated by: "**S**" for seasonal phenology of adults, "**C**" for circadian flight period, "**P**" for synergistic/antagonistic minor pheromone components, "*****" for predicted separation mediated by minor components, and "**?**" for unknown mechanism)

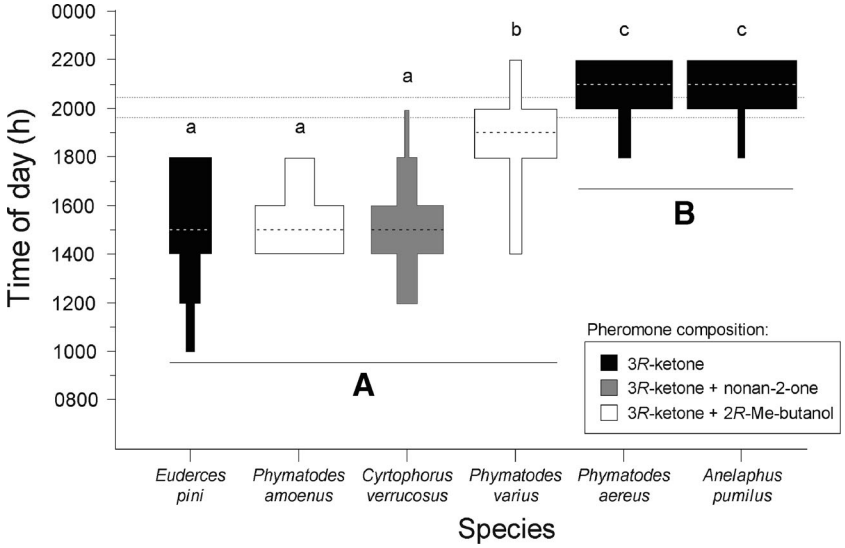

Fig. 4 Numbers of beetles for six species of cerambycids that were captured with pheromone-baited timer traps during 2-h periods, arranged by earliest to latest median hour of capture. Sample sizes for *E. pini*, *P. amoenus*, *C. verrucosus*, *P. varius, P. aereus*, and *A. pumilus* were 11, 4, 23, 10, 9, and 82, respectively. For each species, the total time interval during which specimens were captured is indicated by the height of the graphic block, while the percent capture at a given time interval is indicated by its width. *Dashed lines* within blocks indicate median capture as the midpoint of a 2-h period, and *gray lines* indicate the range in timing of sunset during the experiment. The *shading of the block* indicates pheromone composition (see inset). Chemical abbreviations: 3*R*-ketone = (*R*)-3-hydroxyhexan-2-one, 2*R*-Me-butanol = (*R*)-2-methylbutan-1-ol. *Lowercase letters* indicate significantly different peak activity period (multiple Wilcoxon rank sum; family-wise error controlled at $P<0.05$ by false discovery rate), *uppercase letters* indicate species that overlap in >25 % of flight period

pheromones consist of 3*R*-ketone and (*R*)-2-methylbutan-1-ol are segregated in seasonal flight period (*P. lengi* flies later in the year than both *P. amoenus* and *P. varius*) or circadian flight period (*P. amoenus* flies earlier in the day than *P. varius*). It also follows that the minor pheromone components that serve to segregate synchronous species are likely to have minimal effect on species with which they do not overlap in flight period. Consistent with that hypothesis, *A. pumilus* was unaffected by (*R*)-2-methylbutan-1-ol and nonan-2-one that synergize attraction to 3*R*-ketone in species that fly earlier in the day.

The field bioassays do not suggest a mechanism for avoiding cross attraction between *P. aereus* and *A. pumilus*, adults of which overlap broadly in both seasonal and circadian flight period (Fig. 3). Male *P. aereus* produce primarily 3*R*-ketone (with a small amount of the *S* enantiomer), and male *A. pumilus* produce pure 3*R*-ketone, but adults of both species are attracted by racemic 3-ketone (Fig. 5), suggesting that *A. pumilus* is not inhibited by the 3*S*-ketone in the pheromone of the other species. Nevertheless, the possibility remains that it synergizes attraction of *P. aereus* to the dominant enantiomer. Alternatively, cross attraction could be averted by segregating mechanisms other than those we have considered here. For instance, species that overlap in circadian flight period may differ in the time of day that males call, which could minimize the chances of cross attraction. Volatiles from host plants also are known to strongly synergize responses of some cerambycid species to their pheromones (Allison et al. 2012; Pajares et al. 2010; Teale et al. 2011). Thus, volatiles associated with particular plant species, or plants in a certain physiological state, may mediate segregation among cerambycid species with similar pheromones. However, *Phymatodes aereus* apparently shares larval hosts with *A. pumilus* (species of *Quercus*, *Castanea*; Yanega 1996), so it seems unlikely that host volatiles would serve as a segregating mechanism for these two species. Cerambycid species also may be segregated by their preferences for particular habitats within forests. For example, field studies using traps positioned at different heights have shown that cerambycid species may differ dramatically in their abundance across vertical strata of the forest canopy (Dodds 2014; Graham et al. 2012). We currently are testing traps baited with live males of the study species to better assess the incidence of cross attraction in nature, which may guide future research on mechanisms that inhibit attraction to pheromones of other species with synchronous flight periods.

Although the findings of this study are consistent with strong selection for segregation among species that share pheromones, cross attraction nevertheless may be adaptive in some situations. For example, male cerambycids of many species call from larval host plants (Lacey et al. 2004; Lemay et al. 2010), which may expedite location of hosts for other species that can intercept that chemical signal. Such chemical eavesdropping might explain the weak but significant attraction of adult *C. verrucosus* to 3-ketone alone. That species is polyphagous on hardwoods (Linsley 1964), and attraction to pheromones of other cerambycids therefore could constitute an exploitative method of finding suitable hosts. Such opportunistic cross attraction is most evident in species that are attracted by pheromone

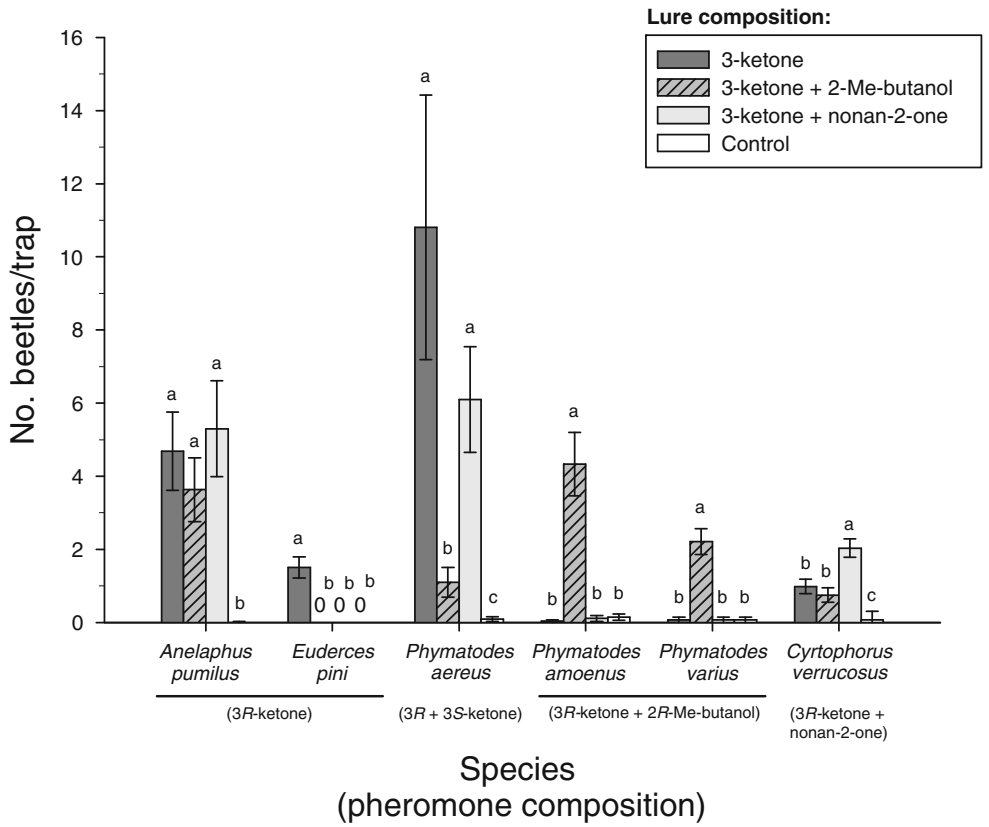

Fig. 5 Mean (±SE) number of beetles of six primary study species that were captured by traps baited with synthetic approximations of their pheromones. Species ordered as in Table 1. Chemical abbreviations: 3-ketone = racemic 3-hydroxyhexan-2-one, 3*R*-ketone = (*R*)-3-hydroxyhexan-2-one, 3*S*-ketone = (*S*)-3-hydroxyhexan-2-one, 2-Me-butanol = racemic 2-methylbutan-1-ol, 2*R*-Me-butanol = (*R*)-2-methylbutan-1-ol. Treatment means significantly different for all species: *A. pumilus* (N=838, Friedman's Q_3=68.6, P<0.001), *E. pini* (N=6, Q_3=12.8, P=0.005), *P. aereus* (N=386, Q_3=27.8, P<0.001), *P. amoenus* (N=386, Q_3=71.8, P<0.001), *P. varius* (N=39, Q_3=40.2, P<0.001), *C. verrucosus* (N=260, Q_3=53.7, P<0.001). *Error bars* indicate standard error, and means with *different letters* within species are significantly different (multiple Wilcoxon rank sum; family-wise error controlled at P<0.05 by false discovery rate)

components of other species, but that they themselves apparently do not produce. A notable example is *Phymatodes grandis* Casey (formerly *P. lecontei*) of western North America: the pheromone of males consists solely of (*R*)-2-methylbutan-1-ol, yet adults of both sexes are strongly attracted by 3-ketone, the dominant pheromone component of sympatric species that share the same hosts (Hanks et al. 2007). Cross attraction, even if rare, would seem to encourage interspecific mating. In fact, research on closely-related species of cerambycids has revealed cases of introgression (Nakamine and Takeda 2008), cross-breeding (Wickman 1969), and apparent species hybrids (Hovore 1983). Nevertheless, there is a growing body of evidence that close-range mate recognition in cerambycids is mediated by unique blends of contact pheromones in the cuticular lipids of females, which likely maintain reproductive isolation (Ginzel 2010).

The ubiquity of 3*R*-ketone as a pheromone component of the primary study species, as well as the later season species, is further evidence of its important role in communities of species within the large subfamily Cerambycinae. This compound is likely the dominant or sole pheromone component of at least another nine species native to eastern North America, which were excluded from the present study due to their scarcity at our study sites. These species are in the same genera or tribes as the study species (*Euderces*, *Neoclytus*, *Xylotrechus*), and males are either known to produce 3*R*-ketone, adults were attracted by 3-ketone in field bioassays, or both (unpub. data). Moreover, there is mounting evidence that 3*R*-ketone plays a similar role in communities of cerambycids in western North America (Hanks et al. 2007; Ray et al. 2009), Europe (Imrei et al. 2013; Schröder et al. 1994), and Asia (Leal et al. 1995; Wickham et al. 2014). The same pattern holds true for other common pheromone motifs of cerambycids, with sympatric species often overlapping in pheromone chemistry, and motifs shared across continents (see Introduction).

The conservation of pheromone chemistries among cerambycid species and the integrated nature of the co-evolved pheromone systems described in the present study have implications for invasion biology. The wood-boring larvae are readily transported by international trade, and introduction of exotic species poses a significant threat

to natural and managed forests (Haack 2006; Haack et al. 2010). However, if an invading species overlaps extensively in phenology and pheromone chemistry with native species, the resulting interference in its pheromone channel may present a significant barrier to its establishment. This natural form of mating disruption would be most effective during the initial stages of an invasion, when the invaders are at very low density, and yet must locate conspecifics to mate. In such cases, native communities of cerambycids may actually serve as a first line of defense against establishment of exotic and potentially invasive species.

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