Environmental Entomology, 2016, 1–10
doi: 10.1093/ee/nvw022
Research article

OXFORD

Community and Ecosystem Ecology

The Role of Multimodal Signals in Species Recognition Between Tree-Killing Bark Beetles in a Narrow Sympatric Zone

Deepa S. Pureswaran,[1] Richard W. Hofstetter,[2,3] Brian T. Sullivan,[4] and Kristen A. Potter[5]

[1]Canadian Forest Service, Laurentian Forestry Centre, 1055, rue du PEPS, Quebec City, QC G1V 4C7, Canada (deepa.pureswaran @canada.ca), [2]School of Forestry, Box 15018, Northern Arizona University, Flagstaff, AZ 86011 (rich.hofstetter@nau.edu), [3]Corresponding author, e-mail: rich.hofstetter@nau.edu, [4]USDA Forest Service, Southern Research Station, Pineville, LA 71360 (briansullivan@fs.fed.us), and [5]Landscape Conservation Initiative, Box 5767, Northern Arizona University, Flagstaff, AZ 86011 (kristen.potter@nau.edu)

Received 20 December 2015; Accepted 29 February 2016

Abstract

When related species coexist, selection pressure should favor evolution of species recognition mechanisms to prevent interspecific pairing and wasteful reproductive encounters. We investigated the potential role of pheromone and acoustic signals in species recognition between two species of tree-killing bark beetles, the southern pine beetle, *Dendroctonus frontalis* Zimmermann, and the western pine beetle, *Dendroctonus brevicomis* LeConte, in a narrow zone of sympatry, using reciprocal pairing experiments. Given the choice of adjacent con- or heterospecific female gallery entrance in a log, at least 85% of walking males chose the entrance of the conspecific, and half the males that initially entered heterospecific galleries re-emerged and entered the conspecific gallery within 15 min. Waveform analysis of female acoustic "chirps" indicated interspecific differences in chirp timing. Males may use information from female acoustic signals to decide whether to enter or remain in the gallery. Individuals in forced heterospecific pairings (produced by confinement of a heterospecific male within the female entrance) did not differ in pheromone production from individuals of conspecific pairs. However, due to the absence of the right species of male, galleries with heterospecific pairs released an abnormal pheromone blend that lacked at least one key component of the aggregation pheromone of either species. The complete aggregation pheromone (i.e., the pheromone blend from entrances with pairs) does not appear to deter interspecific encounters or confer premating reproductive isolation *per se*; however, it may confer selective pressure for the maintenance of other reproductive isolation mechanisms.

Key words: acoustic signal, character displacement, *Dendroctonus*, multispecies aggregation, pheromone

When two or more related species coexist in space and time, reproductive isolation mechanisms must be sufficient to prevent interspecific pairing, as such typically doomed attempts at reproduction are a waste of gametes, energy, and possibly other limiting resources for both members of the pair. Thus, within sympatric zones, natural selection should favor divergence of mate-location behaviors and cues such as pheromones and acoustic signals to ensure effective discrimination against heterospecific individuals (Noor 1999). Such reproductive character displacement would result in the evolution of distinct mate recognition signals between closely related species (Symonds and Elgar 2008).

The genus *Dendroctonus* (Coleoptera: Curculionidae: Scolytinae) consists of conifer-infesting bark beetles that generally must kill their host trees in order to complete their life cycle and which periodically undergo region-wide population outbreaks (Wood 1982b, Six and Bracewell 2015). Outbreaks are characterized by synchronized mass-attacks on individual trees that are mediated by the beetles' relatively sophisticated pheromone communication system (Vega and Hofstetter 2015). Attacking individuals produce an aggregation pheromone that can attract conspecifics of both sexes in sufficient abundance to overcome the preformed resin defenses of vigorous hosts (Wood 1982a). Individual females initiate tunnels called "galleries" in the phloem and are soon joined by a solitary male. Following mating, the pair extends the gallery, and the female lays eggs in the phloem of the gallery walls (Reid 1962). Once successfully established in a host, the pair's release of aggregation pheromone declines, and production of compounds that inhibit attraction ("antiaggregation pheromone") may terminate mass-attack thereby limiting intraspecific competition (Raffa et al. 1993). The tree is killed by disruption of

the phloem tissue by adult and larval feeding and by the pathogenic effects of beetle-inoculated fungi.

Members of the genus *Dendroctonus* have a limited range of host species presumably due to the different adaptations necessary for countering the physical and chemical defenses specific to different conifer taxa (Franceschi et al. 2005). For example, in western North America, the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, is specific to *Pinus* spp., the spruce beetle, *D. rufipennis* Kirby, is specific to *Picea* spp., and the Douglas-fir beetle, *D. pseudotsugae* Hopkins, attacks only *Pseudotsuga* sp. (Wood 1982b, Six and Bracewell 2015), even though all three host and beetle species might occur in the same forest. The pheromone blends of *Dendroctonus* spp. are generally composed of differing combinations of the few same components—an apparent legacy of their common ancestry (Symonds and Elgar 2004b). Not surprisingly, cross-species attraction of pheromone blends is not uncommon in *Dendroctonus* (Lanier and Burkholder 1974). However, pairing attempts between males and females of different *Dendroctonus* spp. may rarely happen because mating occurs within the tissue of the host tree and beetles do not usually land and initiate attack on non-host species (Pureswaran and Borden 2003).

The western pine beetle, *Dendroctonus brevicomis* LeConte, and the southern pine beetle, *D. frontalis* Zimmermann, are closely related species (Bentz and Stock 1986, Lanier et al. 1988). For much of their distribution, the two species are allopatric, except for a narrow band of sympatry in northern Arizona where their ranges overlap (Wood 1982b, Davis and Hofstetter 2009) (Fig. 1). In this zone, the two species attack the same host species, ponderosa pine, *Pinus ponderosa* Douglas ex C. Lawson, and are apparently sympatric in both space (attacking the entire length of the bole of the same tree) and time (adults of both species are active throughout the warm months and attack the bole simultaneously), and their galleries often occur adjacent to one another (Davis and Hofstetter 2009). However, these two species differ in chromosome number and are apparently incapable of producing offspring (Lanier et al. 1988). Since their crosses are likely a genetic dead-end, selection should favor acquisition of effective species recognition mechanisms within the sympatric zone (Noor 1999). This should be especially true for species where males and females pair and remain together for an extended period of time. Male *Dendroctonus* are monogamous and usually remain in the gallery for many days after mating, while the female proceeds with oviposition and tunneling (Hopkins 1909). Following pairing, the male initially defends the gallery from intruding males after which he follows behind the tunneling female while clearing boring dust and excrement from the distal centimeters of the gallery. Additionally, he creates ventilation holes along the gallery that presumably facilitate brood development, and thus he invests in a certain degree of parental care (McGhehey 1968). Thus pairing in *Dendroctonus* spp. involves a large commitment of time and energy, rendering mistakes more costly than for species in which pairing merely involves copulation.

The two species utilize identical aggregation pheromone components (i.e., the sum of attractive components from both sexes; Supp. Table [online only]) with the possible exception of *exo*-brevicomin which is produced in merely minute quantities by *D. frontalis* (Payne et al. 1977, Byers 1983, Pureswaran et al. 2008). While this suggests that *exo*-brevicomin is a suitable cue for mediating species discrimination, there is no evidence that *D. frontalis* utilizes *exo*-brevicomin to avoid *D. brevicomis* and thereby enhance reproductive isolation. Recent studies on cross-attraction between the two species have demonstrated that *exo*-brevicomin baits increased, rather than decreased, *D. frontalis* captures in traps baited with frontalin and host odors (Gaylord et al. 2006, Hofstetter et al. 2008, Pureswaran et al. 2008). The fact that both species appear to colonize the same portions of the bole (Davis and Hofstetter 2009) likewise implies that long-range semiochemicals do not contribute to reproductive isolation, and that, to the extent that it occurs, species discrimination involves beetle interactions on the host, perhaps using short-range olfactory cues, acoustic signals, or contact cues.

Acoustic signals are common among bark beetles, and are used predominantly in short-range communication once beetles have landed on a tree (Barr 1969, Rudinsky et al. 1973, Fleming et al. 2013), and appear to function in mate recognition (Barr 1969, Ryker and Rudinsky 1976), male-male competition (Rudinsky and Michael 1974), mediation of courtship behaviors (Barr 1969, Rudinsky 1969, Rudinsky and Michael 1972, Rudinsky et al. 1973), and avoidance of predation (Lewis and Cane 1990). Bark beetles communicate their presence to prospective mates using acoustic signals, without which they are often not accepted as mates (Barr 1969). However, the role of acoustic cues in species recognition and mate location has not been directly studied in *D. frontalis* and *D. brevicomis* (but see Ryker 1988). *Dendroctonus* produce sound via stridulation from specialized structures located on the abdomen and inside the elytra (Ryker 1984, Lyal and King 1996, Fleming et al. 2013). In *Dendroctonus*, stridulatory structures are sexually dimorphic and typically less elaborate or absent in the gender initiating galleries in host trees (Barr 1969, Rudinsky and Michael 1973), which is the female.

We hypothesized that when the two species occur on the same tree, H1) mate-seeking males would discriminate against heterospecific entrance holes and fail to enter the gallery of the wrong female, H2) differences in species-specific signals produced by females exist and thus have the potential to facilitate female discrimination by males, and H3) when males are paired with heterospecific females, the acoustic calls and pheromone blend emanating from the gallery would differ from that of a reproductively compatible conspecific pair. Heterospecific pairing might result in abnormal pheromone production by the pair or its individual members (including changes in composition, release rates, and timing) that could potentially alter behavioral responses associated with conspecific pairings.

In this paper, we use sample populations of *D. frontalis* and *D. brevicomis* from their sympatric zone in Arizona to: 1) investigate the frequency with which heterospecific pairings occur in the laboratory when males are given a choice of female gallery entrances, 2) compare the quantitative pheromone profiles and female acoustic signals of the two sympatric species, and 3) identify changes in pheromone production by either species following forced heterospecific pairing. We discuss the selection pressures that might shape the evolution and persistence of olfactory and acoustic signals when closely related species are sympatric on the same host tree species.

Materials and Methods

Collection of Beetles, Bolts, and Phloem

Dendroctonus frontalis and *D. brevicomis* were collected alive from aggregation pheromone-baited traps (*D. brevicomis* lure; Synergy Semiochemicals Corp., Burnaby, BC, Canada) deployed in the Northern Arizona University/Arizona State Lands Centennial Forest, located 15 km west of Flagstaff, Arizona, USA (latitude 35° 10' N, longitude 111° 45' E, elevation: 2,080 m) during the summers of 2008, 2009, 2014, and 2015. Beetles were separated by species and sex and stored in plastic bags containing moist paper towels for 2–3 d at 4°C until used. *Pinus ponderosa* (20 cm dbh, between 50

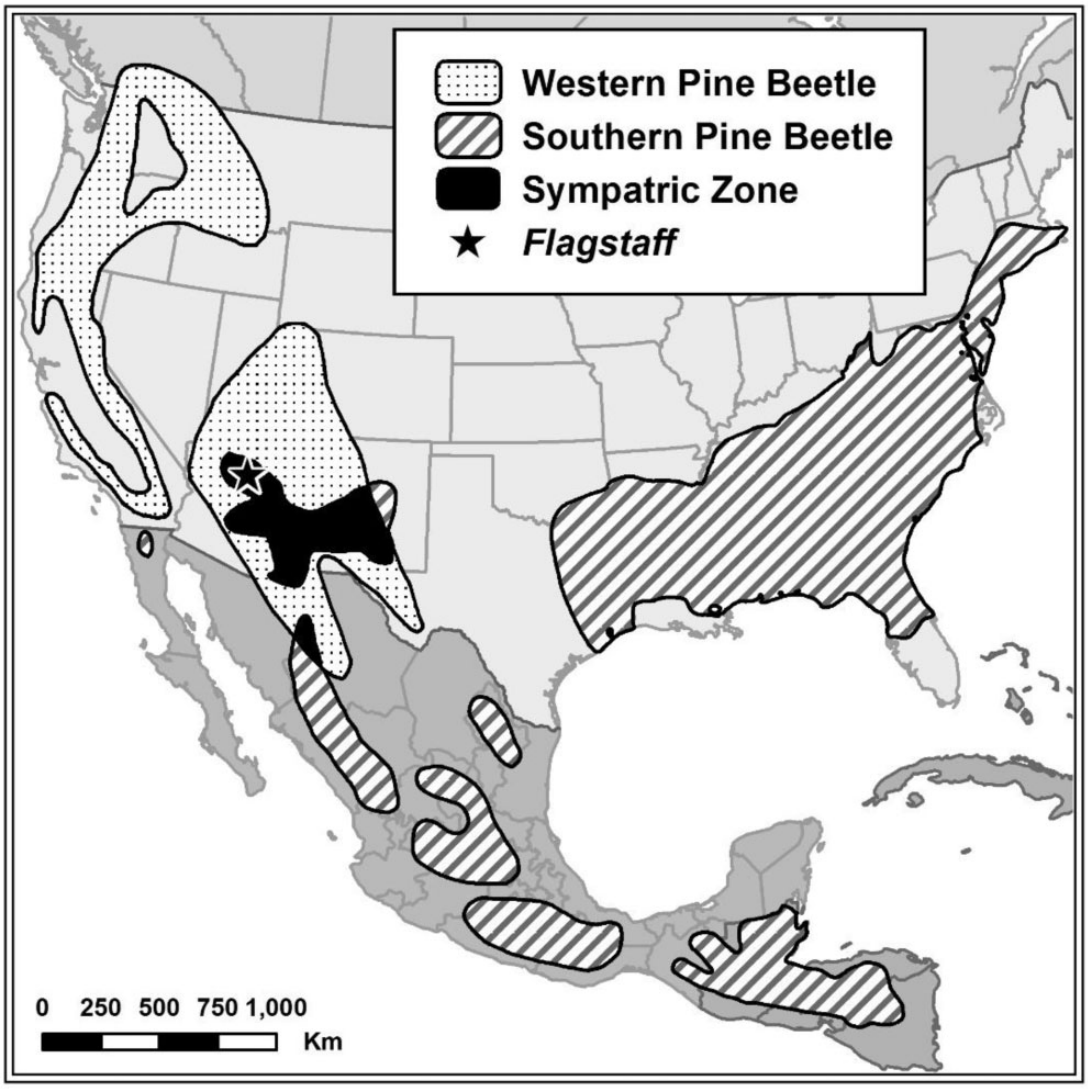

Fig. 1. Geographic ranges of the western pine beetle, *D. brevicomis,* and the southern pine beetle, *D. frontalis,* in North America (Clarke and Nowak 2009, K. Dodds, pers. communication). Shaded region demarks the sympatric zone in northern Arizona where both species commonly occur on the same host trees.

and 60 yr old) were felled (one tree in 2008 and 2009 for phloem sandwich tests, and two trees in 2014 and 2015 for mate selection bioassays) in the same location and during the same time as the bark beetle traps. In 2008 and 2009, phloem tissue was stripped from the trees after removal of corky bark with a draw-knife (Aflitto et al. 2014). Phloem pieces were placed in vacuum-sealed bags and transported on ice packs in a cooler to the laboratory where they were stored at 4°C until used in experiments (methods similar to Aflitto et al. 2014). In 2014 and 2015, bolts between 40 and 50 cm in length were cut from the felled tree and transported to the laboratory for the mate selection bioassay.

Preparation of Phloem Sandwiches

Phloem tissue was cut into 10- by 10-cm swatches and sandwiched between two 12- by 12-cm acrylic sheets (modified from Aflitto et al. 2014). The acrylic was secured firmly against both surfaces of the phloem by a metal spring clamp, and several layers of Parafilm were wrapped tightly around the edges of the sandwich to retard desiccation. A hole (3.2 mm diameter) was drilled in the center of one of the acrylic sheets through which beetles could be introduced into the phloem and emitted volatiles could be captured. During aerations the Parafilm was removed from one of the four edges of the sandwich and a piece of charcoal fabric (4 by 4 cm; Universal Replacement Prefilter, Honeywell #38002, Southborough, MA) was folded and secured in the opening to filter organic volatiles from incoming air. The surface of the phloem did not make an air-tight seal against the acrylic sheets, and thus air could pass freely from the charcoal-filtered opening across the surface of the phloem to the drill hole.

Aerations of Galleries in Phloem Sandwiches

Volatiles were sampled repeatedly from gallery entrances in artificial phloem sandwiches infested either with solitary females of either species or pairs representing all four possible crosses between males and females of the two species (i.e., paired *D. frontalis*, paired *D. brevicomis*, a female *D. frontalis* with a male *D. brevicomis*, a female *D. brevicomis* with a male *D. frontalis*). A female beetle of either species was introduced singly into the drill-hole of a unique phloem sandwich and allowed to bore into the phloem. After 18 h, volatiles arising from the entrance were quantitatively sampled for a

6-h interval (aeration #1; Fig. 2). To accomplish this, the inlet (a piece of 3.2 mm o.d. PFA tubing) of a cartridge filled with conditioned adsorbent for airborne organic compounds (0.1 g Porapak Q, 50–80 mesh, Grace Chromatography, Columbia, MD) was inserted snugly into the drill hole of the sandwich, and a light vacuum was applied to the outlet of the cartridge to maintain a 15 ml/min flow of air through the adsorbent. Shortly following this first aeration (i.e., ~24 h following female introduction), a male of either *D. frontalis* or *D. brevicomis* was introduced into the hole in the acrylic. The entrance was then sampled two further times for 6 h with fresh cartridges: 5 min after introduction of the male (aeration #2), and again ~24 h following introduction of the male (aeration #3; ~48 h after female introduction). The experiment was replicated five times each in 2008 and 2009 to obtain a total of 10 replicates per treatment. Following each sample collection, the ends of each adsorbent cartridge were sealed with Parafilm, and then the cartridges were stored at <−20° C (except during overnight shipment on ice packs to the USDA Southern Research Station Pineville laboratory in Louisiana) until extracted and analyzed. Cartridges were extracted with 1.2 ml redistilled pentane at room temperature, and each extract was spiked with 1.77 µg heptyl acetate as an internal standard. Vials with extract were stored at −80°C prior to analysis.

Recordings of Acoustic Signals

Acoustic recording of the female bark beetles occurred at three time periods (Fig. 2; Acoustics and behavior #1, 2, and 3): (T1) ~24 h after introduction to the phloem sandwiches (prior to male introduction), (T2) within the first 5 min after male introduction, and (T3) at ~24 h after male introduction. Recordings were made using a FG-3329 electret condenser microphone (Knowles Electronics, Itasca, IL) and an HD-P2 TASCAM digital audio recorder at a 96 kHz (24 bit) sampling rate (similar to Yturralde and Hofstetter 2015). Recordings were made ~2 mm from the tunnel entrance, regardless of the proximity of beetles within the phloem. This microphone has low sensitivity to high ultrasonic frequencies regardless of distance and the distance from the microphone to sound source was variable. Therefore, only temporal (not spectral) characteristics were recorded and analyzed. Each female was recorded for 5 min at each time period.

The following parameters were measured for the first five sequential chirps from each female: chirp length, chirp interval, and strikes per chirp (Fig. 3). For recordings after male introduction, female chirps were easily distinguished from male chirps due to their distinct waveform characteristics and lower amplitude. In those recordings, the first five clear (i.e., not overlapping with male chirps) chirps *after* males began to chirp were used, to ensure that females were potentially responding to male stridulation. All measurements were done using Raven Pro (v1.4, Cornell Laboratory of Ornithology, Ithaca, NY). When necessary, sounds were bandpass filtered to exclude non-beetle noise below 1–3 kHz.

Aerations of Individual Beetles Excised From Bolts

In 2008 and 2009, volatiles were collected from individual beetles immediately after they were excised from galleries established in bolts of ponderosa pine in the laboratory. Sampled beetles included solitary, gallery-initiating females of both species as well as males and females from recently established pairs representing all four possible crosses between males and females of the two species. To facilitate attack of bolts, beetles were confined inside empty gelatin capsules (Clear gelatin capsule Size 1, Capsuline, Inc., Pompano Beach, FL) secured over a 5-mm drill-hole that penetrated the outer bark. Gelatin capsules were spaced no closer than 10 cm on the bark surface, and crosses of each category were performed on separate, randomly selected bolts from a single tree. Females in the solitary treatment were allowed to bore into the phloem and excavate galleries for 48 h. For paired beetle treatments, females were first allowed to bore for 24 h, and then either a con- or heterospecific male was introduced to the entrance and the pairs were left undisturbed for another 24 h. Five to nine replicates for each treatment in which beetles were alive and healthy were then carefully dissected from galleries and inserted individually, abdomen-first, into 100-µl-capacity conical glass vials containing 2 mm (~0.3 mg) of conditioned Super Q adsorbent (80–100 mesh; Alltech, Deerfield, IL) in the vial's constricted tip (Sullivan 2005). Beetles were confined to the bottom of the vials beneath a short piece of PFA tubing so that the tip of the abdomen was maintained 1–2 mm from the adsorbent. The PTFE-lined caps of the vials were loosely threaded to allow sufficient gas exchange for beetle respiration, and the vials were maintained in a vertical orientation in a stream of purified, humidified (i.e., blown through sterile water), room-temperature air during a 24-h period. Afterward, beetles and PFA tubing were removed, and the adsorbent was then steeped in 50 µl redistilled pentane (spiked with 3.5 ng/µl heptyl acetate as an internal standard) for 15 min at room temperature. The extraction pentane was then transferred to another vial with a fine-tipped glass pipette (to exclude the adsorbent), and stored at −80°C prior to analysis.

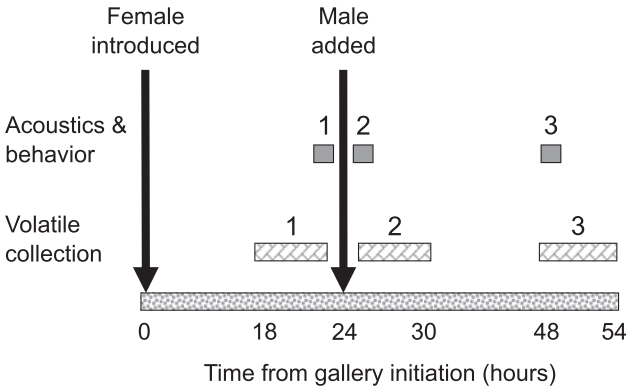

Fig. 2. Timing of insect introductions, aerations of pheromones (i.e. Volatile collection #1, 2, and 3), and recording of acoustic signals and behavior (i.e. Acoustics & behavior #1, 2, and 3) for the phloem sandwich experiment.

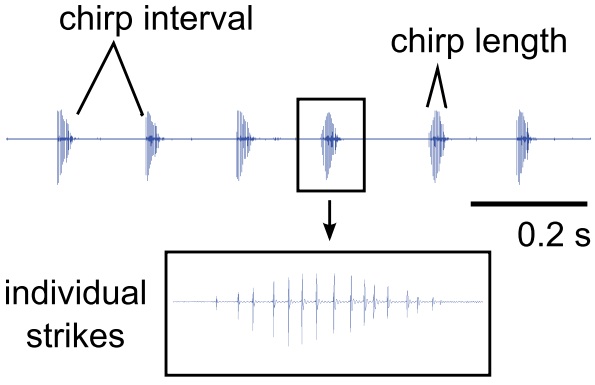

Fig. 3. Example waveform of chirps produced by females of *D. frontalis* and *D. brevicomis*, with descriptions of parameters measured in this study (chirp length, chirp interval, number of strikes per chirp) (Online figure in color).

Chemical Analysis of Volatiles

An aliquot of each extract sample (2 µl portion) was analyzed on an Agilent 6890–5973 coupled gas chromatograph-mass spectral detector (GC-MS) fitted with an HP-INNOWax (Agilent Technologies, Santa Clara, CA; 60 m long by 0.25 mm diam. by 0.25 µm film thickness) polyethylene glycol-phase microcapillary column; the oven temperature program was 40°C for 1 min, then 16°C/min to 80°C, 7°C per min to 230°C, and held 10 min. Quantification of five *Dendroctonus* pheromone components (frontalin, *exo*-brevicomin, *endo*-brevicomin, *trans*-verbenol, and verbenone) was based on standard curves constructed by analyzing serial dilutions of known quantities of commercially obtained synthetic versions of the compounds. The serial dilutions were spiked with the same concentration of the internal standard heptyl acetate as the samples.

Mate Selection Bioassay

Two holes (0.25 cm diameter) were drilled 2 cm apart horizontally on the bark of 40–50 cm long, vertically oriented bolts of ponderosa pine. A female *D. frontalis* and a female *D. brevicomis* were each randomly placed into a hole to initiate gallery construction, and confined under half of a gelatin capsule. After 24 h, if both females successfully entered the bolt (determined by the presence of copious frass within the gelatin capsule), the capsules were removed and either a male *D. frontalis* or a male *D. brevicomis* was released onto the bark surface 2.5 cm below the midpoint between the female entry holes. An open, clear plastic cylinder (5 cm diameter, 6 cm length) was secured around the two female entry holes, to keep the male within the test area. The male was observed continuously while he traversed the bark and eventually entered one of the two holes. If the male did not enter one of the female entry holes within 15 min, the male was removed and replaced by another male. If the second male did not enter within 15 min, the female pair was discarded and not used in the study. Time taken by the male to make his choice and which female was chosen was recorded. The experiment was replicated 41 times for *D. frontalis* and 40 times for *D. brevicomis*. The entry holes were observed for an additional 15 min to determine whether the male remained in the gallery or reemerged and switched female entry holes. The entry holes were then sealed with a fine mesh screening to prevent beetles from escaping. The mesh screening was sealed to the bark with hot glue (Mini Glue Sticks, Michaels Stores, Inc., Flagstaff AZ). After 2 wk, the bark was removed from the logs, and the gallery lengths, presence of male and female beetles, and eggs or larvae were noted.

Statistical Analysis

Analyses of Pheromones

All pheromone amounts (in *n*g, with an approximate threshold of detection of 0.1 *n*g per microliter sample) were transformed using $\log_{10}(x+1)$ to meet the assumptions of normality and homoscedasticity. For aerations of galleries in phloem sandwiches, the amounts of different pheromone components produced by *D. frontalis* and *D. brevicomis* in conspecific versus heterospecific pairs [either 0 h (early) and 24 h (late) after the male was introduced into the female gallery] were compared using a general linear model (PROC GLM) followed by paired contrasts (CONTRAST statement) between con- and heterospecific pairs for each pheromone component.

For aeration of individual beetles excised from bolts, log transformed pheromone amounts were subjected to multivariate analysis of variance (MANOVA) followed by the Ryan-Einot-Gabriel-Welsch (REGW) multiple comparisons procedure. Comparisons were within each species and sex, i.e., single females, females with conspecific males, females with heterospecific males, males with conspecific females, and males with heterospecific females.

All analyses were performed using SAS (SAS 2002–2003) version 9.1 statistical software, and $\alpha = 0.05$.

Analyses of Acoustic Signals

Female acoustic data (i.e., chirp length, strikes per chirp, and chirp interval) were analyzed using linear mixed effect (LME) models, which accounted for repeated observations on females, especially because not all females chirped in all recording periods (T1, T2, and T3). We chose this analysis because T1 and T2 chirps from the same female were not independent. Analyses were implemented in the R statistical package (R Development Core Team 2010).

Mate Selection Bioassay

The proportion of males (*D. frontalis* and *D. brevicomis*) that chose the gallery of a conspecific female when simultaneously offered entrances with females of both species (p, a binomial parameter), was calculated. A chi-square test of equal proportions (PROC FREQ) was performed with a binomial distribution to determine whether the proportion of males choosing a conspecific female significantly differed from 0.5 ($H_o = 50{:}50$). The "exact" statement was used to produce exact *P*-values. Time (in seconds) of first entry into the gallery of the female by male *D. frontalis* and male *D. brevicomis* were log transformed using $\log_{10}$ (time+1) and analyzed with a student *t*-test.

Results

Analyses of Pheromones

Orthogonal contrasts between con- and heterospecific pairs aerated within galleries, revealed significant differences in pheromone release (Fig. 4A–F). Conspecific pairs with female *D. frontalis* produced small amounts of both *exo*- and *endo*-brevicomin which were nearly undetectable in heterospecific pairs. For early and late conspecific pairs respectively, the amounts of *exo*-brevicomin emitted (and the test results of contrast with heterospecific pairs) were 3.6 ($F = 7.2$, $P = 0.008$) and 5.0 ng ($F = 7.3$, $P = 0.008$) and *endo*-brevicomin was 135 ($F = 41$, $P < 0.0001$) and 65 ng ($F = 39$, $P < 0.0001$; Fig. 4B and C). Also, more verbenone was produced by early conspecific pairs than early heterospecific pairs ($F = 4.1$, $P = 0.05$; Fig. 4B). However, the amount of frontalin and *trans*-verbenol produced by con- versus heterospecific pairs with a female *D. frontalis* did not differ significantly in either the early or late aerations (Fig. 4B and C).

For pairs with a female *D. brevicomis*, both early ($F = 107$, $P < 0.0001$) and late ($F = 134$, $P < 0.0001$) conspecific pairs produced more frontalin than heterospecific pairs (Fig. 4E and F), and early conspecific pairs produced more *trans*-verbenol than heterospecific pairs ($F = 4.0$, $P = 0.05$; Fig. 4E). These con- and heterospecific pairs did not differ significantly in release of either brevicomin or verbenone, and the heterospecific pairs did not produce any frontalin at all (Fig. 4E and F).

In aerations of individual beetles excised from bolts, female *D. frontalis* that had paired with a male of either species produced significantly less frontalin and more verbenone than solitary females (Table 1). Female *D. brevicomis* that had paired with a male of either species produced significantly less *endo*-brevicomin than solitary females. Furthermore, significantly more frontalin was isolated from *D. frontalis* males paired with conspecific rather than heterospecific females, although the mean amount detected (3 ng) neared

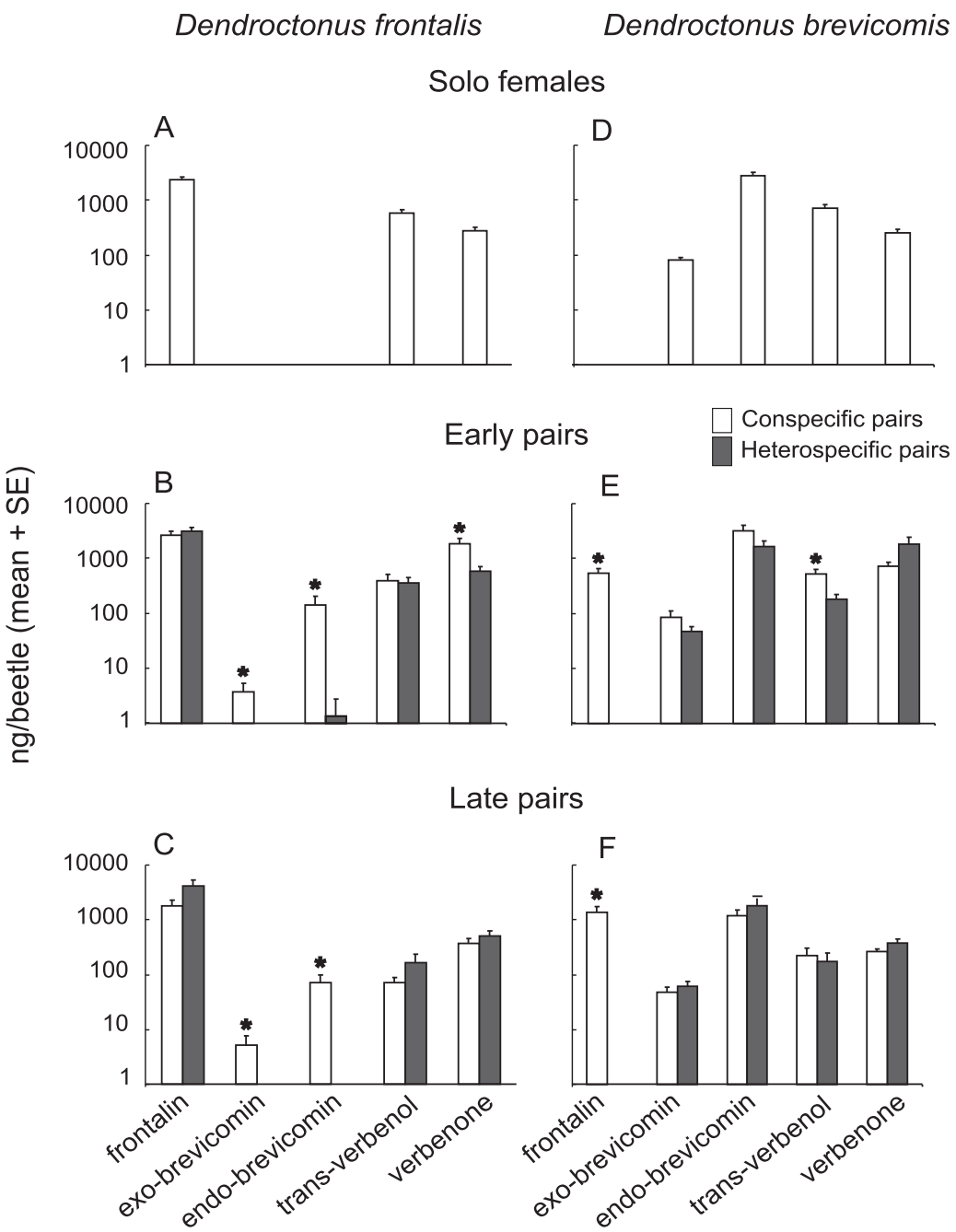

Fig. 4. (**A–F**) Amounts of pheromone components collected at gallery entrances of either *D. frontalis* or *D. brevicomis* foundresses paired <6 h (early) or 48–54 h (late) with conspecific or heterospecific males. Asterisks indicate significant differences between conspecific and heterospecific pairs for a given compound, $P < 0.05$ (MANOVA followed by paired contrasts).

the detection threshold of the instrument. No other significant difference in pheromone production was detected between individuals from conspecific versus heterospecific pairs (Table 1).

Analyses of Acoustic Signals

Not all females chirped in both T1 and T2 recordings, so sample sizes varied for each trial (Fig. 5). T3 was not used in the analysis due to the lack of female chirps produced during that time period. Previous studies also described single clicks made intermittently by tunneling *D. brevicomis* females (Ryker 1988), but we were unable to distinguish these, if any occurred from background noises. When recorded alone (T1), *D. frontalis* and *D. brevicomis* females differed in chirp length, chirp interval, and strikes per chirp (Fig. 5, Table 2; all $P < 0.001$ also in additional pairwise comparisons). When paired with males (regardless of the species), *D. frontalis* females did not modify their chirps relative to chirps when alone whereas *D. brevicomis* females decreased their chirp intervals (Fig. 5, Table 2). Although only 1 of 10 conspecific-paired *D. frontalis* females chirped (T2), those 10 females were also less likely to chirp during T1 than the 10 *D. frontalis* females subsequently paired with heterospecific males. Thus, our results do not imply that *D. frontalis* females are less likely to chirp in conspecific pairings per se.

Table 1. Aerations of individual beetles in conical vials excised from bolts

Treatment	*n*	Frontalin	*Exo*-Brevicomin	*Endo*-Brevicomin	*Trans*-Verbenol	Verbenone
Female *D. frontalis*						
Alone for 48 h	8	1,626 (262) a	0.06 (0.06)	0.38 (0.21)	154 (49)	6.6 (2) b
Conspecific pair: with male *D. frontalis*	5	229 (51) b	0 (0)	3 (3)	568 (215)	81 (23) a
Heterospecific pair: with male *D. brevicomis*	5	521 (281) b	0 (0)	0.4 (0.4)	710 (383)	82 (37) a
Male *D. frontalis*						
Conspecific pair: with female *D. frontalis*	5	3 (1) a	8 (3)	110 (38)	371 (181)	158 (43)
Heterospecific pair: with female*D. brevicomis*	8	0.13 (0.13) b	7 (2)	124 (29)	109 (38)	154 (54)
Female *D. brevicomis*						
Alone for 48 h	8	0.13 (0.1)	69 (14)	3,734 (768) a	505 (142)	4 (1)
Conspecific pair: with male *D. brevicomis*	8	0.1 (0.1)	29 (6)	777 (199) b	601 (171)	8 (2)
Heterospecific pair: with male *D. frontalis*	8	0.1 (0.1)	42 (8)	1,688 (449) b	474 (99)	6 (1)
Male *D. brevicomis*						
Conspecific pair: with female *D. brevicomis*	8	987 (209)	0 (0)	0.13 (0.1)	267 (87)	63 (22)
Heterospecific pair: with female *D. frontalis*	9	532 (71)	0 (0)	0.17 (0.17)	524 (218)	54 (12)

Results of MANOVA followed by REGW multiple range test on $\log_{10}(x+1)$ transformed data. Amount of pheromone detected [mean (SE)] in ng/beetle. Significant differences among means are followed by letters. Means with the same letter within a column in each treatment are not significantly different.

Mate Selection Bioassay

The proportion of male *D. frontalis* selecting a conspecific female (i.e., entering the conspecific entrance first) was 0.90 (37/41) and the proportion of male *D. brevicomis* selecting a conspecific female was 0.85 (34/40). The choices of male *D. frontalis* and *D. brevicomis* differed significantly from 50:50 ($P < 0.02$), suggesting that males did not select entry holes randomly. However, males did not always choose conspecific females. Of the 10 males that entered heterospecific holes (total for both species), half were observed to reemerge from the hole quickly (mean = 170 s ± 72 s) and enter the conspecific hole. No males in conspecific galleries attempted to leave the gallery during the observation period. Eighty-five percent (33/39) of the final *D. frontalis* conspecific pairings and 89% (33/37) of *D. brevicomis* conspecific pairings resulted in the production of offspring. Males that remained in tunnels of heterospecific females started new galleries branching off of the female galleries. Of the females that did not have male partners, 11% (4/37) of the female *D. frontalis* and 28% (11/39) of the female *D. brevicomis* still produced offspring suggesting that these field collected beetles were already mated and are likely re-emergent beetles.

Time until entry for male *D. frontalis* and *D. brevicomis* into galleries of their respective conspecific females was not statistically different ($P = 0.98$), with *D. frontalis* males first entering tunnels at 2 min 9 s ± 29 s (mean ± SE) and *D. brevicomis* entering tunnels at 2 min 12 s ± 19 s. Time until entry (1 min 13 s ± 48 s) for male *D. frontalis* into galleries of heterospecific females was not significantly different (t-ratio = 1.02, $P = 0.19$) from *D. frontalis* entering conspecifics galleries. Time until entry (45 s ± 13 s) of male *D. brevicomis* into galleries of heterospecific females was significantly (t-ratio = 3.36, $P = 0.004$) faster than *D. brevicomis* entering conspecifics galleries.

Discussion

Pheromone Emission Profiles by Conspecific Versus Heterospecific Pairs

Our results indicate that short-range olfactory cues or acoustic signals help to deter interspecific encounters and confer premating reproductive isolation in *D. frontalis* and *D. brevicomis*. Males correctly choose to enter conspecific galleries a majority of the time. When they were confined in the gallery of the wrong species of female, members of the resulting heterospecific pairs do not differ significantly from individuals in conspecific pairings in either the composition or production sequence of their pheromone components (Table 1). Therefore, pairing with the correct species is apparently not necessary for eliciting normal production and timing of release of pheromone components by individual members of beetle pairs. Furthermore, species-specific cues are evidently not involved in governing regulation of pheromone production and release by beetles that form pairs. Our data on pheromone production by individual beetles suggests that differences in the pheromone profiles of galleries of females paired with either hetero- or conspecific males can be attributed to the combination of the normal (i.e., conspecific pair-associated) female pheromone blend with the normal pheromone blend of the respective male. This further supports the conclusion that beetles do not respond to a heterospecific partner by altering pheromone release. Male *D. frontalis* forced to mine alone into logs produced significantly less *endo*-brevicomin than those allowed to enter the gallery of a conspecific virgin female (Sullivan et al. 2007), indicating that pairing does stimulate male release of this pheromone component. However, the use of bolts rather than standing trees could have influenced the chemical profiles and behavior of beetles since these species typically attack standing trees and are infrequently found in slash material and fallen trees (Wood 1982a). Also, logs and beetles in our tests were collected over multiple years and this could have affected patterns and variation in chemical profiles released by beetles (Byers 1983).

Some degree of cross-attraction presumably occurs between these two species since conspecific pairs of both species release the major aggregation pheromone components of the other (Symonds and Elgar 2004b) and both are attracted to the same artificial lures consisting of semiochemicals produced in common (Hofstetter et al. 2008, 2012; Pureswaran et al. 2008). Ironically, this cross-attraction would be terminated or reduced if it did result in heterospecific pairing. That is, both species are apparently attracted to conspecific pairings of either species, whereas neither should be attracted to heterospecific pairings of these same species. This might represent a significant selective disadvantage to heterospecific pairing (namely, the inability to elicit a successful mass-attack) even if it were possible for heterospecific pairs to produce otherwise fit, fertile offspring (they are not; Lanier et al. 1988). However, if both types of heterospecific pairings were to occur beside each other on the same tree (i.e. female *D. frontalis* x male *D. brevicomis*; female *D. brevicomis* x male *D. frontalis*), they would mutually supply the missing

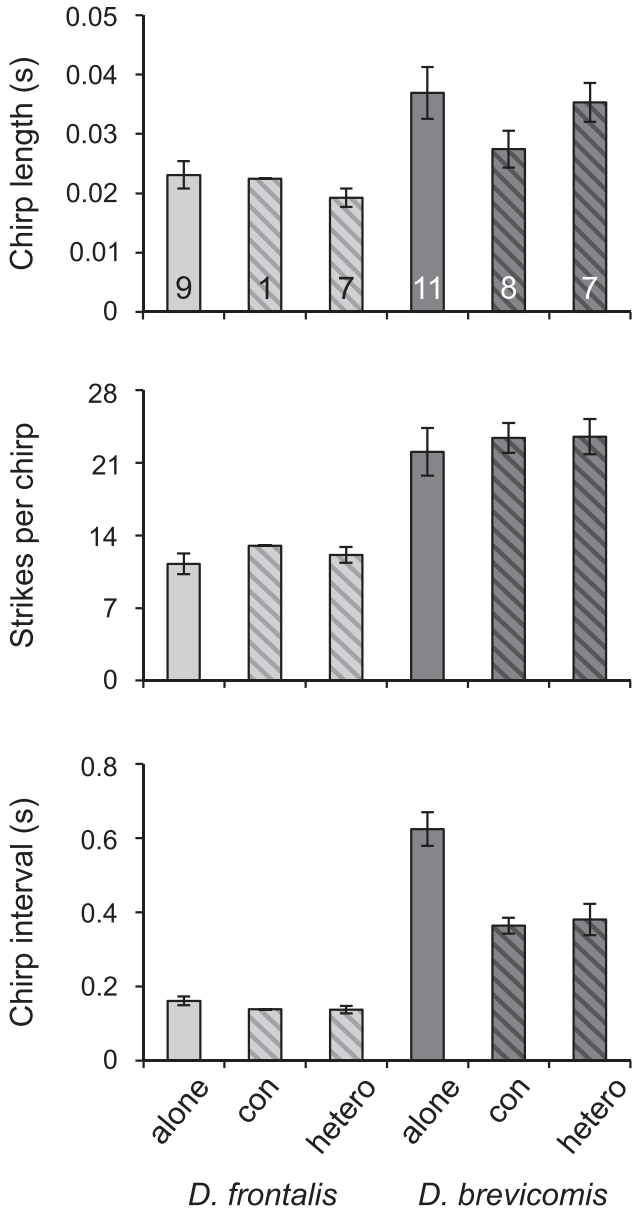

Fig. 5. Comparison of chirps made by female *D. frontalis* or *D. brevicomis* beetles when alone, paired with a conspecific male, or paired with a heterospecific male (mean ± SE; light gray—*D. frontalis* female, dark gray—*D. brevicomis* female; solid bars—recordings of females alone, striped bars—pairings with males). Sample sizes are on the upper bars.

aggregation pheromone components of the other and restore attraction. Thus, the potential attraction handicap of heterospecific pairing may be less important when trees are being attacked by multiple individuals of both species, whereas it might prevent heterospecific pioneer pairs from inducing a mass-attack.

Species-Specificity of Acoustic Cues

In many species of bark beetles, pheromone-mediated communication is complemented by vibro-acoustic communication during pairing (Rudinsky and Michael 1974, Ryker and Rudinsky 1976, Yturralde and Hofstetter 2015). Sound production by female *Dendroctonus* beetles is relatively unstudied, although some evidence suggests that females may chirp to maintain appropriate spacing as they make galleries (Rudinsky and Michael 1973, Rudinsky et al. 1976) and when interacting with a male (Ryker and Rudinsky 1976, Fleming et al. 2013).

Table 2. Summary of linear mixed effect (LME) models for chirps of female bark beetles (*D. frontalis* and *D. brevicomis*)

Chirp Features	Num d.f.	Den d.f.	*F*	*P*
Chirp length				
Intercept	1	183	175.39	<0.0001
Species	1	26	7.98	<0.01
Treatment	2	183	43.36	<0.0001
Species × Treatment	2	183	4.24	0.0159
Strikes per chirp				
Intercept	1	185	346.26	<0.0001
Species	1	26	29.66	<0.0001
Treatment	2	185	8.10	<0.001
Chirp interval				
Intercept	1	140	938.58	<0.0001
Species	1	26	197.41	<0.0001
Treatment	2	140	35.17	<0.0001
Species × Treatment	2	140	12.78	<0.0001

In all cases, species refers to species of the female, and treatment refers to solo, with conspecific male, or with heterospecific male.

Our results indicate that females produce acoustic chirps that differ between the species (Fig. 5; Table 2). However, it is important to point out that our sample sizes are small. The chirps of *D. brevicomis* females were longer, more frequent and contained more tooth strikes than those of *D. frontalis*. These are characteristics that males could use to decide whether or not to enter a gallery. Indeed, 85–90 percent of male beetles correctly chose the galleries of conspecific females. While the relative importance of close-range pheromones versus acoustic signals in a male's decision to enter a gallery is still unknown, female chirps are likely to play a role. Once inside female galleries, males may also use information from female acoustic signals to decide whether to remain in the gallery. Differences in female chirp parameters may therefore be reinforced by selection.

Interestingly, in *D. brevicomis*, solitary female calls, perhaps aimed primarily at other females in the bark, differed from the call made when responding to a male beetle. *D. brevicomis* females chirped more frequently when in the presence of a male. Females of *D. frontalis* gave similar chirps regardless of context. This intraspecific difference suggests the possibility of dual functions for the female call in *D. frontalis*, but perhaps not for *D. brevicomis*.

Evolution of Multimodal Signals: A Cause or Consequence of Sympatry?

Signal diversity and overlap in related species of bark beetles is intriguing from an evolutionary perspective. Character displacement in pheromone signals between sympatric species of bark beetle may not be critical in maintaining reproductive isolation since multimodal (and likely redundant) signals are involved in species recognition and mate location on the infested tree. Pheromone blend production and perception could therefore undergo significant shifts (Symonds and Elgar 2004a) without risking significant reduction in reproductive isolation. In particular, such shifts could enhance beetle fitness during periods of low population density. At these times it might be necessary for two or more related species to coattack the same tree to ensure sufficient attacks to permit depletion of host defenses and successful colonization of otherwise recalcitrant hosts (Davis and Hofstetter 2009). Co-occurring bark beetle species typically segregate temporally or spatially within a tree (Six and Bracewell 2015) and when they do co-occur, usually results in competition and reduced

fitness (Paine et al. 1981). However, *D. frontalis* and *D. brevicomis* in their sympatric zone appear to benefit in terms of host colonization success and have reduced competitive interactions between their respective fungi (Davis and Hofstetter 2009, Six and Bracewell 2015). Reduced competitive interactions among these beetles could result from mutualistic interactions between the differing fungal symbionts associated with each beetle species (Davis and Hofstetter 2009, Hofstetter et al. 2015). Future work should test whether the signals produced by *D. frontalis* and *D. brevicomis* in the allopatric parts of their ranges differ from those in this sympatric zone.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

Acknowledgments

We thank Reagan McGuire, Seth Davis, and Eli Jenson for their assistance in collecting insects and phloem. We thank the Rocky Mountain Research Station in Flagstaff, AZ, for use of their facilities, and P. Duval and S. Bourassa for making Figure 1. This project was funded by Canadian Forest Service O&M funds to D.P. and U.S.D.A. Cooperative Agreement 08-JV-11221633-250 to R.H., and supported by the Northern Arizona University School of Forestry and U.S.D.A. Forest Service.

References

Aflitto, N. C., R. W. Hofstetter, R. McGuire, D. D. Dunn, and K. A. Potter. 2014. Technique for studying arthropod and microbial communities within tree tissues. J. Visualized Exp. 93: e50793.

Barr, B. A. 1969. Sound production in Scolytidae (Coleoptera) with emphasis on the genus *Ips*. Can. Entomol. 101: 636–672.

Bentz, B. J., and M. W. Stock. 1986. Phenetic and phylogenetic relationships among ten species of *Dendroctonus* bark beetles (Coleoptera: Scolytidae). Ann. Entomol. Soc. Am. 79: 527–534.

Byers, J. A. 1983. Influence of sex, maturity and host substances on pheromones in the guts of bark beetles, *Ips paraconfusus* and *Dendroctonus brevicomis*. J. Insect Physiol. 29: 5–13.

Clarke, S. R., and J. T. Nowak. 2009. Southern Pine Beetle, Forest insect and disease leaflet 49. United States Department of Agriculture.

Davis, T. S., and R. W. Hofstetter. 2009. Effects of gallery density and species ratio on the fitness and fecundity of two sympatric bark beetles (Coleoptera: Curculionidae). Environ. Entomol. 38: 639–650.

Fleming, A. J., A. A. Lindeman, A. L. Carroll, and J. E. Yack. 2013. Acoustics of the mountain pine beetle *Dendroctonus ponderosae* (Curculionidae, Scolytinae): Sonic, ultrasonic and vibration characteristics. Can. J. Zool. 91: 235–244.

Franceschi, V. R., P. Krokene, E. Christiansen, and T. Krekling. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytol. 167: 353–375.

Gaylord, M. L., T. E. Kolb, K. F. Wallin, and M. R. Wagner. 2006. Seasonality and lure preference of bark beetles (Curcullionidae : Scolytinae) and associates in a northern Arizona ponderosa pine forest. Environ. Entomol. 35: 37–47.

Hofstetter, R. W., Z. Chen, M. L. Gaylord, J. D. McMillin, and M. R. Wagner. 2008. Synergistic effects of *alpha*-pinene and *exo*-brevicomin on pine bark beetles and associated insects in Arizona. J. Appl. Entomol. 132: 387–397.

Hofstetter, R. W., M. L. Gaylord, S. Martinson, and M. R. Wagner. 2012. Attraction to monoterpenes and beetle-produced compounds by syntopic *Ips* and *Dendroctonus* bark beetles and their predators. Agric. For. Entomol. 14: 207–215.

Hofstetter, R. W., J. Dinkins-Bookwalter, T. S. Davis, and K. D. Klepzig. 2015. Symbiotic associations of bark beetles, pp. 309–245. In Vega and Hofstetter (ed.), Bark beetles: Biology and ecology of native and invasive species. Academic Press, Elsevier Publishing, USA.

Hopkins, A. D. 1909. Contributions toward a monograph of scolytid beeltes, p. 164. In Bark beetles of the genus *Dendroctonus*. USDA Bureau of Entomology, Washington, Goverment Print office, WA.

Lanier, G. N., and W. E. Burkholder. 1974. Pheromones in speciation of Coleoptera, pp. 161–189. In M. C. Birch (ed.), Pheromones. Elsevier, Amsterdam.

Lanier, G. N., J. P. Hendrichs, and J. E. Flores. 1988. Biosystematics of the *Dendroctonus frontalis* (Coleoptera: Scolytidae) complex. Ann. Entomol. Soc. Am. 81: 403–418.

Lewis, E. E., and J. H. Cane. 1990. Stridulation as a primary antipredator defence of a beetle. Anim. Behav. 40: 1003–1004.

Lyal, C. H. C., and T. King. 1996. Elytro-tergal stridulation in weevils (Insecta: Coleoptera: Curculionoidea). J. Nat. Hist. 30: 703–773.

McGhehey, J. H. 1968. Territorial behaviour of bark beetle males. Can. Entomol. 100: 1153.

Noor, M. A. 1999. Reinforcement and other consequences of sympatry. Heredity 83: 503–508.

Paine, T. D., M. C. Birch, and P. Sivhra. 1981. Niche breadth and resource partitioning by four sympatric species of bark beetles (Coleoptera: Scolytidae). Oecologia 48: 1–6.

Payne, T. L., J. E. Coster, and P. C. Johnson. 1977. Effects of slow-release formulations of synthetic endo- and exo-brevicomin on southern pine beetle flight and landing behavior. J. Chem. Ecol. 3: 133–141.

Pureswaran, D. S., and J. H. Borden. 2003. Test of semiochemical mediated host specificity in four species of tree killing bark beetles. Environ. Entomol. 32: 963–969.

Pureswaran, D. S., R. W. Hofstetter, and B. T. Sullivan. 2008. Attraction of the southern pine beetle, *Dendroctonus frontalis*, to pheromone components of the western pine beetle, *Dendroctonus brevicomis* (Coleoptera: Curculionidae: Scolytinae), in an allopatric zone. Environ. Entomol. 37: 70–78.

R Development Core Team 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing computer program, version By R Development Core Team, Vienna, Austria.

Raffa, K. F., T. W. Phillips, and S. M. Salom. 1993. Strategies and mechanisms of host colonization by bark beetles, pp. 103–128. In T. D. Schowalter and G. M. Filip (eds.), Beetle-pathogen interactions in conifer forests. Academic Press, New York, NY.

Reid, R. W. 1962. Biology of the mountain pine beetle, *Dendroctonus monticolae* Hopkins, in the east Kootenay region of British Columbia. I. Life cycle, brood development and flight periods. Can. Entomol. 94: 531–538.

Renwick, J. A. A., and J. P. Vité. 1970. Systems of chemical communication in *Dendroctonus*. Contrib. Boyce Thompson Inst. 24: 283–292.

Rudinsky, J. A. 1969. Masking of the aggregation pheromone in *Dendroctonus pseudotsugae* Hopk. Science 166: 884–885.

Rudinsky, J. A., and R. R. Michael. 1972. Sound production in Scolytidae: Chemostimulus of sonic signal by the douglas-fir beetle. Science 175: 1386–1390.

Rudinsky, J. A., and R. R. Michael. 1973. Sound production in Scolytidae: Stridulation by female *Dendroctonus* beetles. J. Insect Physiol. 19: 689–705.

Rudinsky, J. A., and R. R. Michael. 1974. Sound production in Scolytidae: 'Rivalry' behaviour of male *Dendroctonus* beetles. J. Insect Physiol. 20: 1219–1230.

Rudinsky, J. A., M. Morgan, L. M. Libbey, and R. R. Michael. 1973. Sound production in Scolytidae: 3-methyl-2-cyclohexen-1-one released by female Douglas fir beetle in response to male sonic signal. Environ. Entomol. 2: 505–509.

Rudinsky, J. A., L. C. Ryker, R. R. Michael, L. M. Libbey, and M. E. Morgan. 1976. Sound production in scolytidae: female sonic stimulus of male pheromone release in two Dentroctonus beetles. J. Insect Physiol. 22: 1675–1681.

Ryker, L. C. 1984. Acoustic and chemical signals in the life cycle of a beetle. Sci. Am. 250: 113–123.

Ryker, L. C. 1988. Acoustic studies of *Dendroctonus* bark beetles. Fla. Entomol. 71: 447–461.

Ryker, L. C., and J. A. Rudinsky. 1976. Sound production in Scolytidae: Acoustic signals of male and female *Dendroctonus valens* LeConte. Z. Angew. Entomol. 80: 113–118.

Salom, S. M., R. F. Billings, W. W. Upton, M. J. Dalusky, D. M. Grosman, T. L. Payne, C. W. Berisford, and T. N. Shaver. 1992. The effect of verbenone enantiomers and recemic endo-brevicomin on response of *Dendroctonus frontalis* (Coleoptera: Scolytidae) to attractant-baited traps. Can. J. For. Res. 22: 925–931.

SAS 2002-2003. SAS/STAT® Users Guide computer program, version 9.1. By SAS, Cary, NC.

Silverstein, R. M., R. G. Brownlee, T. E. Bellas, D. L. Wood, and L. E. Brown. 1968. Brevicomin: Principal sex attractant in the frass of the female western pine beetle. Science 159: 889–891.

Six, D. L., and R. R. Bracewell. 2015. Dendroctonus, pp. 305–350. In Vega and Hofstetter (ed.), Bark Beetles: Biology and Ecology of Native and Invasive Species. Academic Press, Elsevier Publishing, USA.

Sullivan, B. T. 2005. Electrophysiological and behavioral responses of southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae), to volatiles isolated from conspecifics. J. Econ. Entomol. 98: 2067–2078.

Sullivan, B. T., W. P. Shepherd, D. S. Pureswaran, and K. Mori. 2007. Evidence that (+)-endo-brevicomin is a male produced aggregation pheromone component of the southern pine beetle, *Dendroctonus frontalis*. J. Chem. Ecol. 33: 1510–1527.

Symonds, M. R. E., and M. A. Elgar. 2004a. The mode of pheromone evolution: evidence from bark beetles. Proc. R. Soc. Lond. Ser. B. 271: 839–846.

Symonds, M. R. E., and M. A. Elgar. 2004b. Species overlap, speciation and the evolution of aggregation pheromones in bark beetles. Ecol. Lett. 7: 202–212.

Symonds, M. R. E., and M. A. Elgar. 2008. The evolution of pheromone diversity. Trends Ecol. Evol. 23: 220–228.

Vega, F. E., and R. W. Hofstetter. 2015. Bark beetles: biology and ecology of native and invasive species. Academic Press, Elsevier Publishing, USA.

Vité, J. P., R. F. Billings, C. W. Ware, and K. Mori. 1985. Southern pine beetle: enhancement or inhibition of aggregation response mediated by enantiomers of endo-brevicomin. Naturwissenschaften 72: 99–100.

Wood, D. L. 1982a. The role of pheromones, kairomones and allomones in the host selection and colonization of bark beetles. Ann. Rev. Entomol. 27: 411–446.

Wood, S. L. 1982b. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph, Bringham Young University, Provo, UT.

Yturralde, K. M., and R. W. Hofstetter. 2015. Characterization of stridulatory structures and sounds of the larger mexican pine beetle, *Dendroctonus approximatus* (Coleoptera: Curculionidae: Scolytinae). Fla. Entomol. 98: 516–527.