

Discrimination of Odors Associated With Conspecific and Heterospecific Frass by Sibling Species *Dendroctonus frontalis* (Coleoptera: Curculionidae: Scolytinae) and *Dendroctonus mesoamericanus* (Coleoptera: Curculionidae: Scolytinae)

Alicia Niño-Domínguez,¹ Brian T. Sullivan,^{2,3} Jose H. Lopez-Urbina,¹ and Jorge E. Macías-Sámano¹

¹El Colegio de la Frontera Sur (ECOSUR), Carretera Antigua Aeropuerto km 2.5, Tapachula, Chiapas, CP 30700, México, ²USDA Forest Service Southern Research Station, 2500 Shreveport Highway, Pineville, LA 71360, and ³Corresponding author, e-mail: briansullivan@fs.fed.us

Subject Editor: Dong H. Cha

Received 11 May 2018; Editorial decision 28 August 2018

Abstract

In the Central American region, the aggressive, sibling bark beetles *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae: Scolytinae) and *Dendroctonus mesoamericanus* Armendáriz-Toledano & Sullivan (Coleoptera: Curculionidae: Scolytinae) commonly colonize pines concurrently, and in nature they avoid heterospecific pairing, although it can be produced in the lab. We performed walking arrestment bioassays in the lab to examine the capacity of both sexes of both species to discriminate odors from frass expelled from gallery entrances of either solitary females or conspecific pairs of either species. Males of both species strongly preferred odors of frass from solitary, conspecific females over those of heterospecific females or pairs of either species. Female *D. frontalis* did not discriminate among these frass categories, whereas female *D. mesoamericanus* preferred frass of conspecific females. In gas chromatography–electroantennographic detection and gas chromatography–mass spectrometry analyses, we determined that males of both species could sense a nearly identical spectrum of approximately 16 host- and beetle-produced compounds present in frass of females of one or both species. Only two of these compounds, *endo*-brevicomine and ipsdienol, which were present in frass of female *D. mesoamericanus* and pairs of either species but absent in frass of solitary *D. frontalis* females, qualitatively distinguished these categories. Several known attractants and synergists for either species declined in concentration postpairing. Our results complement earlier research and indicate how semiochemical composition and concentration in frass might mediate male discrimination of attack sites of conspecific, unpaired females. Furthermore, our data indicate that semiochemical responses for walking females differ from those of males and between species.

Key words: syntopy, pheromone, electroantennogram, reproductive isolation, olfactometer

Bark beetles in the genera *Dendroctonus*, *Ips*, and *Scolytus* include some of the most significant biotic disturbance agents and economic pests to forests worldwide (Wood 1982, Grégoire et al. 2015). The more aggressive species can kill healthy trees when attack densities reach sufficient numbers to overcome host defenses (Seybold et al. 2006, Raffa et al. 2016). Many of these aggressive species utilize pheromones to attract and aggregate conspecifics on a single host during a mass attack that sometimes involves thousands of insects. Syntopic coexistence on a single host by sibling species is not uncommon in bark beetles (Zuñiga et al. 1995, 1999; Ayres et al. 2001; Moser et al. 2005; Hofstetter et al. 2012), although classic models of

competition state that simultaneous use of an identical resource by multiple species is unsustainable (Denno et al. 1995). It has been suggested that there may be benefits to syntopy in bark beetles, insofar as, due to the greater overall numbers of attacking beetles, multiple species participating simultaneously in a mass attack event may be more successful in overcoming host defenses than any one species attacking singly (Švihra et al. 1980, Ayres et al. 2001, Økland et al. 2009, Pureswaran et al. 2016). Nonetheless, after mass-attack by coattacking species has rendered a host suitable for colonization, competition for the newly available phloem resource can be severe (Anderbrant et al. 1985, Davis and Hofstetter 2009). Association

of sibling species of bark beetles on the same host can be stable if mechanisms exist to reduce interspecific competition and ensure reproductive isolation. Pheromones have been shown to play a role in achieving both ends. Pheromone components and blends unique to particular species function in discrimination of mates (Lanier and Burkholder 1974, Lanier and Wood 1975, Schlyter et al. 2015, Symonds and Gitau-Clarke 2016). Additionally, pheromone components of competing bark beetle species may be repellent or inhibit attraction, causing avoidance of portions of the bark colonized by competitors and thereby promoting partitioning of the host bole (Byers and Wood 1980, Byers 1989, Ayres et al. 2001, Birgersson et al. 2012).

Dendroctonus frontalis Zimmermann (the southern pine beetle; Coleoptera: Curculionidae: Scolytinae) and *Dendroctonus mesoamericanus* Armendariz-Toledano & Sullivan (Coleoptera: Curculionidae: Scolytinae) are aggressive bark beetle species that attack *Pinus* and are sympatric in parts of Mexico (states of Michoacán, Oaxaca, and Chiapas), Guatemala, Belize, El Salvador, Honduras, and Nicaragua (Armendariz-Toledano et al. 2014, 2015). Both species are commonly encountered in the same hosts and can participate simultaneously in mass attacks. Although there is species overlap in colonization over the bole and crown of the host, *D. frontalis* attacks are concentrated in the middle and upper portions of the host, and *D. mesoamericanus* in the lower three meters (Moreno 2008; authors' unpublished data). Although hybrid crossings that result in larval brood can be produced in the laboratory, such crosses have not been found in nature (Armendariz-Toledano et al. 2014). These observations imply that coexistence of these species is mediated by behavioral mechanisms that reduce competition and promote reproductive isolation.

Evidence indicates that conspecific attraction and avoidance of heterospecific pairing within syntopic populations of *D. frontalis* and *D. mesoamericanus* are mediated by semiochemicals, and, furthermore, these species possess similarities and differences in their systems of chemical communication that likely favor their coexistence (Sullivan et al. 2012; Niño et al. 2015, 2016). In *Dendroctonus*, females initiate attacks on hosts by boring into the bark and releasing one or more attractive pheromone components that function in procuring a mate (i.e., by attracting a male into the female-initiated gallery) and aggregating conspecifics of both sexes for promoting a host-killing mass-attack (Skillen et al. 1997, Sullivan 2011, Six and Bracewell 2015). Arriving males commonly produce their own, sometimes distinct, pheromone components that may either enhance or inhibit conspecific aggregation (Skillen et al. 1997). Analyses of odors from gallery entrances or derived from excrement and hindgut extracts of beetles indicate that female *D. frontalis* produce the pheromone component frontalin, and female *D. mesoamericanus* additionally produce *endo*-brevicommin and ipsdienol; males of both species produce *endo*-brevicommin and some ipsdienol (although the latter is found in *D. mesoamericanus* males in merely trace amounts) (Sullivan et al. 2012, Niño-Domínguez et al. 2015; ipsdienol has not been detected in *D. frontalis* of the southeastern United States). These three compounds are likely essential to intraspecific and interspecific interactions of these species. In walking olfactometer tests, males of both species were attracted to the combination specifically produced by conspecific but not heterospecific females (Niño-Domínguez et al. 2015): frontalin was much more attractive to *D. mesoamericanus* males in the presence of *endo*-brevicommin and ipsdienol, whereas these two compounds greatly reduced attraction of *D. frontalis* males to frontalin alone (all combinations included *alpha*-pinene as a host component). It is proposed that this difference in response explains the greater attraction of walking

males of either species to odors of gallery entrances of conspecific but not heterospecific females (Niño-Domínguez et al. 2015), and thereby restricts heterospecific pairings and promotes reproductive isolation.

The research presented here was intended to complement a previous laboratory study on the semiochemical interactions between *D. frontalis* and *D. mesoamericanus* (Niño-Domínguez et al. 2015). In the present study, we examined specifically expelled frass as a source of semiochemicals influencing behavior of walking beetles. Frass consists of a mixture of fecal pellets containing pheromone (Borden et al. 1968, Silverstein et al. 1968) and host odor-releasing fragments of phloem tissue, and it is encountered by alighted beetles walking on the bark surface of attacked trees, particularly near gallery entrances. Frass and frass extracts have been used previously in laboratory assays to study semiochemical responses in *Dendroctonus* (Hunt and Borden 1988, Paine et al. 1999), and frass can be conveniently manipulated for experiments. The present study was intended to: 1) Establish the reproducibility of earlier olfactometer observations of species discrimination by mate-seeking males (Niño-Domínguez et al. 2015) when using an olfactometer of substantially different design. In the earlier research, the olfactometer delivered odor in a continuous airstream and stimulated anemotaxis. In the olfactometer of the present study, odor arose passively from the substrate and elicited arrestment, and this latter design may better reflect naturally occurring encounters of males with pheromone sources while searching the bark. 2) Characterize behavioral responses of either species to semiochemicals associated with paired beetles and determine the composition of these compounds. Male *Dendroctonus* may produce pheromone components postpairing that modify conspecific attraction to female components (Skillen 1997) and can play a role in terminating attraction of males to females that have paired, including in *D. frontalis* (Rudinsky 1973, Rudinsky et al. 1974, Mccarty et al. 1980). 3) Investigate whether females also may use frass odors for close-range orientation on the bark surface. Having alighted on the bark surface, males primarily are seeking mates, whereas females are seeking sites for initiating galleries. Thus, we would expect semiochemical responses to differ between the sexes during walking and close-range encounter of odor sources even though they might not differ in-flight during host seeking. Our general hypothesis was that semiochemical production and response in *D. frontalis* and *D. mesoamericanus* have evolved to promote successful host colonization while reducing conflicts possibly resulting from cohabitation on the same host.

Materials and Methods

Logs (15–20 cm diam.) of *Dendroctonus*-infested and uninfested *Pinus oocarpa* Schiede (Pinales: Pinaceae) were collected in Parque Nacional Lagunas de Montebello, Chiapas, Mexico (16° 07' N, 91° 44' W). Infested logs were placed into cloth bags, and emerging adult beetles were collected daily and held in plastic Petri dishes with moist Kimwipe (Kimberly-Clark, Roswell, GA) at 10°C. Adults were ≤3 d old when used in bioassays. Prior to bioassays, uninfested logs were stored at 10°C for ≤10 d.

Adult *D. frontalis* and *D. mesoamericanus* were distinguished by external diagnostic characters (Sullivan et al. 2012, Armendariz-Toledano et al. 2015). Following bioassays, a subsample of males was dissected to confirm identifications by using diagnostic characters of genitalia (Armendariz-Toledano et al. 2014, 2015). All work was performed in a laboratory at 24°C, ~48% RH, and with fluorescent ceiling lighting.

Olfactometer Trials

Frass was amassed from individual females or conspecific pairings of either species forced to attack bark of uninfested logs. A single female was placed into a hole (1.5-mm diam.) drilled into the bark and subsequently covered with a piece of plastic screen secured in place with tape. For sampling of beetle pairs, a male was added to the hole 4 h following introduction of the female. After female penetration into the log or addition of the male, the screen barrier was eliminated and the entrance covered by the opening of a glass vial (2 ml) into which was collected frass ejected from the entrance. The gallery entrance was covered by two vials consecutively for 24 h each. Following their removal, vials were closed with PTFE-lined caps and stored at -20°C for <5 d.

The olfactometer (Supp Fig. 1 online only) consisted of an ELISA plate (polystyrene, 96 wells of 360- μl volume each, 0.7-cm well diameter, 1.2-cm depth; Corning Inc., Corning NY, part # M0661) whose surface was covered by a rectangle of fine white screen (nylon cloth, 40 threads per cm) held taught by an acrylic frame to form a flat arena surface. The screen surface was divided into four equal, rectangular quadrants (4 \times 6 cm; each covering 4 \times 6 = 24 total wells) within the area of the plate surface, with a single sample well within each quadrant (i.e., B4, B9, G4, and G9; the center of these four wells was located 31 mm from the common vertex of the quadrants; Supp Fig. 1 online only) designated for filling with frass (or left empty as controls). Frass combined from both vials of a single entrance hole (a mean of 16 and 42 mg of frass was produced by *D. frontalis* and *D. mesoamericanus* females, respectively; pairs produced slightly more) was added to the designated well of one (for single odor assays) or two opposite or all four (for odor choice assays) quadrants. (*D. mesoamericanus* are on average larger than *D. frontalis* and generate frass at a higher rate; hence, the amount of frass added to wells [i.e., the total produced in 48 h] differed for the two species.) Both the frass origin and the quadrants receiving frass were chosen randomly for each set of trials. During each bioassay trial, a single beetle was released at the common vertex of the quadrants, and the total time spent subsequently by the beetle in each of the four quadrants was recorded for 5 min following release. Thus, the response variable was the duration of arrestment caused by odors of frass within the given quadrant.

Frass from a single female or pair was used for a set of 8–12 sequential trials (40–60 min duration). The ELISA plates were used for one set and then discarded, and the order of species and sexes of beetles bioassayed for each set and experiment were randomized. Two groups of experiments were performed. The first (June 2012) measured male arrestment by frass of solitary females of either species presented singly or as a two-way choice (frass portions were first tested singly and then transferred to a clean ELISA plate and used in the two-way choice trials), whereas the second group (May 2017) measured both male and female preferences in a four-way choice among frass of solitary females and intraspecific pairings of either species. Assays were performed on a lab bench under ambient laboratory conditions.

Volatiles Sampling From Frass

Immediately after a set of bioassay trials with a single unit of frass, volatiles from the frass were sampled for 3 h onto adsorbent cartridges (117-mg Porapak-Q; SKC Inc., Eighty Four, PA). Frass from each individual ELISA well was placed into a 12-cm (0.4 cm i.d.) length of glass tubing and confined between glass wool plugs. One end of the frass-holding tube was connected by PTFE tubing to a source of purified nitrogen (50 ml/min) and the other end to an

adsorbent cartridge. To enhance the release of volatiles from the frass, the frass-containing tube was secured inside an aluminum block heated to 60°C . Cartridges were desorbed with 1.5-ml pentane (HPLC grade, Sigma-Aldrich Co., Milwaukee, WI), and extracts were spiked with 3.8 μg of cycloheptanone (98%, Sigma-Aldrich) as an internal standard. In total 12–15 extracts were collected (each derived from frass of a different female or pair) for each species from the first (solitary females) and second (solitary females and pairs) groups of experiments.

Olfactory Responses of Male Antenna

Male olfactory sensitivity to components within volatiles collected from frass of solitary females (first group of experiments) was assessed by coupled gas chromatography–electroantennographic detection (GC-EAD). The GC-EAD apparatus and procedures for preparing antenna were same as Cano-Ramírez et al. (2012). The GC had a helium ionization detector (HID) and an HP-INNOWax microcapillary column (Agilent Technologies, Wilmington, DE; polyethylene glycol; 30-m long, 0.25-mm diam., 25- μm film thickness) and operated with a temperature program of 50°C for 1 min, $16^{\circ}\text{C}/\text{min}$ to 80°C , and then $7^{\circ}\text{C}/\text{min}$ to 200°C held 10 min. Aliquots (100 μl) from each of 10–12 of the cartridge-derived samples were combined within species and concentrated ~ 10 -fold passively on a bench-top. Antenna of five male *D. frontalis* and six male *D. mesoamericanus* were each tested with pooled aeration samples of female frass of either species (2 μl injected into GC). An EAD impulse detected at the same retention time in four or more GC-EAD runs was considered to represent a genuine olfactory response stimulated by a compound in the sample. Putative olfactory stimulants (i.e., HID peaks coinciding with EAD spikes and later identified by gas chromatography–mass spectrometry [GC-MS]) were in most cases confirmed in their activity through GC-EAD analyses performed with authentic standards. (Compounds lacking such confirmation are indicated in the results.) Antennal preparations were used once and discarded.

Quantification of Odorants

Volatiles collections from frass were analyzed by GC-MS (Hewlett-Packard model G1800C, Palo Alto, CA) by using an identical column, temperature program, and carrier gas flow as used for GC-EAD analyses. Compounds were identified by matches of retention times and mass spectra with those obtained from authentic standards (suppliers: Sigma-Aldrich; Synergy Semiochemicals, Burnaby, BC; Bedoukian Research, Danbury, CT). Identified olfactory stimulants detected in the samples were quantified against a calibration curve constructed from a dilution series of standards (both the dilution sequence and each sample were spiked with an identical quantity of internal standard).

Statistical Analyses

Male occupancy times in each olfactometer quadrant could not be transformed to meet test assumptions due to the large number of zeroes; therefore, we used a GLM with a negative binomial distribution (Lindén and Mäntyniemi 2011) and a logarithmic link function for all pairwise comparisons. A Wilcoxon one-sample test having a hypothetical median of zero and a 95% confidence interval was employed to contrast frass-containing and frass-free quadrants when the latter had zero occupancy times. The overdispersion was evaluated from deviance residual values respecting degrees of freedom of the model. In the single-odor olfactometer bioassays, a significant response to frass was recorded when mean occupancy

time in the frass-containing quadrant exceeded the average occupancy of the three frass-free quadrants; relative responsiveness to different frass types was established by comparing occupancy times of the frass-treated quadrants in each type of single-choice assay. For choice tests (different frass types in different quadrants), preferences were identified by comparison of occupancy times among quadrants. In GC-EAD analyses, the mean height of the EAD voltage deflections associated with single HID peaks present in odors from females of either species was contrasted within species of responding male. Contrasts were performed with a *t*-test for normally distributed data or with a Kolmogorov–Smirnov test otherwise. For quantitative GC-MS analyses, data were log₁₀-transformed and within each compound were compared among frass categories with either a *t*-test (first group of experiments) or a two-way ANOVA whose factors were the frass category and set of trials in which the frass was used (second set of experiments), followed by a Tukey test for all-pairwise contrasts. When the quantity detected for a particular compound and frass category was zero, this treatment was removed from the parametric analyses and all-pairwise contrasts between the zero-quantity and positive quantity treatments were performed with a Fisher's exact test with Bonferroni correction for multiple contrasts on the proportions of samples with or without the specific compound. For all statistical tests, $\alpha = 0.05$.

Results

Olfactometer Bioassays

In single-odor tests of male responses to female frass odors (the first group of bioassays), both *D. frontalis* (Fig. 1A) and *D. mesoamericanus* (Fig. 1B) males spent significantly more time in the quadrant above the single, frass-filled ELISA plate well than the other quadrants (i.e., without frass-filled wells) when the frass was from conspecific females (for *D. frontalis* males: $D = 71.5$, $gl = 142$; $X^2_{0.05,1} = 4.06$, $P = 0.044$; for *D. mesoamericanus* males: $D = 43.2$, $gl = 186$;

$X^2_{0.05,1} = 9.04$, $P = 0.003$) but not heterospecific females (for *D. frontalis*: $D = 102$, $gl = 142$; $X^2_{0.05,1} = 3.50$, $P = 0.061$; for *D. mesoamericanus*: $D = 81.9$, $gl = 174$; $X^2_{0.05,1} = 3.43$, $P = 0.064$). For males of both species, time spent within the frass-containing quadrant was significantly greater when the frass was from conspecific rather than heterospecific females (for *D. frontalis*: $D = 70.0$, $gl = 70$; $X^2_{0.05,1} = 6.90$, $P = 0.009$; for *D. mesoamericanus*: $D = 89.0$, $gl = 89$, $X^2_{0.05,1} = 6.64$, $P = 0.001$). In an odor choice test (i.e., when frass-filled wells were available simultaneously in opposite quadrants, Fig. 2), males of both species spent significantly more time in the quadrant with frass of conspecific females than heterospecific females (for *D. frontalis*: $D = 62.7$, $gl = 101$; $X^2_{0.05,2} = 37.3$, $P < 0.001$; for *D. mesoamericanus*: $D = 137$, $gl = 161$, $X^2_{0.05,2} = 13.0$, $P = 0.002$). In the second set of bioassays (four-odor tests with frass from solitary females as well as pairs), males of both species discriminated among quadrants (for *D. frontalis*: $D = 240$, $gl = 240$; $X^2_{0.05,3} = 109.4$, $P < 0.001$; for *D. mesoamericanus*: $D = 164$, $gl = 164$; $X^2_{0.05,3} = 150.4$, $P < 0.001$). Male *D. frontalis* were arrested for greater time in the quadrant with conspecific female frass than any other frass category, whereas there were no significant differences in arrestment time among quadrants with frass from pairs of either species or solitary female *D. mesoamericanus* (Fig. 3A). Similarly, *D. mesoamericanus* males were arrested over quadrants with frass of solitary, conspecific females longer than with other frass categories; they preferred frass of *D. frontalis* pairs over conspecific pairs but did not prefer either over frass of solitary female *D. frontalis*. Responses by females of both species differed from those of males. Female *D. frontalis* did not discriminate among frass odors ($D = 72$, $gl = 72$; $X^2_{0.05,3} = 2.537$, $P = 0.469$), whereas *D. mesoamericanus* females displayed preferences ($D = 72$, $gl = 72$; $X^2_{0.05,3} = 23.127$, $P < 0.001$). Residence time by *D. mesoamericanus* females was significantly greater over frass of conspecific females than over other frass categories, and less over frass of solitary *D. frontalis* females than frass of pairs of either species (and the latter did not differ) (Fig. 3B).

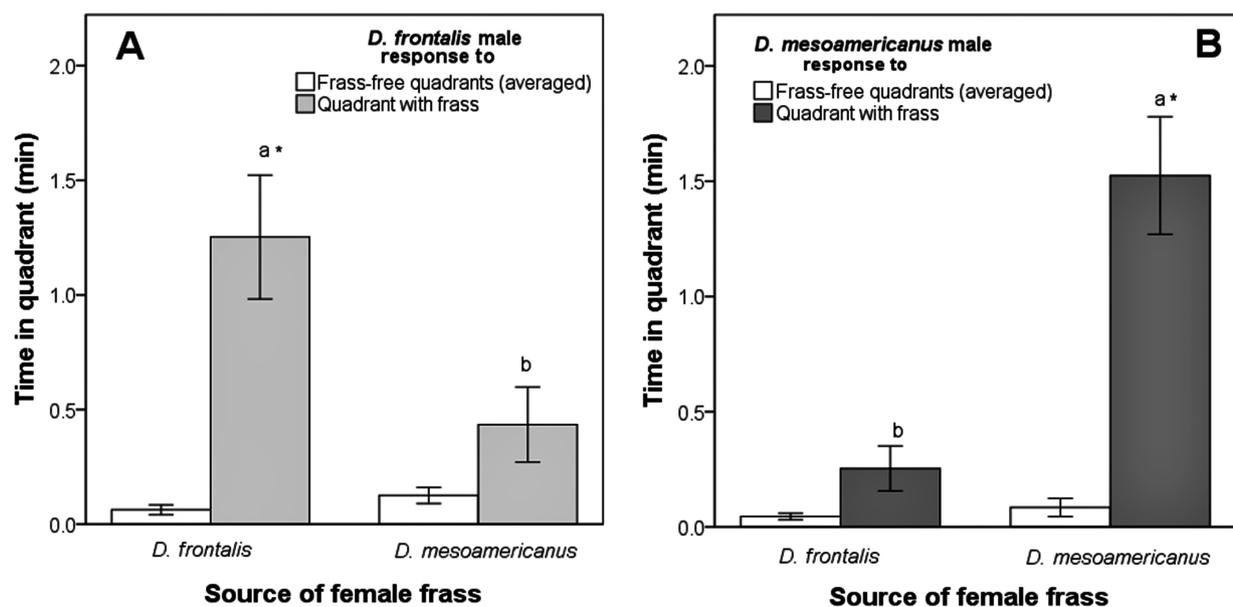


Fig. 1. Mean (\pm SE) time spent by walking male *D. frontalis* (A) and *D. mesoamericanus* (B) within quadrants dividing the arena of a screen-floored platform olfactometer. Female frass was placed into a plastic well underneath one of the four (5.4×7.3 cm) quadrants, and a male beetle was then released at the common vertex of the quadrants. The times spent by the males within the three quadrants without frass (controls) were averaged for statistical comparisons. An asterisk indicates that the arrestment time within the frass-treated quadrant was significantly greater than the average time in the control quadrants; treatments labeled with different lower-case letters differed significantly in mean arrestment duration (GLM with negative binomial distribution and logit function, with $\alpha = 0.05$).

Electrophysiological Responses (GC-EAD)

We detected antennal responses in males of one or both species at 15 different retention times in GC-EAD analyses of frass odors of females of either species (Fig. 4). All but one of these retention times coincided with HID peaks that GC-MS analyses indicated were single compounds. There were at least three coelutents at this retention time (Fig. 4, peak 13): ipsdienol (in *D. mesoamericanus* frass),

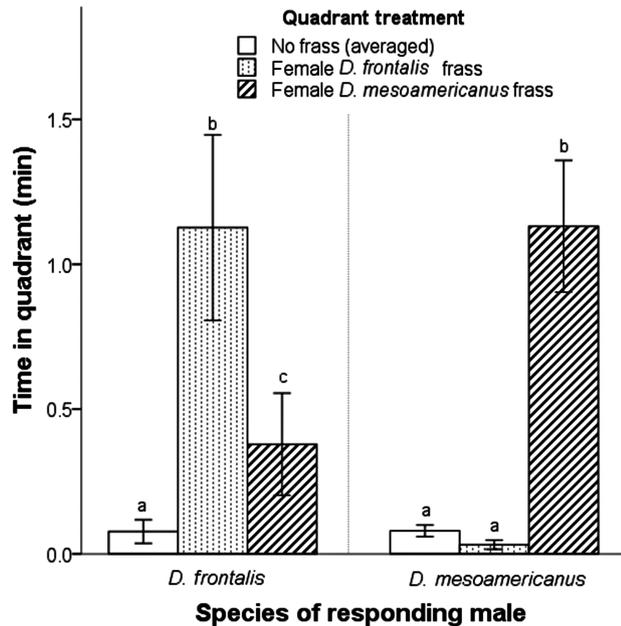


Fig. 2. Mean (\pm SE) time spent by walking male *D. frontalis* and *D. mesoamericanus* within quadrants dividing the arena of a screen-floored platform olfactometer. Frass from females of either species was placed into single plastic wells beneath two opposite quadrants, and a male beetle was then released at the common vertex of the quadrants. The times spent by the males within the two quadrants without frass (controls) were averaged for statistical comparisons. Within each species of male, treatments labeled with different lower-case letters differed significantly in mean arrestment duration (GLM with negative binomial distribution and logit function, with $\alpha = 0.05$).

trans-verbenol, and 4-allylanisole (standards of which produced EAD responses in both species). Males of both species generally responded to the same compounds albeit with somewhat different EAD response amplitudes. For males of both species, olfactory stimulants present in female frass of both species did not apparently differ qualitatively except at the retention time of *endo*-brevicomin, which occurred only with frass of *D. mesoamericanus* females. A weak EAD response at the retention time of nonanal occurred only for male *D. frontalis* and only with odors from frass of *D. mesoamericanus* females.

For males of both species, significant variation was observed in the amplitude of EAD responses to compounds collected from frass of *D. frontalis* females (for *D. frontalis* males: $F_{0.05,10} = 4.90$, $P < 0.001$; for *D. mesoamericanus* males: $F_{0.05,12} = 3.26$, $P = 0.002$) and *D. mesoamericanus* females (for *D. frontalis* males: $F_{0.05,15} = 3.89$, $P < 0.001$; for *D. mesoamericanus* males: $F_{0.05,12} = 20.8$, $P < 0.001$). In female frass of both species, *alpha*-pinene, frontalinal, *endo*-brevicomin (only from frass of female *D. mesoamericanus*), and peak 13 generally elicited the strongest antennal responses in males of both species (Fig. 4).

Quantification of Olfactory Stimulants

Compounds indicated as olfactory stimulants present in female frass (Fig. 4) were quantified in volatiles samples (Figs. 5 and 6). Of these, the host compounds *alpha*-pinene, longifolene, and 4-allylanisole were the most abundant in all samples. In all contrasts between frass of solitary females of either species (i.e., from both the first [females only] and second [females and pairs] groups of experiments), there were no significant differences in concentrations of any of the presumably host-derived compounds (Figs. 5A and 6A). However, for the second bioassay group, frass of *D. mesoamericanus* pairs generally had lower concentrations of host compounds than frass of solitary *D. mesoamericanus* females (i.e., for *alpha*-pinene, *beta*-pinene, myrcene, limonene, longifolene, terpinen-4-ol, and 4-allylanisole). Frass of solitary female and paired *D. frontalis* did not differ significantly from each other or solitary/paired *D. mesoamericanus* in concentrations of any of the host compounds.

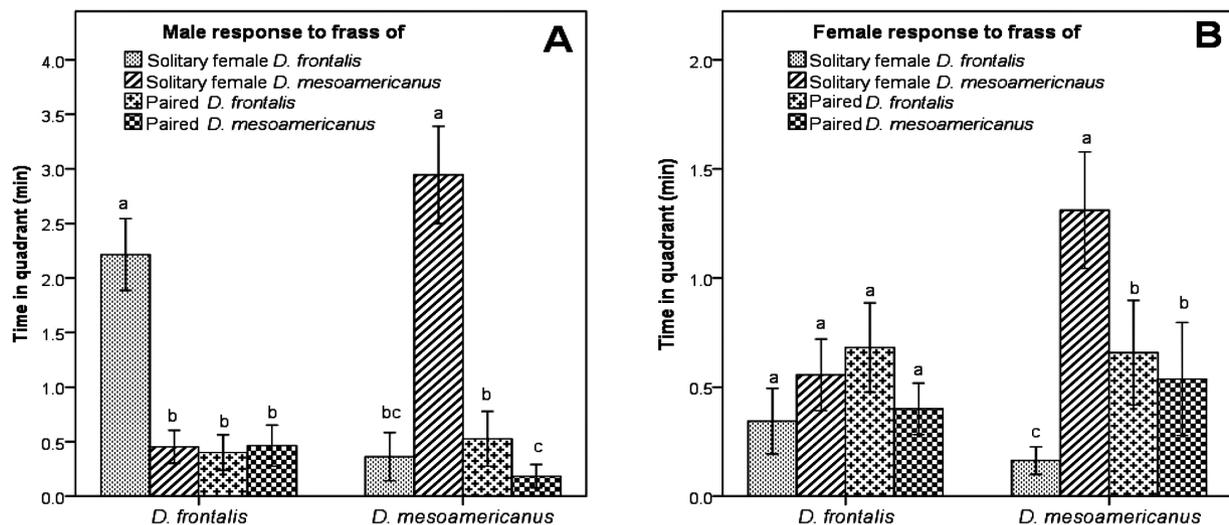


Fig. 3. Mean (\pm SE) time spent by walking males (A) and females (B) of *D. frontalis* and *D. mesoamericanus* within quadrants dividing the arena of a screen-floored platform olfactometer. Frass produced by single females and conspecific pairs from each species was placed into wells beneath each quadrant, and a single beetle was then released at the common vertex of the quadrants. Treatments labeled with different lower-case letters differed significantly in mean arrestment duration (GLM with negative binomial distribution and logit function, with $\alpha = 0.05$).

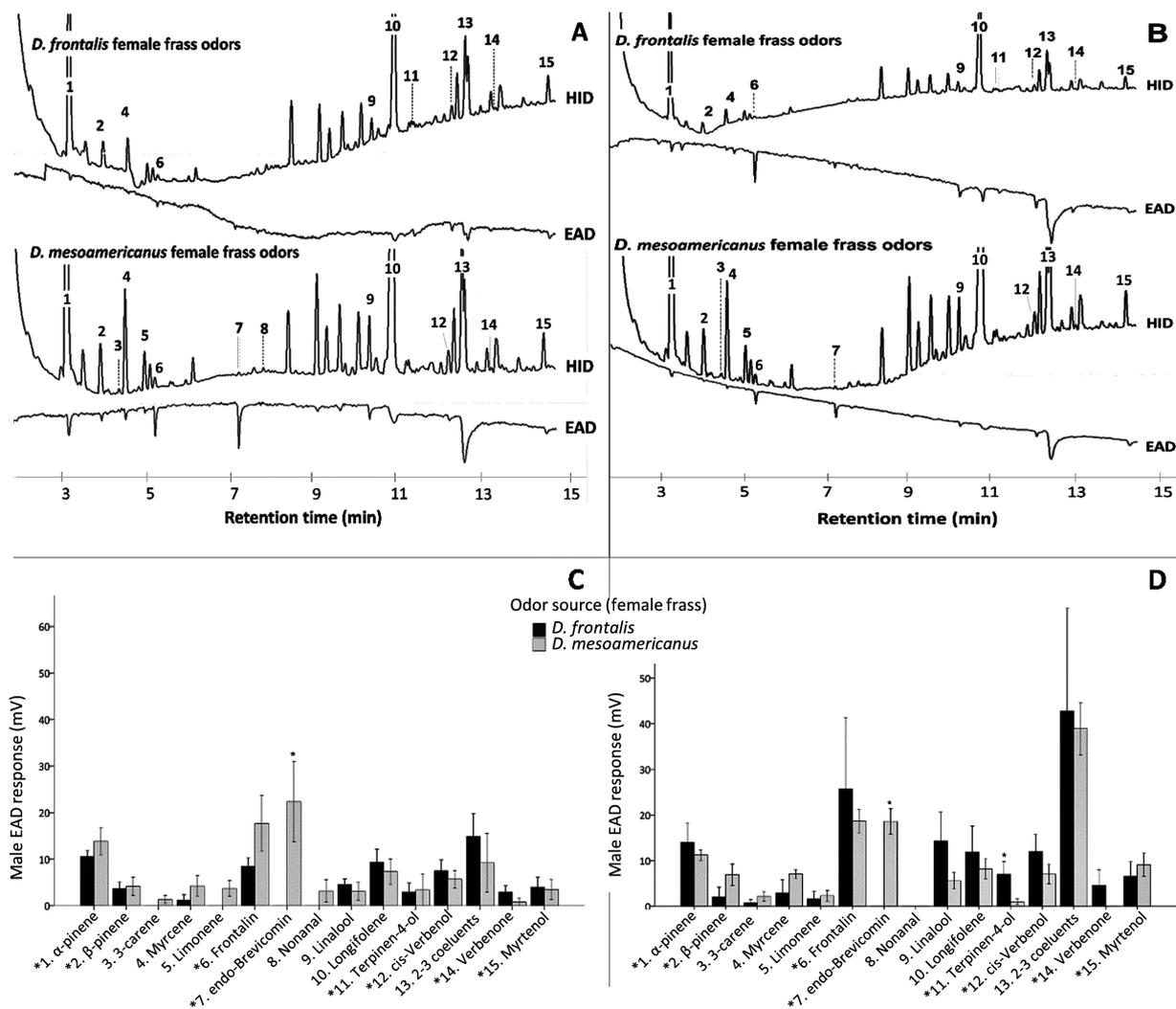


Fig. 4. Coupled GC-EAD analyses with the antennae of male *D. frontalis* (A and C) and *D. mesoamericanus* (B and D) responding to volatiles collected from frass generated by solitary females of either species mining in a log. In (A) and (B), the upper trace of each pair is the output of a universal GC detector (a helium ionization detector, HID) and the bottom trace is the antenna response (electroantennographic detector, EAD). Asterisks associated with columns indicate a statistically significant difference (*t*-test or Kolmogorov–Smirnov test; $\alpha = 0.05$) in EAD response amplitudes to odors of females of either species for the indicated compound. Asterisks associated with a compound name indicate that its electrophysiological activity was confirmed in GC-EAD trials with commercially obtained standards of the compound. Peak 13 contained coeluting *trans*-verbenol, 4-allylanisole, and ipsdienol (present only in *D. mesoamericanus* samples).

With respect to putatively beetle-produced olfactory stimulants identified in frass of solitary females, all were detected (with a threshold of 0.01 $\mu\text{g}/\text{sample}$) in frass of at least some of the solitary female and paired *D. mesoamericanus* and paired *D. frontalis* (Figs. 5B and 6B). However, *endo*-brevicomine and ipsdienol were not detected in frass of solitary female *D. frontalis*, and these compounds were detected in a significantly greater proportion of samples in all three of the other treatment categories except for *endo*-brevicomine in frass of *D. mesoamericanus* pairs (detected in only five of 15 samples; Fisher exact test, $P = 0.126$). Frass of solitary female and paired *D. mesoamericanus* and paired *D. frontalis* did not differ in quantities of *endo*-brevicomine (Fig. 6B); however, frass of solitary female *D. mesoamericanus* had significantly higher quantities of ipsdienol than did frass of paired *D. frontalis* or paired *D. mesoamericanus* (Fig. 6B), and the latter two did not differ significantly. In the first group of bioassays (Fig. 5B), frass of solitary female *D. mesoamericanus* contained more frontalin than frass of solitary female *D. frontalis*, but these categories did not differ in the second set of tests (Fig. 6B). Frass of solitary female *D. frontalis* (but not solitary

female *D. mesoamericanus*) had more frontalin than frass of pairs of either species (Fig. 6B). Frass of solitary females of both species did not differ significantly in quantities of *cis*-verbenol, *trans*-verbenol, verbenone, or myrtenol (Figs. 5B and 6B). However, in the second group of bioassays, frass of *D. mesoamericanus* pairs contained significantly less *cis*- and *trans*-verbenol than the other three categories, and less myrtenol than *D. frontalis* pairs or solitary female *D. mesoamericanus* (Fig. 6B).

Discussion

The results of our tests for arrestment responses of walking males to frass odors arising from the substrate are essentially consistent with those of our previous study in which walking males of both species were assayed in the laboratory for response to a moving stream of air that had been passed across a female gallery entrance (Niño-Domínguez et al. 2015). This consistency suggests that presence of odors in moving versus ostensibly stationary air does not alter response of walking beetles, and that isolated frass generates

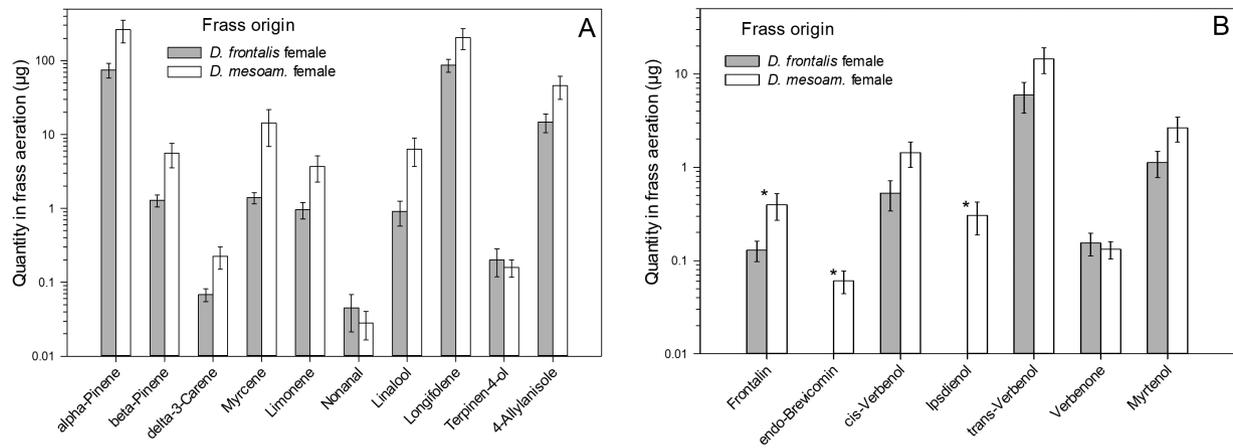


Fig. 5. Mean (\pm SE) quantities of compounds identified in volatiles from frass of solitary, mining females of either *D. frontalis* or *D. mesoamericanus*. Odors were sampled immediately after use of frass in the bioassays testing odors of female frass (Figs. 1 and 2). Volatiles are graphed separately for (A) compounds presumably arising from the host and (B) known *Dendroctonus* pheromone components (all have shown behavioral activity at least with *D. frontalis*; Sullivan 2011; Niño-Domínguez 2015, 2016). Asterisks indicate a significant difference in quantities detected from either species ($\alpha = 0.05$). Statistical contrasts were with a *t*-test on log-transformed quantities except for *endo*-brevicomin and ipsdienol for which proportions of beetles from which the compound was detected were contrasted with a Fisher's exact test.

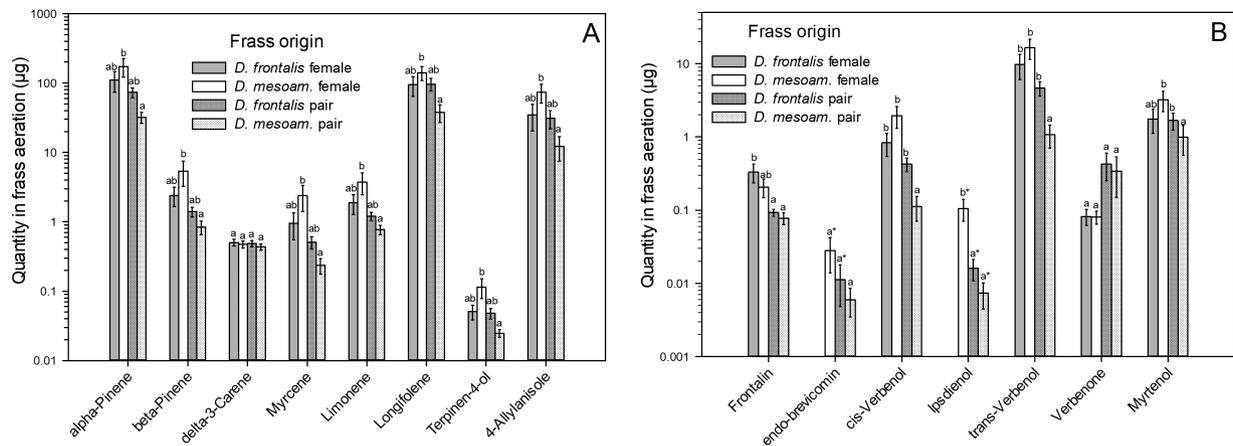


Fig. 6. Mean (\pm SE) quantities of compounds identified in volatiles from frass of solitary females or intraspecific pairings of either *D. frontalis* or *D. mesoamericanus*. Odors were sampled immediately after use of frass in the bioassays with all four categories of frass (Fig. 3). Host (A) and beetle (B) compounds are graphed separately (Fig. 5 legend). Means associated with the same lower-case letter did not differ significantly ($P \leq 0.05$) among frass categories within compound according to an ANOVA followed by an all-pairwise Tukey test on the log-transformed data. Because ipsdienol and *endo*-brevicomin were beneath the threshold of detection for all samples of solitary female *D. frontalis* frass, only the three remaining frass categories could be analyzed in the ANOVA, and these categories were contrasted with solitary female *D. frontalis* by using Fisher's exact tests of proportions of beetles from which the compound was or was not detected (asterisk indicates $P \leq 0.05$ for the contrast).

a similar response as odors derived directly from the active gallery entrance. In both the present and past studies, males of both species responded positively to odors associated with conspecific females, and they preferred odors of conspecific females over those of heterospecific females (this study, Figs. 1, 2, and 3; Niño-Domínguez et al. 2015, Figs. 1 and 2). Additionally, a weak cross-attractive response was evident in both studies (e.g., male *D. frontalis* were arrested to some extent by odors of *D. mesoamericanus* females; present study, Fig. 2; Niño-Domínguez et al. 2015, Fig. 2). Furthermore, the present study indicates that males of both species discriminate between frass from gallery entrances containing solitary versus paired, conspecific females through olfaction since they were more strongly arrested by odors of the former. Thus, our data indicate that semiochemicals in frass allow males of both species to distinguish locations of females that are both available (unpaired) and suitable (correct species) for pairing.

Females of both species were apparently less discriminating of frass odors than males (Fig. 3). This difference is consistent with females being the gallery-initiating and 'calling' sex in *Dendroctonus* (Wood 1982, Six and Bracewell 2015). Although female *D. frontalis* exhibited no discrimination among frass of different origins (and generally had a weak response), *D. mesoamericanus* females displayed the same preference as conspecific males for odors of frass from solitary, conspecific females. This contrasting behavior between females of the two species is not reflected in odor discrimination by flying beetles, as females (and males) of both species are more attracted to traps baited with logs infested with *D. mesoamericanus* than *D. frontalis* females (Niño-Domínguez et al. 2016). Responses distinctive to walking rather than flying bark beetles may reflect behaviors postlanding on the host or otherwise at very close range to the odor source (Byers 2012). Accordingly, it is possible that females of the two species have divergent strategies postlanding

for locating sites for establishment, with pheromones of previously established beetles playing a stronger role for *D. mesoamericanus* females.

The behavior of the beetles in response to the different frass categories was associated with differences in composition of olfactory stimulants present in frass volatiles. Qualitatively, this difference was the presence of ipsdienol and *endo*-brevicomin in frass of female *D. mesoamericanus* and pairs of either species but not *D. frontalis* females. (Our failure to detect these compounds in frass from some galleries of female *D. mesoamericanus* and pairs of either species could be attributed to the volatilization of these compounds from the frass during the behavioral bioassays which preceded volatiles collections. Also, individual variation in pheromone production by bark beetles can be quite large [Schlyter and Birgersson 1989, Pureswaran et al. 2008].) This difference was observed previously for odors from entrances of solitary females of the two species (Niño-Domínguez et al. 2016). Both of these compounds have been isolated from newly established female *D. mesoamericanus* and male *D. frontalis*, and newly established male *D. mesoamericanus* produce *endo*-brevicomin and trace ipsdienol (Sullivan et al. 2012, Niño-Domínguez et al. 2016). Thus, the pheromone components we detected in frass of pairs reflected the sum of those shown to be produced separately by either sex of each species (Pureswaran and Sullivan 2012, Pureswaran et al. 2016).

endo-Brevicomin and ipsdienol inhibit responses by walking male *D. frontalis* to conspecific female-associated attractants (Rudinsky et al. 1974, Niño-Domínguez et al. 2015), but enhance attraction of walking male *D. mesoamericanus* (Niño-Domínguez et al. 2015). It is possible that differences in the presence of these compounds mediated discrimination of frass odors by males of both species in our bioassays. In particular, it could explain discrimination of frass of solitary, female *D. frontalis*, which was the only frass category which completely lacked these compounds. However, we had hypothesized that production of *endo*-brevicomin and ipsdienol by *D. frontalis* males after pairing with females (Sullivan et al. (2012) and the present study) might stimulate attraction by male *D. mesoamericanus* to *D. frontalis* pairs. Such incidental, one-way cross-attraction presumably would interfere with mate location by *D. mesoamericanus* males and be a factor that might destabilize coexistence of the two species. However, male *D. mesoamericanus* were not more attracted to frass from *D. frontalis* pairs than to frass from solitary *D. frontalis* females (which was not significantly attractive), and thus, we saw no evidence of this possible interference. Likewise, frass of *D. mesoamericanus* pairs contained all three attractive pheromone components for this species but did not apparently stimulate a response from males.

Levels of pheromone components with demonstrated attractiveness to walking males (i.e., frontalin for both species; ipsdienol and *endo*-brevicomin for *D. mesoamericanus*; Niño-Domínguez et al. 2015) were generally lower in frass of pairs compared with frass of solitary females. Production of pheromones by *Dendroctonus* has been observed generally to decline rapidly following pairing and establishment (Byers et al. 1984, Pureswaran and Sullivan 2012). This pattern of decline in our data also included *cis*-verbenol and *trans*-verbenol which have been shown to be attractant synergists for *D. frontalis* populations in the southeastern United States (Sullivan 2011). This decline could have been an important factor in the loss of attractiveness of frass following pairing. Once a mass attack has rendered the host available for colonization and the sexes have paired, there is ostensibly no further benefit to their continuing to release attractive pheromone components. It is also possible that pairs produce attraction inhibitors (Byers 1989). Mean levels of verbenone, an attraction inhibitor for *D. frontalis* (Sullivan 2011),

increased several-fold in frass of both species postpairing, although this apparent change was not statistically significant. Curiously, female *D. mesoamericanus* did respond more strongly to frass of both *D. frontalis* and *D. mesoamericanus* pairs than frass of solitary female *D. frontalis*, a result consistent with female attraction to ipsdienol and *endo*-brevicomin in the former.

In conclusion, our experiments indicated that semiochemical discrimination by walking males should reduce the potential for interspecific semiochemical interference during mate finding, and they provide further evidence that semiochemical responses by the two species are concordant with syntopy. Additionally, we found no evidence for the existence of candidate mate and host location semiochemicals for either *D. frontalis* or *D. mesoamericanus* that may have been overlooked in previous studies.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

Acknowledgments

CONACYT provided the doctoral fellowship to A. Niño (grant 316670), and research support was from USDA Forest Service Cooperative Agreement 11-IC-11330129-046. Thanks to Gerardo Zúñiga, Pablo Liedo Fernandez and Leopoldo Cruz López for their guidance as committee members to A. Niño; and the Comisión Nacional de Áreas Naturales Protegidas (CONANP) and officials and staff of Parque Nacional Lagunas de Montebello for use of facilities and cooperation in obtaining insects for experiments. Special thanks to Don Roberto Castellanos for being an amazing forester and friend. We thank William Shepherd for helpful comments on an earlier draft of this manuscript.

References Cited

- Anderbrant, O., F. Schlyter, and G. Birgersson. 1985. Intraspecific competition affecting parents and offspring in the bark beetle *Ips typographus*. *Oikos*. 45: 89–98.
- Armendáriz-Toledano, F., A. Niño, B. T. Sullivan, J. Macías-Sámamo, J. Víctor, S. R. Clarke, and G. Zúñiga. 2014. Two species within *Dendroctonus frontalis* (Coleoptera: Curculionidae): Evidence from morphological, karyological, molecular, and crossing studies. *Ann. Entomol. Soc. Am.* 107: 11–27.
- Armendáriz-Toledano, F., A. Niño, B. T. Sullivan, L. R. Kirkendall, and G. Zúñiga. 2015. A new species of bark beetle, *Dendroctonus mesoamericanus* sp. nov. (Curculionidae: Scolytinae), in southern Mexico and Central America. *Ann. Entomol. Soc. Am.* 108: 403–414.
- Ayres, B. D., M. P. Ayres, M. D. Abrahamson, and S. A. Teale. 2001. Resource partitioning and overlap in three sympatric species of *Ips* bark beetles (Coleoptera: Scolytidae). *Oecologia*. 128: 443–453.
- Birgersson, G., M. J. Dalusky, K. E. Espelie, and C. W. Berisford. 2012. Pheromone production, attraction, and interspecific inhibition among four species of *Ips* bark beetles in the Southeastern USA. *Psyche*. 2012: 532652.
- Borden, J. H., R. Silverstein, and R. Brownlee. 1968. Sex pheromone of *Dendroctonus pseudotsugae* (Coleoptera: Scolytidae): production, bioassay, and partial isolation. *Can. Entomol.* 100: 597–603.
- Byers, J. A. 1989. Behavioral mechanisms involved in reducing competition in bark beetles. *Holarct. Ecol.* 12: 466–476.
- Byers, J. A. 2012. Bark beetles, *Pityogenes bidentatus*, orienting to aggregation pheromone avoid conifer monoterpene odors when flying but not when walking. *Psyche*. 2012: 940962.
- Byers, J. A., and D. L. Wood. 1980. Interspecific inhibition of the response of the bark beetles, *Dendroctonus brevicomis* and *Ips paraconfusus*, to their pheromones in the field. *J. Chem. Ecol.* 6: 149–164.
- Byers, J. A., D. L. Wood, J. Craig, and L. B. Hendry. 1984. Attractive and inhibitory pheromones produced in the bark beetle, *Dendroctonus brevicomis*, during host colonization: regulation of inter- and intraspecific competition. *J. Chem. Ecol.* 10: 861–877.

- Cano-Ramírez, C., F. Armendáriz-Toledano, J. E. Macías-Sámamo, B. T. Sullivan, and G. Zúñiga. 2012. Electrophysiological and behavioral responses of the bark beetle *Dendroctonus rhizophagus* to volatiles from host pines and conspecifics. *J. Chem. Ecol.* 38: 512–524.
- 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.
- Denno R. F., M. S. McClure, and J. R. Ott. 1995. Interspecific interactions in phytophagous insects: competition reexamined and resurrected. *Annu. Rev. Entomol.* 40: 297–331.
- Grégoire, J.-C., K. F. Raffa, and B. S. Lindgren. 2015. Economics and politics of bark beetles, pp. 585–613. *In* F. E. Vega and R. W. Hofstetter (eds.), *Bark beetles: biology and ecology of native and invasive species*. Elsevier, London.
- Hofstetter, R. W., M. L. Gaylord, M. 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.
- Hunt, D. W. A., and J. H. Borden. 1988. Response of mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and pine engraver, *Ips pini* (Say), to ipsdienol in southwestern British Columbia. *J. Chem. Ecol.* 14: 277–293.
- Lanier, G. N., and W. E. Burkholder. 1974. Pheromones in speciation of Coleoptera., pp. 161–189. *In* M. C. Birch (ed.), *Pheromones*. North-Holland Publishing, Amsterdam.
- Lanier, G., and D. Wood. 1975. Specificity of response to pheromones in the genus *Ips* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 1: 9–23.
- Lindén A., and S. Mäntyniemi. 2011. Using the negative binomial distribution to model overdispersion in ecological count data. *J. Ecol. Soc. Am.* 92: 1414–1421.
- Mccarty, F. A., P. M. Billings, J. V. Richerson, T. L. Payne, and L. J. Edson. 1980. Response of the southern pine beetle to behavioral chemicals in the laboratory. *J. Georgia Entomol. Soc.* 15: 307–317.
- Moreno, C. B. 2008. Diferenciación morfológica y comportamental de dos morfos de *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytinae) en Montebello, Chiapas. M.S. thesis, El Colegio de la Frontera Sur, Chiapas, MX.
- Moser, J. C., B. A. Fitzgibbon, and K. D. Klepzig. 2005. The Mexican pine beetle, *Dendroctonus mexicanus*: first record in the United States and co-occurrence with the southern pine beetle – *Dendroctonus frontalis* (Coleoptera: Scolytidae or Curculionidae: Scolytinae). *Entomol. News.* 116: 235–244.
- Niño-Domínguez, A., B. T. Sullivan, J. H. López-Urbina, and J. E. Macías-Sámamo. 2015. Pheromone-mediated mate location and discrimination by two syntopic sibling species of *Dendroctonus* Bark beetles in Chiapas, Mexico. *J. Chem. Ecol.* 41: 746–756.
- Niño-Domínguez, A., B. T. Sullivan, J. H. López-Urbina, and J. E. Macías-Sámamo. 2016. Responses by *Dendroctonus frontalis* and *Dendroctonus mesoamericanus* (Coleoptera: Curculionidae) to semiochemical lures in Chiapas, Mexico: possible roles of pheromones during joint host attacks. *J. Econ. Entomol.* 109: 724–731.
- Økland B., O. Skarpaas, and K. Kausrud. 2009. Threshold facilitations of interacting species. *Popul. Ecol.* 51: 513–523.
- Paine, T., J. Millar, C. Hanlon, and J. S. Hwang. 1999. Identification of semiochemicals associated with Jeffrey pine beetle, *Dendroctonus jeffreyi*. *J. Chem. Ecol.* 25: 433–453.
- Pureswaran, D. S., and B. T. Sullivan. 2012. Semiochemical emission from individual galleries of the southern pine beetle, (Coleoptera: Curculionidae: Scolytinae), attacking standing trees. *J. Econ. Entomol.* 105: 140–148.
- Pureswaran, D. S., B. T. Sullivan, and M. P. Ayres. 2008. High individual variation in pheromone production by tree-killing bark beetles (Coleoptera: Curculionidae: Scolytinae). *Naturwissenschaften.* 95: 33–44.
- Pureswaran, D. S., R. W. Hofstetter, B. T. Sullivan, and K. A. Potter. 2016. The role of multimodal signals in species recognition between tree-killing bark beetles in a narrow sympatric zone. *Environ. Entomol.* 45: 582–591.
- Raffa, K., M. N. Andersson, and F. Schlyter. 2016. Chapter one: host selection by bark beetles: playing the odds in a high-stakes game. *Adv. Insect Physiol.* 50: 1–74.
- Rudinsky, J. A. 1973. Multiple functions of the southern pine beetle pheromone verbenone. *Environ. Entomol.* 2: 511–514.
- Rudinsky, J. A., M. E. Morgan, L. M. Libbey, and T. B. Putnam. 1974. Antiaggregative-rivalry pheromone of the mountain pine beetle, and a new arrestant of the southern pine beetle. *Environ. Entomol.* 3: 90–97.
- Schlyter, F., and G. Birgersson. 1989. Individual variation in bark beetle and moth pheromones - a comparison and an evolutionary background. *Holarct. Ecol.* 12: 457–465.
- Schlyter, F., R. Jakuš, F. Z. Han, J. H. Ma, B. Kalinová, P. Mezei, J. H. Sun, L. Ujhelyiová, and Q. H. Zhang. 2015. Reproductive isolation of *Ips nitidus* and *I. shangrila* in mountain forests of Western China: responses to chiral and achiral candidate pheromone components. *J. Chem. Ecol.* 41: 678–688.
- Seybold, S. J., D. P. W. Huber, J. C. Lee, A. D. Graves, and J. Bohlmann. 2006. Pine monoterpenes and pine bark beetles: a marriage of convenience for defense and chemical communication. *Phytochem. Rev.* 5: 143–178.
- Silverstein, R. M., R. G. Brownlee, T. E. Bellas, D. L. Wood, and L. E. Browne. 1968. Brevicomins: principal sex attractant in the frass of the female western pine beetle. *Science.* 159: 889–891.
- Six, D., and R. Bracewell. 2015. *Dendroctonus*, pp. 305–350. *In* F. E. Vega and R. W. Hofstetter (eds.), *Bark beetles: biology and ecology of native and invasive species*. Elsevier, London.
- Skillen, E. L., C. W. Berisford, M. A. Camaan, and R. C. Reardon. 1997. Semiochemicals of forest and shade tree insects in North America and management applications. pp. 13–46. USDA Forest Service Forest Health Technology Enterprise Team publication FHTET-96-15, Morgantown, WV.
- Sullivan, B. T. 2011. Southern pine beetle behavior and semiochemistry, pp. 25–50. *In* R. N. Coulson and K. D. Klepzig (eds.), *Southern pine beetle II*. USDA forest service southern research station gen. Tech. Rep. SRS-140, Asheville, NC.
- Sullivan, B. T., A. Niño, B. Moreno, C. Brownie, J. Macías-Sámamo, S. R. Clarke, L. R. Kirkendall, and G. Zúñiga. 2012. Biochemical evidence that *Dendroctonus frontalis* consists of two sibling species in Belize and Chiapas, Mexico. *Ann. Entomol. Soc. Am.* 105: 817–831.
- Švihra, P., T. D. Paine, and M. C. Birch. 1980. Interspecific olfactory communications in southern pine beetles. *Naturwissenschaften.* 67: 518–520.
- Symonds, M., and C. Gitau-Clarke. 2016. Chapter five: the evolution of aggregation pheromone diversity in bark beetles. *Adv. Insect Physiol.* 50: 195–234.
- Wood, S. L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Nat. Mem.* 6: 1359.
- Zúñiga, G., Y. Salinas-Moreno, and R. Cisneros. 1995. Coexistencia en simpatria de *Dendroctonus frontalis* Zimmermann y *Dendroctonus mexicanus* Hopkins (Coleoptera: Scolytidae) sobre un mismo hospedero. *Acta Zool. Mex. (n.s.)* 64: 59–62.
- Zúñiga, G., G. Mendoza-Correa, R. Cisneros, and Y. Salinas-Moreno. 1999. Zonas de sobre posición en las áreas de distribución geográfica de las especies mexicanas de *Dendroctonus* Erichson (Coleoptera: Scolytidae) y sus implicaciones secológico-evolutivo. *Acta Zool. Mex.* 77: 1–22.