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Pheromone-Mediated Mate Location and Discrimination by Two Syntopic Sibling Species of *Dendroctonus* Bark Beetles in Chiapas, Mexico

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Abstract Where their geographic and host ranges overlap, sibling species of tree-killing bark beetles may simultaneously attack and reproduce on the same hosts. However, sustainability of these potentially mutually beneficial associations demands effective prezygotic reproductive isolation mechanisms between the interacting species. The pine bark beetle, *Dendroctonus frontalis* Zimmermann, is syntopic in the Central American region with a recently described sibling species, *Dendroctonus mesoamericanus* Armendáriz-Toledano and Sullivan, but mechanisms for their reproductive isolation are uncertain. We investigated whether semiochemicals mediate species discrimination by mate-seeking males of both species. In olfactometer bioassays, walking males of both species strongly preferred odors from gallery entrances of conspecific females. Coupled gas chromatography-electroantennographic detection and gas chromatography-mass spectrometry isolated 16 olfactory stimulants for males in these odors, but only two, ipsdienol and *endo*-brevicomin (both from *D. mesoamericanus* females), differed in quantity in female-associated odors between the species. In olfactometer bioassays, with 10, 1, or 0.1 female entrance equivalents of synthetic semiochemicals, the combination of ipsdienol and *endo*-brevicomin inhibited responses of male *D. frontalis* and enhanced responses of male *D. mesoamericanus* to two compounds associated with female entrances of both species (the pheromone component frontalin and host odor α-pinene). We conclude that ipsdienol and *endo*-brevicomin, pheromone components produced by females of just one of the two species (*D. mesoamericanus*), mediate interspecific mate discrimination by males of both species and provide an apparently symmetrical reproductive isolation mechanism.

Keywords Coevolution · Bark beetle · Walking bioassay · Short-range attraction · Reproductive isolation mechanisms · Pheromone · Coleoptera · Curculionidae

Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae) feed and reproduce primarily within the phloem of trees and include some of the most significant biotic mortality agents of trees worldwide. The majority of species colonize only dead, diseased, or severely weakened hosts (Raffa et al. 1993); however, a minority of species, particularly in the genera *Scolytus* Geofroy, *Dendroctonus* Erichson, and *Ips* DeGeer, can colonize and kill healthy trees (Wood 1982b). Successful colonization of vigorous trees by these aggressive bark beetle species relies on group release of aggregation pheromones that mediate synchronous attacks in numbers (typically thousands of individuals) sufficient to overwhelm host defenses that, otherwise, would kill or expel smaller numbers of invaders (Raffa and Berryman 1983; Seybold et al. 2006). The bark beetle *Dendroctonus frontalis* Zimmermann is a major mortality agent of *Pinus* L. and ranges throughout the southeastern USA, and from Arizona (USA) south to Nicaragua (Billings et al. 2004; Clarke and Nowak 2010). It has been found in apparent syntopy with certain other primary *Dendroctonus*

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species (Davis and Hofstetter 2009; Moser et al. 2005; Wood 1982a, b; Zúñiga et al. 1995). Within the Central American region, *D. frontalis* attacks trees apparently simultaneously with *Dendroctonus mesoamericanus* Armendáriz-Toledano and Sullivan, a newly recognized sibling species (Armendáriz-Toledano et al. 2015). Prior to its recognition as a distinct species, *D. mesoamericanus* was synonymous with *D. frontalis*. Arrivals of flying beetles and attacks by either species tend to be concentrated on different sections of host bole but overlap substantially in time and space, such that entry sites and galleries are intermixed (Moreno 2008).

Since colonization success requires sufficient abundance of attacking beetles to deplete a host's defenses, there may be selective benefits to joint attack by multiple primary bark beetle species, especially when local abundance of any single species is insufficient (Okland et al. 2009). Consequently, the negative effects of competition between species may be outweighed by the greater host availability that is rendered by communal mass attack (Ayres et al. 2001; Okland et al. 2009; Svihra et al. 1980; Wagner et al. 1985). This hypothesis is supported by evidence that the aggregation pheromones of syntopic, primary *Dendroctonus* species can be cross-attractive (Gaylord et al. 2006; Hofstetter et al. 2008). There is at least some degree of attraction by flying *D. frontalis* and *D. mesoamericanus* to blends of shared pheromone components, although low responses by *D. mesoamericanus* to synthetic lures in trapping trials suggest that the aggregation-mediating semiochemicals for this species have not yet been fully characterized (Sullivan et al. 2012).

Pheromones function in many insects to confer premating reproductive isolation between species, and this has been proposed for bark beetles (Lanier and Wood 1975; Raffa 2001; Sturgeon and Mitton 1982; Wood 1982a). The intermixing of closely related species, which presumably occurs during joint mass attacks by bark beetles, should promote the evolution and persistence of effective pre-mating reproductive isolation mechanisms (Pfening 2012). Otherwise, unproductive interspecific sexual interactions might consume time and energy resources of individual beetles and reduce their lifetime reproductive capacity (Gröning and Hochkirch 2008). Furthermore, the greater time in which males would be exposed on the bark surface in pursuing and rejecting (or being rejected by) heterospecific females would render beetles more susceptible to predation (Bunt et al. 1980). However, evidence of species cross-attraction to pheromones and overlap in pheromone blend composition among sympatric bark beetle species has created uncertainty about the importance of pheromones in reproductive isolation in bark beetles (Lanier and Burkholder 1974; Symonds and Elgar 2004).

In laboratory crossing studies, confinement of males of *D. frontalis* and *D. mesoamericanus* over gallery entrances of heterospecific females resulted in pairing, sperm transfer, gallery formation, egg laying, and development of larvae, although measures of pairing success, such as frequency of sperm transfer, gallery length, and brood production were generally less in heterospecific than conspecific pairings (Armendáriz-Toledano et al. 2014). Hybrid viability and fertility were not tested because the authors have, as yet, been unable to rear brood of either conspecific or heterospecific pairings to adulthood in the laboratory. However, differing chromosome numbers between the two species suggest that post-zygotic reproductive isolation exists (Armendáriz-Toledano et al. 2014). Despite the ability to force heterospecific pairings in the laboratory, dissections of naturally infested pines in Chiapas, Mexico have failed to reveal the presence of heterospecific pairs within zones of species overlap on the bark (Niño et al. unpublished data). These data suggest that effective premating reproductive isolation mechanisms are present that deter entry of males into gallery entrances of heterospecific females or otherwise deter pairing. Sustained coexistence of the two species and the possibility of "cooperative" mass attack behavior between them likely depend upon the existence of such mechanisms.

The objective of this work was to investigate responses by males of these two species to semiochemicals produced by both con- and hetero-specific females. Previous research indicated that there are differences in the composition of volatiles produced by females of the two species (Sullivan et al. 2012). We hypothesized that male discrimination of female pheromones could be a mechanism that deters interspecific pairing and thus reduces possible negative fitness consequences of joint mass attack by closely related species of *Dendroctonus* bark beetles.

Methods and Materials

Biological Material Logs (15–20 cm diam) of both naturally infested and uninfested, apparently healthy pines were cut throughout the year from standing *P. oocarpa* within Parque Nacional Lagunas de Montebello, Trinitaria, Chiapas, México (16° 07′ N, 91° 44′ W). Bark beetle adults used in bioassays were collected daily in the laboratory, as beetles emerged from infested logs enclosed in cloth bags. Adults were housed in plastic Petri dishes with moistened Kimwipe® (Kimberly-Clark, Roswell, GA, USA) and held at 10 °C. Adult beetles used in experiments were no more than 3 days-old. The ends of uninfested logs were sealed with paraffin and retained no more than 10 day at 10 °C prior to use in bioassays.

For bioassays, *D. frontalis* and *D. mesoamericanus* were distinguished by the presence of fine ridges on the pre-episternal area of the prothorax of *D. mesoamericanus* (Armendáriz-Toledano et al. 2015; Sullivan et al. 2012). Following bioassays, species identity of a subsample of males was confirmed through dissection and examination of genitalia (Armendáriz-Toledano et al. 2014, 2015). All research

operations (insect rearing, bioassays, volatile collections) were performed in the laboratory at a mean temperature of 24 °C and RH 48 %. Behavioral bioassays were performed under fluorescent ceiling lighting.

Responses of Walking Males to Volatiles from Female Gallery Entrances We assayed walking responses of individual males of each species to volatiles from a gallery entrance of a solitary female of either species that had been mining in an uninfested log (30 cm long × 15–20 cm diam) for 1 day. Each female was confined inside an artificial pit made into the bark surface using a drill bit (3–4 mm diam); only a single female was infested onto any log. A disk of fine-mesh plastic screen was secured over the pit with duct-tape to prevent female escape. After 4 h, and if boring dust was apparent, the screen cover was removed and the mouth (27 mm diam) of a modified glass aeration funnel was secured over the entrance using a ring of paraffin wax. The wax, applied when melted, produced an air-tight seal between the bark surface and the mouth of the funnel. The funnel modification consisted of a 4 mm i.d., 30 mm-long piece of glass tubing fused with and penetrating ~10 mm into the funnel cone. Inside the cone, the tubing curved toward the center of the funnel mouth so that its opening was centered over, and 5 mm distant from, the gallery entrance (Supplemental 1A). By forcing air into the funnel stem and simultaneously drawing it from this tubing, it was possible to pass air directly across the gallery entrance while maintaining a closed air path. For walking bioassays, we used an acrylic four-arm olfactometer (Vet et al. 1983) adapted for work with bark beetles (description and illustration in Supplemental 1).

For odor-receiving arms, the modified funnel attached to the infested log was connected between the humidified/purified air supply and the olfactometer arm by PTFE tubing (Supplemental 1A). In 'single odor' bioassays, the test odor was delivered to only one of the four olfactometer arms, selected at random, whereas in 'odor choice' bioassays, two opposite arms, selected randomly, each received a different odor treatment; in both cases, the remaining arms received clean air. Treatment assignment to the four arms was re-randomized every 4–6 trials. For the single odor bioassays, there were two odor treatments (either *D. frontalis* or *D. mesoamericanus* female gallery entrances) and two subject classes (males of either species) tested in all four possible combinations. For the odor choice bioassays, a single choice combination (entrances of *D. frontalis* vs *D. mesoamericanus* females) was tested with males of both species (separately).

At the beginning of each trial, a solitary male was released on the screen covering the air outlet at the center of the olfactometer arena floor, and the arena immediately was covered with a clear acrylic plate. During each 5 min trial, we recorded whether a male crossed into any of four response circles (a 1 cm diam circle drawn on the floor of the arena and immediately in front of the odor inlets of each arm) and the time it spent within each circle. If the male contacted the arena wall, the cover of the olfactometer was removed briefly and the male re-released at the air outlet. Males were used in a single trial and discarded. A single measure was used in statistical comparisons: the time spent by each male inside the response circle (with a failure to enter any circle included in the analysis as a zero-time response). Since this time was influenced by whether the subject entered the response circle (i.e., located the odor source) and the time spent in the circle once entered, our 'response' measurement was an indication of two types of behaviors: attraction to the odor release point and arrestment there.

Collection of Volatiles from Entrances Immediately after behavioral bioassays were completed, volatiles from gallery entrances were sampled for 3 h using glass-enclosed adsorbent cartridges (117 mg Porapak-Q; SKC Inc. Eighty Four, PA, USA). A cartridge was attached with PTFE tubing to the outlet of the aeration funnel secured over the gallery entrance (with the funnel inlet receiving purified/humidified air), and air from the funnel drawn through the cartridge at 50 ml.min^{-1}. Cartridges were extracted with 1.5 ml pentane (HPLC grade, Sigma-Aldrich Co., Milwaukee, WI, USA), with 3.8 μg of cycloheptanone (98 %, Sigma-Aldrich) added to each extract as an internal standard. A total of 13–14 extracts were collected (each from a different female) from gallery entrances of each species.

Electrophysiological Responses of Male Antennae Olfactory sensitivity of male beetles to compounds within volatiles collections from female entrances was assayed by coupled gas chromatography-electroantennographic detection (GC-EAD). The GC-EAD apparatus and antennal preparation procedures were identical to those in Cano-Ramirez et al. (2012). The GC was fitted with an HP-INNOwax capillary column (Agilent Technologies, Wilmington, DE, USA; polyethylene glycol phase; 30 m long, 0.25 mm diam, 25 μm film thickness) and used a temperature program of 50 °C for 1 min, 16 °C.min^{-1} to 80°, 7 °C.min^{-1} to 200 °C, and then held for 10 min. Subsamples (100 μl) from 10 to 12 of the cartridge extracts were pooled by species and concentrated ca. 10-fold by evaporation on the laboratory bench. Antennae of male *D. frontalis* and *D. mesoamericanus* (11 preparations each) were tested with these concentrated extracts (2 μl injected into GC in splitless mode). A genuine olfactory response was recorded as such if an EAD deflection were detected at a particular retention time in at least four GC-EAD runs. Putative olfactory stimulants [i.e., helium ionization detection (HID) peaks coincident with EAD responses] were, in general, confirmed for activity by performing GC-EAD analyses with synthetic mixtures that included these compounds. Compounds without this additional step are noted in the results.

Quantification of Olfactory Stimulants HID peaks coinciding with EAD responses were identified by analyzing the concentrated, pooled extracts by gas chromatography/mass spectrometry (GC/MS; Hewlett-Packard model G1800C, Palo Alto, CA, USA) utilizing the same column, temperature program, and carrier flow rates as used in the GC-EAD analyses. Compound identifications were accomplished by matching both retention times and mass spectra to those of commercially obtained compounds. The identified compounds then were quantified in individual samples from aerations of single female gallery entrances. Quantifications were derived from single ion abundance ratios between the target compound and the internal standard; these ratios then were converted to ng per sample using a calibration curve produced from analyzing serial dilutions of known quantities of standard compounds.

Responses by Walking Males to Synthetic Volatile Blends In order to identify compounds mediating species discrimination by mate-seeking males, we assayed males in the four-armed olfactometer for responses to different synthetic combinations. Test compounds were dissolved in hexane and released from vertically oriented, 5 μl microcapillaries (Drummond Scientific, Broomall, PA, USA), with the bottom opening positioned within the airstream flowing to the inlets of each olfactometer arm (modified from Browne et al. 1974; Supplemental 2). At the beginning of each trial, capillaries were filled with lure solution (or pure solvent for arms not receiving a lure mixture, i.e., controls).

We investigated the behavioral effects of two male olfactory stimulants, *endo*-brevicomin and ipsdienol, that were produced in different quantities by females of the two species. These were tested in combination with α-pinene and/or frontalin, as the latter two compounds were released in similar quantities by female entrances of both species and have previously been identified as semiochemicals attractive to *D. frontalis* (Moreno et al. 2008; Sullivan 2011). α-Pinene was included in all blends, as this host odor is normally present in association with bark beetle attacks on pines. Blends were tested in single-odor assays with the same procedures used for tests of gallery entrance odors. Treatments were α-pinene, 1) alone, 2) with frontalin, 3) with ipsdienol, 4) with *endo*-brevicomin, 5) with frontalin and ipsdienol, 6) with frontalin and *endo*-brevicomin, 7) with frontalin, ipsdienol, and *endo*-brevicomin, 8) with ipsdienol and *endo*-brevicomin (Table 1). Concentrations of compounds in mixtures were adjusted to produce release rates from capillaries that approximated the average release rates of the compounds measured from gallery entrances of female *D. mesoamericanus* (ipsdienol and *endo*-brevicomin) or both species (α-pinene and frontalin). Tests also were performed at one-tenth and ten-fold the single-entrance equivalent rate (Table 1). Additionally, we performed a choice assay in which opposite arms of the olfactometer received either treatment 2 or 7 at a release rate of one entrance equivalent. These treatments were chosen specifically because treatment 2 was an approximate mimic of the odor profile of *D. frontalis* female entrances, whereas treatment 7 served the same role for *D. mesoamericanus*. *endo*-Brevicomin was the pure (+)-enantiomer (>99 %; Sullivan et al. 2007) as produced by female *D. mesoamericanus* in the investigated population (Supplemental 3) and male *D. frontalis* in the southeastern USA (Sullivan et al. 2007). The other semiochemicals in the synthetic blends were racemates (our use of specific enantiomeric compositions of the semiochemicals and its implications are discussed more fully in Supplemental 3).

Statistical Analyses Behavioral bioassay data for time spent within response circles contained an excess of zeroes and could not be transformed to meet assumptions of normality. Therefore, these data were analyzed by GLM with a negative binomial distribution (Lindén and Mäntyniemi 2011)

Table 1 Composition of lure mixtures tested in walking olfactometer assays

				Concentration (μg/μl)[b]			Approx. release rate (μg/min)[d]			Lure blend composition							
Semiochemical	Source[a]	Purity	Enant. Ratio[c]	0.1 X	1 X	10 X	0.1 X	1 X	10 X	1	2	3	4	5	6	7	8
α-pinene	Aldrich	99 %	racemic	0.41	4.1	41	0.23	2.1	17	X	X	X	X	X	X	X	X
Frontalin	ChemTica	>99 %	racemic	0.0012	0.0121	0.121	0.00085	0.0073	0.068		X			X	X	X	
Ipsdienol	Bedoukian	>93 %	racemic	0.00014	0.0014	0.014	0.00006	0.00048	0.0044			X		X		X	X
endo-brevicomin	(Sullivan et al. 2007)	95 %	(+)	0.00015	0.0015	0.015	0.00007	0.00060	0.0053				X		X	X	X

[a] Sigma-Aldrich Co., Milwaukee, WI, USA; ChemTica Internacional, Heredia, Costa Rica; Bedoukian Research Inc., Danbury, CT, USA

[b] Basis for the enantiomeric ratios chosen for tests is explained in the methods text and Supplemental 3

[c] Concentration (in hexane) of components in lure solution added to release capillary

[d] Release rate from capillary based on quantity added to capillary, average time of loss of all liquid from capillary, and average proportions of release among compounds (determined by quantitative analysis of aerations of releasers). The 1x rate mimicked the release rate of compounds from a single gallery entrance of a female *Dendroctonus mesoamericanus* (see text)

with logarithmic link function for pairwise comparisons of means. A Wilcoxon one-sample test with a hypothetical median value equal to zero and a 95 % confidence interval was applied to contrast between odor and control arms when the latter had zero counts. The overdispersion was evaluated from deviance residual values respecting degrees of freedom of the model. For all statistical tests, α=0.05. In the single odor olfactometer bioassays, we tested whether males responded to an odor source or synthetic blend by determining whether the time spent within the response circle exceeded the average time spent within the three control circles for each trial.

For statistical analysis of quantities of individual compounds in entrance volatiles, compounds were first classified as either host volatiles or pheromones (Skillen et al. 1997), and then their quantities were analyzed within these groupings. Data were normalized with a $\sqrt[3]{(X+0.05)}$ transformation and then analyzed by a one-way ANOVA. Additionally, a *t-test* was used to compare quantities of single compounds produced by females of either species. All statistical analyses were performed with SPSS Statistics Vol. 21.

Results

Responses by Walking Males to Volatiles from Female Gallery Entrances In single odor tests with the four arm olfactometer, males of both *D. frontalis* (Fig. 1a) and *D. mesoamericanus* (Fig. 1b) responded to odors of conspecific female entrances (i.e., spent more time at the inlet of the arm receiving odors from the female entrance than inlets of the arms receiving clean air; for *D. frontalis*: D=93.4, gl=266; $X^2_{0.05,\,1}$=5.1, P=0.024; for *D. mesoamericanus*: D=19.6, gl=238; $X^2_{0.05,\,1}$=25.4, P<0.001), whereas males of both species showed no response to odors of heterospecific females (for *D. frontalis*: D=48.4, gl=266; $X^2_{0.05,\,1}$=2.5, P=0.112; for *D. mesoamericanus*: D=58.3, gl=238; $X^2_{0.05,\,1}$=0.363, P=0.547). Furthermore, the average time spent by *D. frontalis* males at the odor-receiving inlet was longer (approximately seven-fold) for gallery entrance odors of female conspecifics than for female heterospecifics (D=130, gl=132; $X^2_{0.05,\,1}$=68.7, P<0.001, Fig. 1a). Similarly, mean response duration by *D. mesoamericanus* males was approximately 12 times longer to odors of female conspecifics than to odors of heterospecifics (D=110, gl=118; $X^2_{0.05,\,1}$=12.3, P<0.001, Fig. 1b). When odors of female entrances of the two species (separately) were released from opposite arms of the olfactometer (Fig. 2), males of both species spent more time (greater than seven-fold on average) at the inlet with odor of a conspecific female entrance than the at the inlets with odor of a heterospecific female entrance or clean air (for *D. frontalis*: D=46.6, gl=157; $X^2_{0.05,\,2}$=69.2, P<0.001; for *D. mesoamericanus*: D=61.1, gl=129; $X^2_{0.05,\,2}$=9.13, P<0.001).

Electrophysiological Responses of Male Antennae At least 16 compounds present in volatiles from gallery entrances of females elicited responses in antennae of males (Fig. 3a and b; Supplemental 4). Ten EAD responses that occurred in males of both species were produced by odors from entrances of females of both species. Nine of these ten EAD responses occurred at retention times corresponding to α-pinene, ß-pinene, myrcene, limonene, frontalin, linalool, longifolene, *cis*-verbenol, and verbenone.

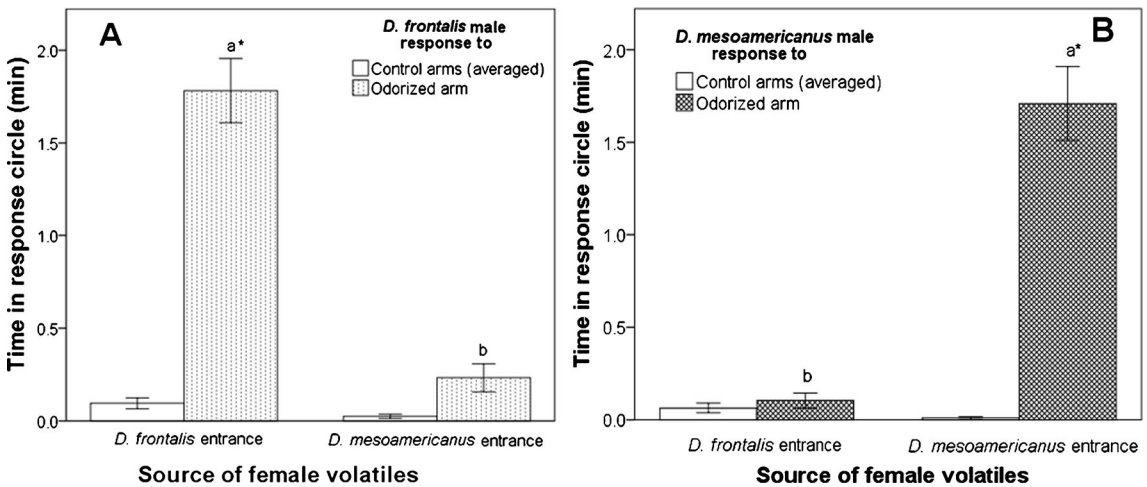

Fig. 1 Mean (±SE) time spent by walking male *Dendroctonus frontalis* (**a**) and *D. mesoamericanus* (**b**) within 1 cm diam circles located immediately in front of odor inlets of a four-arm olfactometer. A single, randomly chosen, arm received volatiles from a gallery entrance occupied by either a *D. frontalis* or *D. mesoamericanus* female, and the other three arms received clean air (controls). Times spent by males within the response circles of the three control arms were averaged (*open bars*) for comparisons. An *asterisk* indicates that arrestment time for an odor treatment was greater than for clean air; treatments labeled with different lower-case letters differed in mean arrestment duration (GLM with negative binomial distribution and logit function, α=0.05)

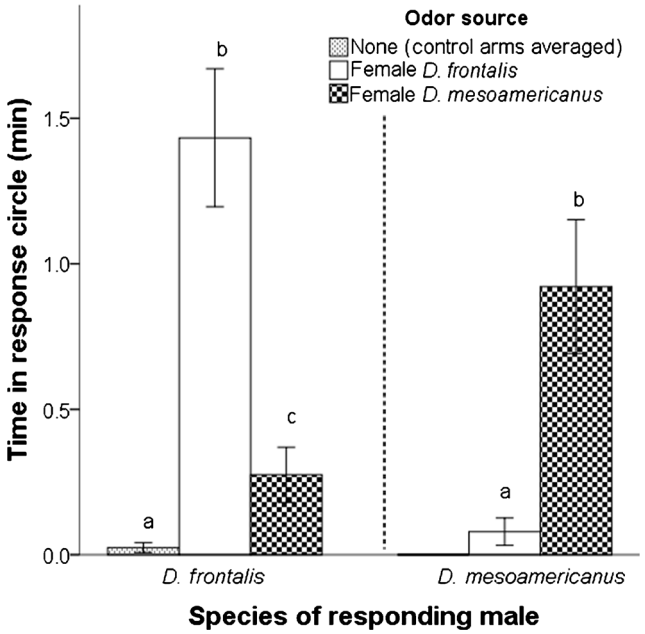

Fig. 2 Mean (±SE) time spent by walking male *Dendroctonus frontalis* and *D. mesoamericanus* within 1 cm diam circles located immediately in front of the odor inlets of a four-arm olfactometer. Two opposite arms received volatiles from gallery entrances occupied by either a *D. frontalis* or *D. mesoamericanus* female, and the other two arms received clean air (controls). Times spent by males within the response circles of the two control arms were averaged (*open bars*) for comparisons. For males of each species, treatments labeled with different lower-case letters differed in mean arrestment duration (GLM with negative binomial distribution and logit function, α=0.05). No male *D. mesoamericanus* entered the control circles (response of zero), and these data were excluded from the analysis

Additionally, one of these ten EAD responses (peak 12; Fig. 3a and b) was at the retention time of coeluting 4-allylanisole and *trans*-verbenol, as well as ipsdienol in *D. mesoamericanus* samples. A strong EAD response at the retention time of *endo*-brevicomin and a weak response at the retention time of terpinen-4-ol were stimulated in males of both species by volatiles from *D. mesoamericanus* females but not *D. frontalis* females. A moderate response at the retention time of myrtenol was elicited from male *D. mesoamericanus* antennae exposed to volatiles from females of both species, whereas this response was weak or absent from male *D. frontalis* antennae. Additionally, male *D. frontalis* antennae had a weak EAD response to an unidentified compound (peak #7) from female *D. mesoamericanus* entrances. Olfactory sensitivities by both species to α-pinene, ß-pinene, frontalin, *endo*-brevicomin, terpinen-4-ol, *cis*-verbenol, 4-allylanisole, ipsdienol, *trans*-verbenol, verbenone, and myrtenol were confirmed in GC-EAD tests with synthetic compounds.

Quantification of Olfactory Stimulants Of the compounds that elicited EAD responses from males, the hydrocarbon monoterpene α-pinene, the hydrocarbon sesquiterpene longifolene, the phenylpropanoid 4-allylanisole, and several other ostensibly host-produced compounds were the most abundant compounds collected from female entrances of both species (Fig. 3c). Host compounds were not present in different quantities in odors from attacks of either species (*t-tests*; $P>0.05$). We quantified seven ostensibly insect-produced compounds detected by GC-EAD (Fig. 3d): two bicyclic ketals (frontalin and *endo*-brevicomin), and five oxygenated monoterpenes (*cis*-verbenol, ipsdienol, *trans*-verbenol, verbenone, and myrtenol). Only two insect-produced compounds from female entrances differed in quantity between species: *endo*-brevicomin (Z=2.20, $P<0.001$) and ipsdienol (Z=1.20, P=0.001), and these were detected only from entrances of *D. mesoamericanus* females.

Responses by Walking Males to Synthetic Volatile Blends In single odor olfactometer assays (Fig. 4), males of the two species exhibited preferences among the different synthetic lure combinations at one tenth (for *D. frontalis*: D=127, gl=271, $X^2_{0.050,\ 7}$=24.6, P=0.001; for *D. mesoamericanus*: D=244, gl=272, $X^2_{0.050,\ 7}$=18.0, P=0.012), one (for *D. frontalis*: D=63.7, gl=233, $X^2_{0.050,\ 7}$=32.4, $P<0.001$; for *D. mesoamericanus*: D=146, gl=255, $X^2_{0.050,\ 7}$=42.4, $P<0.001$), and ten (for *D. frontalis*: D=78.3, gl=272, $X^2_{0.050,\ 7}$=65.6, $P<0.001$; for *D. mesoamericanus*: D=150, gl=272, $X^2_{0.050,\ 7}$=38.7, $P<0.001$) female entrance equivalents. At the 0.1x and 10x concentrations, *D. mesoamericanus* males responded more strongly to the complete four-component blend (the host odor α-pinene with three compounds, frontalin, *endo*-brevicomin, and ipsdienol, produced by *D. mesoamericanus* females) than to all other odor combinations which lacked at least one of the female-produced components (Fig. 4a and c). However, at the 1x concentration, elimination of ipsdienol from the four-component blend did not reduce male *D. mesoamericanus* response, and both this three-component lure and the complete blend were more attractive than any other combination tested (Fig. 4b). Addition of frontalin to α-pinene did not increase responses by male *D. mesoamericanus*, whereas addition of either ipsdienol or *endo*-brevicomin to α-pinene enhanced, reduced, or had no effect on responses at the three concentrations tested.

At all three concentrations, male *D. frontalis* responded more strongly to the α-pinene/frontalin combination than to any other combination of components (Fig. 4d, e, and f). Addition of *endo*-brevicomin and/or ipsdienol to the attractive α-pinene/frontalin combination reduced male *D. frontalis* responses. At the 1x and 10x concentrations, ipsdienol and the ipsdienol/*endo*-brevicomin combination reduced attraction of male *D. frontalis* to α-pinene/frontalin more than did *endo*-brevicomin alone (Fig. 4d and e). However, the combination of *endo*-brevicomin and ipsdienol did not reduce attraction

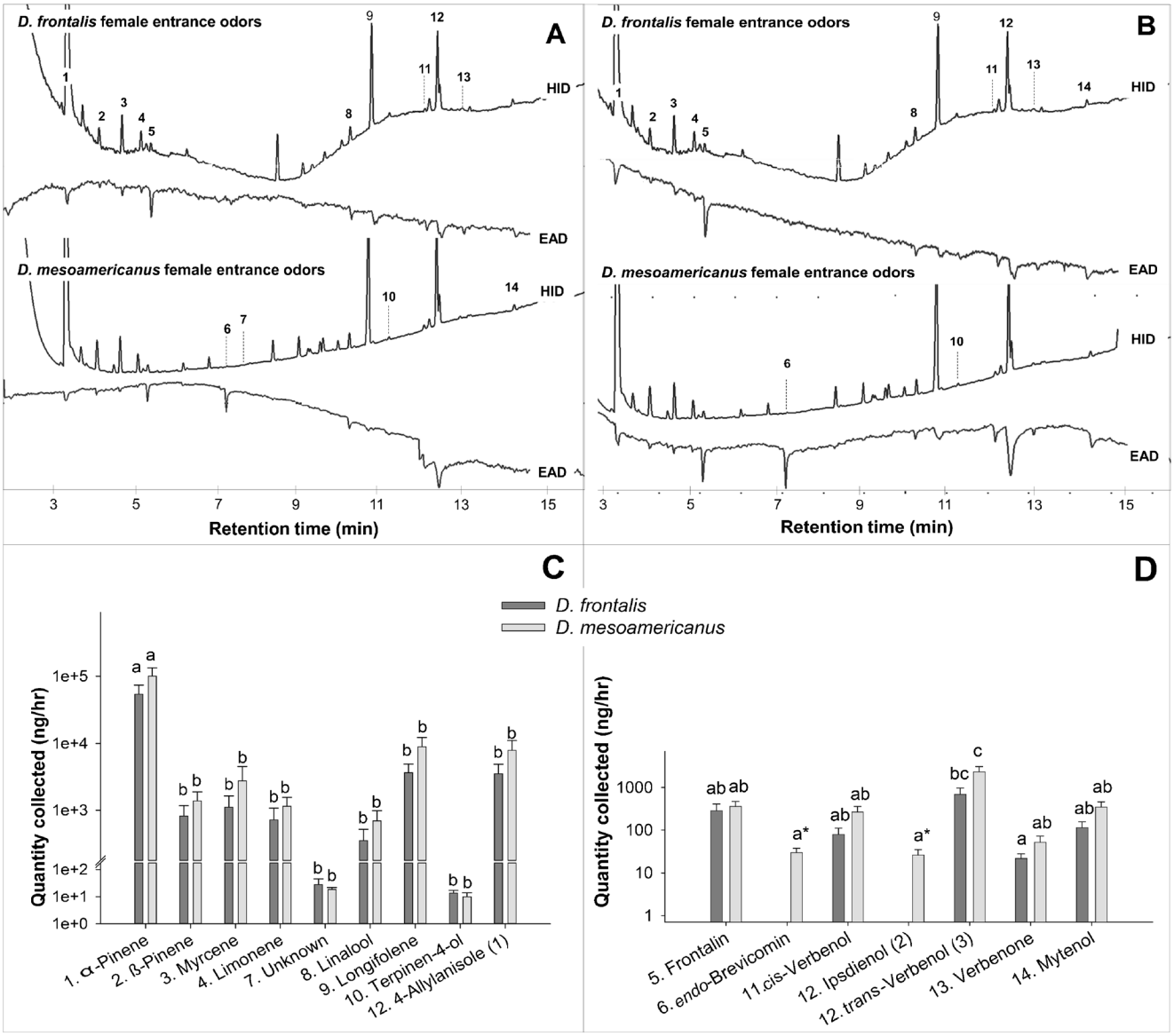

Fig. 3 Coupled gas chromatography–electroantennographic detection (GC-EAD) analyses, using helium ionization detection (HID) and antennae of male *Dendroctonus frontalis* (**a**) and *D. mesoamericanus* (**b**), with volatiles collected from gallery entrances occupied by a female of either species. *Bar graphs* (**c** and **d**) display average quantities (mean±SE) of compounds collected during dynamic headspace aerations of entrances. Compounds originating ostensibly from either host tree tissue (**c**) or the beetle itself (**d**) are displayed separately. Quantities associated with the same lower case letters were not different (one-way ANOVA, α=0.05). An *asterisk* indicates that quantities of a particular compound produced by female entrances differed between species (*t-test* with α=0.05). Number labels of peaks in A and B correspond to numbers and compound identifications of C and D. HID/EAD peak 14 was composed of 2–3 coeluting compounds

more than ipsdienol alone at the 1x and 10x concentrations. In general, both species exhibited lower discrimination of lure combinations at the 0.1x concentration.

When males were presented a choice of two lures that each approximated the odor blend associated with gallery entrances of females of the two species (for *D. frontalis* females, α-pinene/frontalin; for *D. mesoamericanus* females, α-pinene/frontalin/*endo*-brevicomin/ipsdienol; Fig. 5), males of both species strongly preferred synthetic lures approximating the odors of conspecific over heterospecific female entrances (for *D. frontalis*: D=59.2, gl=237, $X^2_{0.050,\ 2}$=54.4, P<0.001; for *D. mesoamericanus*: D=44.1, gl=237, $X^2_{0.050,\ 2}$=44.8, P<0.001).

Discussion

Previous evidence indicated that semiochemicals produced by females of the genus *Dendroctonus* (including *D. frontalis*) mediate male location of, and entry into, female gallery

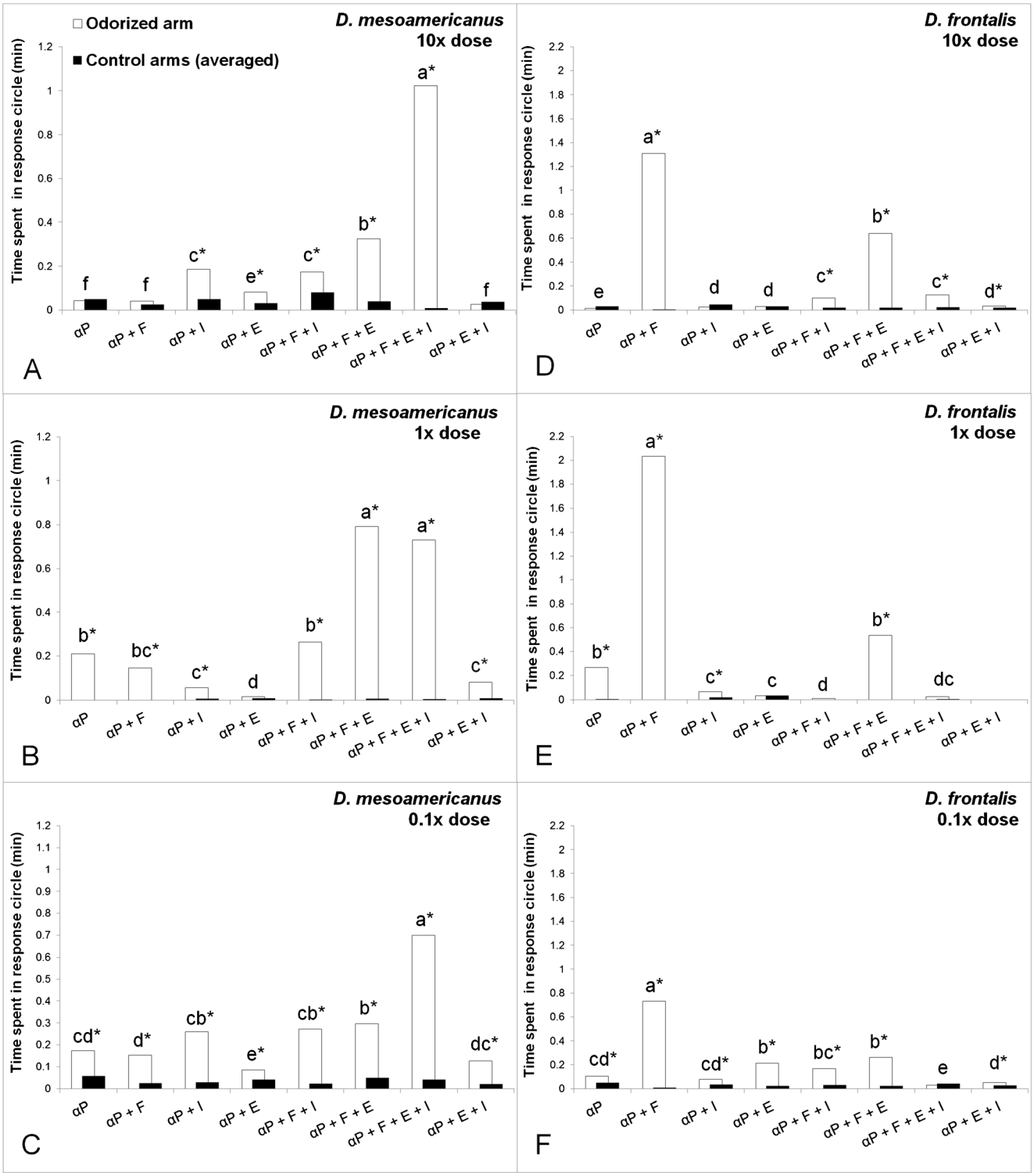

Fig. 4 Mean (±SE) arrestment time of male *Dendroctonus mesoamericanus* (**a**–**c**) and *D. frontalis* (**d**–**f**) in front of the inlets of a four-arm olfactometer. A single arm received synthetic combinations of α-pinene (αP), frontalin (F), ipsdienol (I), and *endo*-brevicomin (E), whereas the other three arms received clean air (controls). Results for the three control arms were averaged (*filled bars*). Odor concentrations were 10 (**a**,**d**), 1 (**b**,**e**), or 0.1 (**c**,**f**) female gallery entrance equivalents (see text for additional details). An *asterisk* indicates that arrestment by odors was greater than by controls; odor treatments labeled with the same lower-case letters did not differ in mean arrestment duration (GLM with negative binomial distribution and logit function or a *Wilcoxon test* when controls had a zero response, α=0.05)

entrances (Libbey et al. 1974; McCarty et al. 1980; Rudinsky 1973; Rudinsky et al. 1974) but also suggested that semiochemicals may not play a significant role in preventing heterospecific pairings between sibling species of

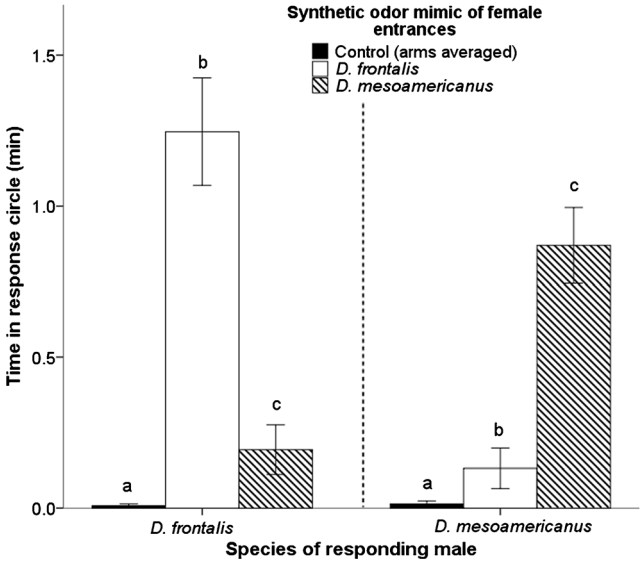

Fig. 5 Mean (±SE) time spent by walking male *Dendroctonus frontalis* and *D. mesoamericanus* within 1 cm diam circles located immediately in front of the odor inlets of a four-arm olfactometer. Two opposite arms each received a single entrance equivalent of a synthetic blend of volatiles associated with female entrances of either species (for *D. frontalis*, α-pinene and frontalin; for *D. mesoamericanus*, α-pinene, frontalin, ipsdienol, and *endo*-brevicomin). Time spent by males within response circles of the two control arms were averaged for comparisons. For males of each species, treatments labeled with different lower-case letters differed in mean arrestment duration (GLM with negative binomial distribution and logarithmic function, α=0.05)

Dendroctonus (Pajares and Lanier 1990). In our study, males of both *D. frontalis* and *D. mesoamericanus* responded more strongly to volatiles from gallery entrances of conspecific rather than heterospecific females. Thus, for males of these two sympatric sibling species, discrimination of the locations of females of the correct species is probably mediated by female-associated semiochemicals, and semiochemicals likely provide at least a partial reproductive isolation mechanism.

Among the EAD-stimulating, ostensibly beetle-produced compounds from female entrances, only two (*endo*-brevicomin and ipsdienol) quantitatively or qualitatively distinguished the odors of the respective species, with both present in *D. mesoamericanus* female entrances but absent from entrances of *D. frontalis* females. *endo*-Brevicomin and ipsdienol are pheromone components that occur commonly in the genera *Dendroctonus* and *Ips,* respectively (Skillen et al. 1997), and they have been reported previously to distinguish odors produced by mining female *D. frontalis* and *D. mesoamericanus* (Sullivan et al. 2012). The qualitative difference between species in production of these compounds by females indicates their suitability as cues by which males might distinguish potential mates. Furthermore, these two compounds, either individually or in combination, had opposite behavioral effects on males of the two species, enhancing attraction/arrestment of *D. mesoamericanus* and inhibiting that of *D. frontalis*. These behavioral changes occurred when these compounds were added to the lure combination of *alpha*-pinene and frontalin, components released in roughly similar quantities by entrances of females of both species. Frontalin was evidently a key component of the attractant for both species, as elimination of frontalin reduced responses to the most attractive combination to each respective species (i.e., frontalin and α-pinene for *D. frontalis* and the complete four-component blend for *D. mesoamericanus*).

The degree of male discrimination of odors in choice bioassays was similar whether the odor sources were volatiles from female entrances of the two species or synthetic mixtures differing in the presence of *endo*-brevicomin and ipsdienol in approximately the same concentrations as released by *D. mesoamericanus* female gallery entrances. Although additional semiochemicals may be involved, our data imply that ipsdienol and *endo*-brevicomin from female *D. mesoamericanus* were the cues that allowed discrimination by males of odors of female entrances of the respective species. However, we note that the ipsdienol used in our tests was racemic, whereas female *D. mesoamericanus* produce >95 % (+)-ipsdienol (Supplemental 3); this difference could have impacted the responses of males.

Although *endo*-brevicomin is not produced by *D. frontalis* females, it is produced by *D. frontalis* males, both before and after pairing with a female (Sullivan et al. 2007, 2012; Vité and Renwick 1971), and ipsdienol also may be produced by some *D. frontalis* males (Sullivan et al. 2012). Rudinsky et al. (1974) found that *endo*-brevicomin inhibited arrestment of walking male *D. frontalis* by female-associated odors and induced them to produce the "rivalry chirp" associated with male-male encounters. Thus, in the context of intraspecific interactions (i.e., as a pheromone), *endo*-brevicomin apparently signals to walking *D. frontalis* males that a gallery entrance contains a paired female and is not suitable for entry. Our new data imply that *endo*-brevicomin also functions in an interspecific context, as a kairomone or synomone, by deterring male *D. frontalis* from entering gallery entrances of syntopic *D. mesoamericanus* females. Such dual functionality of semiochemicals mediating both intra- and interspecific interactions is common in bark beetles (Byers 1989) and is an example of semiochemical parsimony (Blum 1996). However, since male *D. frontalis* apparently produce key components of the *D. mesoamericanus* female pheromone that are lacking in *D. frontalis* females (i.e., *endo*-brevicomin and possibly also ipsdienol), our data suggest that entrances with *D. frontalis* pairs may be attractive to *D. mesoamericanus* males.

In addition to frontalin, *endo*-brevicomin, and ipsdienol, four more compounds (*cis*-verbenol, *trans*-verbenol, verbenone, and myrtenol) that elicited EAD responses from the two species, and that have been demonstrated to function as pheromone components in the genus *Dendroctonus* (Skillen et al. 1997), were detected in female gallery

entrances. All four compounds were detected in both *D. frontalis* and *D. mesoamericanus* female entrances and in a previous study also were isolated from female adults of both species in Chiapas (Sullivan 2011). The quantities detected in female entrances were likely derived both from the beetles themselves and from host-released α-pinene being oxidized either spontaneously or by the beetles' microbial associates (Hughes 1973; Renwick et al. 1973; Seybold et al. 2006). There is evidence that these compounds are pheromone components for *D. frontalis* (Sullivan 2011) and may affect close-range behavior of mate-seeking males. Both verbenone and myrtenol apparently influence acceptance of gallery entrances by male *D. frontalis* and may, like *endo*-brevicomin, mediate the avoidance of entrances already occupied by a male (Rudinsky 1973; Rudinsky et al. 1974). However, in the present study, there was no difference between the species in the amounts of these compounds associated with female gallery entrances, hence it is unlikely these compounds play a role in species discrimination by males. For this reason, they were not included in our tested synthetic lure mixtures, although they may merit additional study for a possible role in mediating interactions between the two species.

The apparent absence of heterospecific pairings in nature suggests that pre-mating reproductive isolation between *D. frontalis* and *D. mesoamericanus* is complete, and it is likely that the semiochemical mechanisms examined in our study are complemented by mechanisms involving other sensory modalities and types of behaviors. Both *Dendroctonus* and *Ips* bark beetles stridulate during interactions within and between sexes and these acoustic cues apparently mediate pairing to some extent (Ryker 1988). Evidence of taxonomically distinct sound patterns has suggested a role for acoustic cues in reproductive isolation in bark beetles (Michael and Rudinsky 1972; Rudinsky and Michael 1973), however, there is as yet no direct evidence for this function (Lewis and Cane 1992). Partitioning (albeit incompletely) of the host bole between *D. frontalis* and *D. mesoamericanus* (Moreno 2008) and a possible difference in timing of peak arrival on the host likely reduce the amount of direct, cross-species interactions between the sexes and, therefore, opportunities for interspecific pairings. Differences in host species preferences, if they exist, might have a similar effect (Lanier and Burkholder 1974). In addition, differences in composition of the aggregation pheromone (which affects flying individuals of both sexes and may include contributions of pheromone components by both sexes, as is the case for *D. frontalis*) may enhance spatial/temporal separation of species (Symonds and Elgar 2004). However, it is not yet apparent whether aggregation pheromone compositions differ for *D. frontalis* and *D. mesoamericanus* (Sullivan et al. 2012), and this is a topic of ongoing investigation.

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