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Southern Pine Beetle, *Dendroctonus frontalis*, Antennal and Behavioral Responses to Nonhost Leaf and Bark Volatiles

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Abstract A growing body of evidence suggests that bark beetles detect and avoid release points of volatile compounds associated with nonhost species, and thus such nonhost volatiles may have potential utility in the management of bark beetles. We used a coupled gas chromatograph-electroantennographic detector (GC-EAD) to assay the olfactory sensitivity of the southern pine beetle, *Dendroctonus frontalis* Zimmermann, to volatiles from leaves and bark of eight species of nonhost angiosperm trees that are common in the range of *D. frontalis*. Tree species sampled were red maple (*Acer rubrum* L.), mockernut hickory [*Carya alba* (L.) Nutt. ex Ell.], sweetgum (*Liquidambar styraciflua* L.), black tupelo (*Nyssa sylvatica* Marsh.), black cherry (*Prunus serotina* Ehrh.), southern red oak (*Quercus falcata* Michx.), blackjack oak [*Quercus marilandica* (L.) Muenchh.], and water oak (*Quercus nigra* L.). Beetle antennae responded to a total of 28 identifiable compounds in these samples. The relative olfactory responsiveness to 14 of these, as well as to nonanoic acid and four additional volatiles reported to be associated with nonhost angiosperms, was assessed in GC-EAD analyses of synthetic dilutions spanning six orders of magnitude. The largest response voltage amplitudes were obtained with *trans*-conophthorin, nonanoic acid, terpinen-4-ol, phenylethyl alcohol, and eucalyptol, whereas the lowest response thresholds were to nonanoic acid, nonanal, linalool, (*E*)-2-hexen-1-ol, and phenylethyl alcohol. Funnel traps baited with various combinations of eleven antennally-active angiosperm volatiles along with a standard attractant captured significantly fewer male and female *D. frontalis* than traps baited with the standard attractant alone. Our data suggest that a diversity of semiochemicals may be involved in host species discrimination by *D. frontalis*, and several may have utility in their management.

Keywords Inhibitor · Bark beetle · GC-EAD · Scolytinae · Nonhost

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Introduction

Coniferophagous bark beetles (Coleoptera: Curculionidae: Scolytinae) have relatively narrow, typically monogeneric host ranges (Wood, 1982) and can discriminate among suitable and unsuitable hosts (Borden, 1996). Suitable host trees are often unevenly distributed in forest landscapes, and inefficient host-finding behaviors can waste a beetle's limited energy reserves (Atkins, 1966; Gries et al., 1990; Kinn et al., 1994), as well as increase its risk of predation (Stephen and Dahlsten, 1976; Dahlsten, 1982) and exposure to harsh environmental conditions (McMullen and Atkins, 1962; Gries et al., 1989; Byers et al., 1998). There is an accumulating body of evidence suggesting that foraging coniferophagous bark beetles detect and avoid volatile compounds released from the leaves and/or bark of angiosperms and other nonhost taxa ('nonhost volatiles'), and thereby may improve foraging efficiency by avoiding nonhost-dominated habitats and landings on unsuitable trees. Numerous individual nonhost volatiles have been identified and shown to inhibit coniferophagous bark beetle responses to traps baited with attractant pheromones and/or host kairomones (e.g., Dickens et al., 1992; Huber and Borden, 2003; summarized in Zhang and Schlyter, 2004; Zhang et al., 2007; Dodds and Miller, 2010; Fettig et al., 2012). Furthermore, nonhost volatiles have been used with some success to protect individual trees from bark beetle attacks (Borden et al., 2003; Jakuš et al., 2003; Fettig et al., 2009; Schiebe et al., 2011; Schlyter, 2012).

The southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae: Scolytinae), is a specialist on *Pinus* spp. and causes greater economic losses to forestry in the southern United States than any other organism (Price et al., 1998). Semiochemicals from both the beetle and host allow *D. frontalis* to locate hosts being attacked by conspecifics, and can mediate 'mass attacks' on resistant trees that overwhelm host defenses and thereby allow colonization and beetle reproduction (Sullivan, 2011). Odors associated with the host and, in particular, the volatile fraction of pine resin (i.e., pine turpentine) appear to be important to this semiochemical blend. Pine turpentine components are released in high concentrations from beetle attack sites together with beetle pheromones (Pureswaran and Sullivan, 2012), and can enhance responses to the beetle's attractive pheromone components by one to two orders of magnitude (Billings, 1985; Sullivan et al., 2007b; authors' unpublished data). α-Pinene, the predominant constituent of turpentine of the major host species of *D. frontalis* within the southeastern United States, is the main active component of the host's odor blend (Renwick and Vité, 1969). Certain volatiles associated with hardwood nonhosts have shown some capacity to inhibit *D. frontalis* aggregation on pine hosts. The angiosperm-associated green leaf volatiles, hexanal and hexanol, alone (Dickens et al., 1992) or in combination with the nonhost volatiles, nonanal, (*Z*)-3-hexen-1-ol, guaiacol, and benzaldehyde (Sullivan et al., 2007a), have been reported to significantly reduce catches of *D. frontalis* in traps baited with components of the aggregation attractant, frontalin and α-pinene. Such evidence suggests that *D. frontalis* requires an olfactory cue of an appropriate host in order to respond to its aggregation pheromone, and that nonhost volatiles may provide a cue for an inappropriate host.

Given the diversity of nonhost tree species within the range of *D. frontalis*, we hypothesized that the compounds potentially involved in signaling an inappropriate host should be likewise diverse. In order to measure this potential diversity, we used a coupled gas chromatograph-electroantennograph (GC-EAD) to detect all olfactory stimulants for *D. frontalis* arising from eight species (six genera) of angiosperm trees that commonly co-occur with host pines of *D. frontalis* in the southeastern United States. Furthermore, we quantified the relative olfactory sensitivity of *D. frontalis* to selected angiosperm volatiles by means of GC-EAD dose–response tests, under the assumption that greater olfactory responsiveness is a probable correlate of relatively greater behavioral activity and biological importance of an olfactory cue. Finally, we performed field trials with selected groups of antennally-active angiosperm volatiles to determine their capacity to alter *D. frontalis* attraction to pheromone-baited traps. Due to their potential low cost and toxicity, aggregation-inhibiting nonhost volatiles could prove to be ideal semiochemical tools for managing *D. frontalis*.

Methods and Materials

Collection of Insects and Nonhost Plant Material Adult *D. frontalis* were collected daily from a rearing chamber (Browne, 1972) housing bolts of naturally-infested loblolly pine, *Pinus taeda* L., cut in the Homochitto National Forest, Mississippi, USA (31° 23′ N, 91° 10′ W). Emerged beetles were maintained at 8 °C on moistened filter paper in glass Petri dishes for up to 1 d, and later sexed by the presence of a protruding pronotal callus (females) or a deep groove on the frons (males) (Wood, 1982).

Eight nonhost hardwood species were selected for analysis based on their relative abundance in host pine forests within the range of *D. frontalis* in the Gulf Coast region of the United States: red maple (*Acer rubrum* L.), mockernut hickory (*Carya alba* (L.) Nutt. ex Ell.), sweetgum (*Liquidambar styraciflua* L.), black tupelo (*Nyssa sylvatica* Marsh.), black cherry (*Prunus serotina* Ehrh.), southern red oak (*Quercus falcata* Michx.), blackjack oak (*Quercus marilandica* (L.) Muenchh.), and water oak (*Quercus nigra* L.). All trees were located in Camp Livingston, Kisatchie National Forest, Louisiana, USA (31° 25′ N, 92° 22′ W). Leaves were clipped from three standing trees of each species (mean dbh=9.4–24.6 cm) and a single additional tree of each species (dbh=12.0–17.5 cm) was felled and its trunk stripped of bark (Table 1). All materials were placed into zippered plastic bags and transported in ice-pack containing coolers to the laboratory.

Volatile Entrapment and Extract Preparation for GC-EAD Analysis Wearing nitrile gloves, we manually shredded leaves from the 8 nonhost hardwoods and placed them inside a sealed 7500-ml glass desiccator within 2 hr following collection. A silicone rubber stopper with two polytetrafluoroethylene (PTFE) tubes passing through it was inserted in the hole at the top of the lid. Prior to insertion into the desiccator lid, the stopper was wrapped with a PTFE sheet through which the tubes also penetrated. This sheet limited contamination of the desiccator headspace with volatiles from the stopper. Inside the desiccator, the opening of one of the tubes was positioned level with the rim of the desiccator (i.e., above the sampled material), whereas the opening of the other was positioned at the bottom of the desiccator and beneath the sampled material. Outside the desiccator, one tube was connected to an activated charcoal filter (a glass trap filled with approximately 20 g activated charcoal, 6–16 mesh) and the other to a PTFE cartridge filled with 500 mg of Super-Q® adsorbent (50–80 mesh, Waters Inc., Milford, MA, USA) in turn connected to a vacuum pump. The leaves were aerated for 48 hr at 500 ml/min and a room temperature of 20–22 °C. Afterward, trapped compounds were desorbed from the cartridges with 7.5 ml of redistilled pentane, concentrated to approximately 1/20 with

Table 1 Tree size and total dry weights of nonhost hardwood leaves and bark aerated to yield volatile compounds used in GC-EAD tests on *Dendroctonus frontalis*

Tree species (common name)	DBH ± SE (cm)[a]	Leaf dry weight (g)[b]	Bark dry weight (g)[b]
Acer rubrum (red maple)	16±4; 13	83	433
Carya alba (mockernut hickory)	9±1; 15	84	448
Liquidambar styraciflua (sweetgum)	14±3; 15	66	497
Nyssa sylvatica (black tupelo)	13±1; 12	84	521
Prunus serotina (black cherry)	10±1; 18	84	620
Quercus falcata (southern red oak)	17±2; 17	131	713
Quercus marilandica (blackjack oak)	25±4; 14	96	669
Quercus nigra (water oak)	16±3; 12	79	643

[a] *DBH* diameter at breast height (approx. 1.5 m). Values are the mean DBH of three trees from which leaves were collected followed by the DBH of the tree from which bark was collected

[b] Leaves and bark were collected from trees felled in June, 2005 and November–December, 2006, respectively. All sample material collected in Louisiana, USA

a micro Kuderno-Danish sample concentrator (Supelco Inc., Bellefonte, PA, USA) with a three-ball Snyder column, and stored at −80 °C. The aerated materials were oven-dried at 60 °C and weighed daily until weight was constant and dry weights could be determined (Table 1).

Bark strips from each hardwood species were sampled for volatiles inside PTFE bags (63.5×30.5 cm). PTFE bags were used instead of the glass desiccator due to greater available volume. Headspace volatiles were conducted out of each bag through a PTFE tube inserted into the bag's mouth so that the tube's opening was positioned near the bag's closed end. The tubing exited the mouth of the bag by passing through a roll of activated charcoal mesh fabric (Universal Replacement Prefilter, Honeywell #38002, Southborough, MA, USA) around which the mouth of the bag was constricted with adhesive tape; this arrangement allowed odor-filtered air to pass into the interior of the bag to replace the air drawn out through the tube. An adsorbent cartridge (500 mg of Super-Q®) was attached to the exterior opening of the tube, and the bag contents were aerated for 24 hr at 100 ml/min and 20–22 °C (*A. rubrum* was aerated for 48 hr due to low concentrations of volatiles). Volatiles adsorbed onto the cartridges were extracted and concentrated as described above for the leaf aeration samples.

GC-EAD Analyses—Leaf/Bark Aerations Electrophysiological responses of the antennae of five male and five female *D. frontalis* to compounds in each of the concentrated foliage and bark aeration samples were assayed on a GC-EAD apparatus as described in Asaro et al. (2004). However, due to accidental loss of two of the bark aeration samples during the course of the experiment, only two male beetles were tested on *L. styraciflua* bark, and no male beetles were tested on *Q. marilandica* bark. Each beetle was assayed with a single foliage or bark sample. Electrodes consisted of an Ag/AgCl wire inserted into a sharpened glass capillary filled with Beadle-Ephrussi saline and 0.5 % polyvinylpyrrolidone. A reference electrode was inserted into the base of the excised head of each beetle. The tip of the recording electrode was removed so its opening matched the diameter of the antennal club, and then one side of the antennal club was laid flat against the opening so its entire surface was in contact with the saline (leaving the opposite side of the club exposed). Each preparation was positioned in a humidified, charcoal-purified airstream (400 ml/min), which received half the effluent from the Agilent 5890 GC. The GC had an HP-INNOWax column (60 m×0.25 mm×0.25 μm film; Agilent Technologies, Wilmington, DE, USA) with helium as the carrier gas. The temperature program was 35 °C for 1 min, 16 °C to 80 °C, 8 °C/min to 230 °C, and then held for a final 8 min. Aeration samples (1 μl each) were introduced into the GC in splitless mode with an injector temperature of 200 °C. EAD voltages (recorded five times per second) were entered into Microsoft Excel and summed across multiple runs to produce composite EAD traces for all analyses with each sex and odor source type. This enhanced our ability to distinguish small responses from background noise. Olfactory stimulants in the GC-EAD traces were tentatively identified by coupled gas chromatography–mass spectrometry (GC-MS) on an Agilent 6890–5973 instrument operating with the same column and operating parameters as the GC-EAD. Sample peaks were identified by matching their mass spectra and retention times to those of commercially-obtained synthetic standards. If a compound produced a detectable response for a particular sex at the same retention time in at least four of the five traces, it was classified as antennally-active.

GC-EAD Analyses—Synthetic Nonhost Volatiles Twenty synthetic volatile compounds—14 olfactory stimulants identified from angiosperm tissue aerations in the above experiment, four compounds reported in the literature to be associated with angiosperm nonhosts (Huber et al., 2000b; Zhang and Schlyter, 2004), nonanoic acid, and (−)-verbenone—were combined into three mixtures at an approximate concentration of 40 μg/μl and diluted serially (4, 0.4, 0.04, 0.004, and 0.0004 μg/μl) in redistilled hexane (Table 2). Nonanoic acid was tested because in the previous experiment an extremely low concentration compound in the *P. serotina* foliage aeration sample with a mass spectrum similar to nonanoic acid (major ions 41, 43, 55, 57, 60, 73,

Table 2 Synthetic compounds tested on male and female *Dendroctonus frontalis* in GC-EAD bioassays

Compound	Source[a]	Purity (%)
Synthetic Mixture 1[b]		
benzyl alcohol	Sigma-Aldrich Corp.	>99
heptanal	Sigma-Aldrich Corp.	95
1-hexanol	Sigma-Aldrich Corp.	98
methyl salicylate	Sigma-Aldrich Corp.	>99
nonanal	Sigma-Aldrich Corp.	95
salicylaldehyde	Sigma-Aldrich Corp.	98
Synthetic Mixture 2[b]		
benzaldehyde	Sigma-Aldrich Corp.	>99
guaiacol	Sigma-Aldrich Corp.	98
hexanal	Sigma-Aldrich Corp.	98
(*Z*)-3-hexen-1-ol	Sigma-Aldrich Corp.	98
(*E*)-2-hexenal	Sigma-Aldrich Corp.	98
(−)-verbenone	Pherotech International Inc.	95
Synthetic Mixture 3[b]		
trans-conophthorin	Pherotech International Inc.	87
eucalyptol	Sigma-Aldrich Corp.	99
(*E*)-2-hexen-1-ol	Sigma-Aldrich Corp.	96
linalool	Sigma-Aldrich Corp.	97
myrcene	Sigma-Aldrich Corp.	90
nonanoic acid	Sigma-Aldrich Corp.	97
phenylethyl alcohol	Sigma-Aldrich Corp.	98
terpinen-4-ol	Acros Organics	97

All chiral compounds were racemic unless otherwise indicated

[a] Sigma-Aldrich Corp., St. Louis, MO, USA; Pherotech International Inc., Delta, British Columbia, Canada; Acros Organics, Geel, Belgium

[b] Each mixture also contained 98 % *cis*-verbenol (Aldrich, Milwaukee, WI, USA) as an internal standard used to calculate percent EAG responses

115, and 129) elicited antennal responses in male and female beetles. However, the retention time of this compound did not precisely match the retention time of a nonanoic acid standard. Although the identity of the unknown compound could not be verified, we assayed nonanoic acid as a possible close analog to this antennally-active unknown. (−)-Verbenone, an aggregation-disrupting pheromone component for *D. frontalis*, was used as a reference compound in this experiment. All mixtures were formulated to insure a minimum 50-second gap between individual compounds in retention time and thus in antennal exposure (i.e., co-eluting and closely-eluting compounds were placed into separate mixtures). This was done to minimize the potential effects of habituation on antenna responsiveness to successively eluting compounds. Each dilution also contained 4 μg/μl *cis*-verbenol (Aldrich, Milwaukee, WI, USA) as an electrophysiologically-active internal standard.

Apparatus and procedures for GC-EAD were similar to those described above for analyses of aeration samples; however, the oven program for synthetic mixtures 1 and 2 was 40 °C for 1 min, 16 °C/min to 80 °C, then 7 °C/min to 230 °C and held for 10 min; for synthetic mixture 3, the oven program was 35 °C for 1 min, 16 °C to 80 °C, then 8 °C/min to 230 °C and held for 8 min. One microliter of each mixture/dilution was injected in split mode (20:1) into the GC with an injector temperature of 200 °C. Since we used a 1:1 split between the EAD and the flame ionization detector, antennae were exposed to roughly 1000, 100, 10, 1, 0.1, or 0.01 ng of each compound with each of the respective dilutions. A total of four beetles were tested for each sex, dilution, and mixture (each beetle exposed to a single mixture/dilution) with these categories assayed in random order.

The voltage amplitudes recorded from the EAD at the retention time for each compound were tabulated. If in two or more of the four replicate analyses this voltage was greater than the 90th percentile of the background noise level, then the response was classed as non-random ($P<0.052$, table of cumulative binomial probabilities; Bhattacharyya and Johnson, 1977) and considered evidence of genuine electrophysiological sensitivity to the compound at the tested dilution. In each run, the level of background noise was determined by calculating the average of the noise during a randomly selected period of approx. 2 min. of the EAD output prior to the introduction of the first sample stimulus. The 'response threshold' for a particular compound was identified as the lowest concentration that still produced electrophysiological activity. All signal voltage deflections from the GC-EAD runs were corrected for loss of antennal sensitivity over the course of a run. This was accomplished by first calculating the responses to the *D. frontalis* pheromone, *endo*-brevicomin (10 μl in 1 ml of mineral oil), introduced to each beetle's antenna at the beginning and end of each GC-EAD run by "puffing" the compound into the air stream from a Pasteur pipette. The assumed linear decline in the height of responses to the two "puffs" then was applied to responses to test compounds at corresponding retention times. These corrected response amplitude values were normalized by dividing the voltage produced at the retention time of each test compound by the response voltage produced by the internal standard *cis*-verbenol, and then means and standard errors were calculated for these values. Differences in mean response voltages between males and females for each compound and dilution (for concentrations at or above the response threshold) were analyzed using a *t*-test with Bonferroni correction ($\alpha=0.05$).

Trapping Study Dispensers releasing eleven angiosperm compounds that elicited antennal responses in GC-EAD experiments were deployed on 12-unit Lindgren multiple-funnel traps in *D. frontalis* infestations in the Homochitto

National Forest, Mississippi, USA (Table 3). We tested the following eight treatments: (1) "attractant" (racemic frontalin and racemic α-pinene); (2) attractant with "green leaf volatile blend" [four 6-carbon alcohols and aldehydes: hexanal, 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexenal]; (3) attractant with "bark volatiles blend" [seven compounds in the bark aerations: benzaldehyde, benzyl alcohol, guaiacol, heptanal, methyl salicylate, nonanal, and salicylaldehyde]; (4) attractant with "alcohols blend" [four alcohols: benzyl alcohol, guaiacol, 1-hexanol, and (*Z*)-3-hexen-1-ol]; (5) attractant with "aldehydes blend" [six aldehydes: benzaldehyde, heptanal, hexanal, (*E*)-2-hexenal, nonanal, and salicylaldehyde]; (6) attractant with "nonhost volatiles blend" [all nonhost compounds used in experiment: benzaldehyde, benzyl alcohol, guaiacol, heptanal, 1-hexanol, (*Z*)-3-hexen-1-ol, hexanal, (*E*)-2-hexenal, methyl salicylate, nonanal, and salicylaldehyde]; (7) attractant with the aggregation-inhibiting pheromone component (−)-verbenone (Salom et al., 1992); and (8) an unbaited control. The sources and chemical purities of the nonhost volatiles and (−)-verbenone are identical to those in the GC-EAD analyses (Table 2). Each compound was released from a separate dispenser attached between the sixth and seventh funnels from the bottom of each trap. We used gravimetric analysis to formulate dispensers to all release approximately 10 mg per day of each nonhost volatile at approx. 22 °C (Table 3). Each dispenser initially was filled with 300 µl of compound, and was replaced with a newly filled dispenser every 2 wk (except for the frontalin dispenser, which was replaced every 4 wk), when approximately half of the original amount of compound remained.

Three randomized complete blocks of traps (8 traps with 1 of the 8 treatments assigned randomly to each without duplication) were established within the leading edges of two active *D. frontalis* infestations located >4 km apart. Traps were spaced ≥10 m apart within blocks, and ≥30 m

Table 3 Synthetic compounds used to trap *Dendroctonus frontalis*

Compound	Dispenser	Release rate (mg/d)[a]	Blend[b]						
			Attractant	Green leaf volatiles	Bark volatiles	Alcohols	Aldehydes	Nonhost volatiles	Attractant + (−)-verbenone
benzaldehyde	4-ml glass vial (9-mm diam. polyethylene lid) X 4	10.0			X		X	X	
benzyl alcohol	polyethylene bag (5×10 cm) X 2	13.0			X	X		X	
guaiacol	polyethylene bag (2.5×2.5 cm)	9.6			X	X		X	
heptanal	polyethylene Epi tube (0.4 ml) X 2	9.6			X		X	X	
hexanal	polyethylene Epi tube (0.4 ml) X 2	11.4		X			X	X	
1-hexanol	polyethylene bag (5×10 cm)	9.3		X		X		X	
(*Z*)-3-hexen-1-ol	polyethylene bag (5×10 cm)	9.6		X		X		X	
(*E*)-2-hexenal	polyethylene Epi tube (0.4 ml) X 4	10.8		X			X	X	
methyl salicylate	polyethylene Epi tube (0.4 ml) X 5	10.5			X			X	
nonanal	polyethylene Epi tube (0.4 ml) X 4	11.2			X		X	X	
salicylaldehyde	polyethylene Epi tube (0.4 ml) X 6	10.2			X		X	X	
frontalin[c]	polyethylene Epi tube (0.4 ml)	2.0	X	X	X	X	X	X	X
α-pinene[d]	polyethylene Epi tube (0.4 ml)	6.7	X	X	X	X	X	X	X
(−)-verbenone	polyethylene bag (5×10 cm)	8.8							X

All chiral compounds were racemic unless otherwise indicated

[a] Release rates determined using gravimetric analysis at approx. 22 °C

[b] *X* compound present in treatment blend

[c] 99% purity; ChemTica International, San Jose, Costa Rica

[d] 97% purity; Aldrich, Milwaukee, WI, USA

between traps of the two blocks located within the single infestation. Trap collections were made approximately twice per week, and after each collection treatment positions were re-randomized without replacement within each block. During July–October, 2005, eight and nine collections, respectively, were made at the two blocks in the larger infestation, and ten collections were made at the block in the smaller infestation. The mean numbers of *D. frontalis* males and females per trap per day were averaged by block and treatment and then log transformed to meet ANOVA assumptions. Suitability of the data transformation was determined through examination of the residuals plots. For each sex, treatments were compared using a 2-way ANOVA (factors block and treatment) followed by Dunnett's test (α=0.05) for comparing each baited treatment to the unbaited control and Tukey's HSD (α=0.05) for comparing the remaining seven baited treatments to each other (SAS Institute, 2003).

Results

GC-EAD Analyses—Leaf/Bark Aerations Forty-six volatile compounds were identified in aerations of leaves and bark of eight nonhost angiosperm species, with 22–30 compounds identified from any single species (Table 4). Male and/or female *D. frontalis* antennae responded to 28 different identified compounds present in one or more of the aeration samples. Of these antennally-active compounds, 22 were detected more often in leaves than bark, four more often from bark than leaves, and two in the same frequency in bark and leaves.

Fifteen antennally-active compounds were isolated from five or more nonhost trees, with nine (1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, caryophyllene, α-*humulene, limonene,* α-pinene, and β-pinene) occurring in the aerations of all of the sampled nonhosts (Table 4). The six remaining common antennally-active volatiles were benzaldehyde, benzyl alcohol, hexanal, phenylethyl alcohol, β-cubebene, and linalool. Four compounds (eucalyptol, geraniol, *trans*-verbenol, and verbenone) excited *D. frontalis* antennae, but were isolated from only a single sampled nonhost species.

GC-EAD Analyses—Synthetic Nonhost Volatiles The 19 tested synthetic angiosperm compounds and reference compound, (−)-verbenone, all elicited antennal responses in male and female *D. frontalis* at some dose (Fig. 1). The largest response amplitudes (relative to the internal standard) were produced by *trans*-conophthorin, nonanoic acid, terpinen-4-ol, phenylethyl alcohol, and eucalyptol. At the maximum tested dose (1000 ng per antenna), hexanal, heptanal, and (*E*)-2-hexenal produced the three lowest response amplitudes. For all dilutions and types of synthetic nonhost volatiles, no significant differences were observed between male and female antennal responses.

Figure 2 summarizes the beetles' antennal response thresholds to the assayed angiosperm volatiles and (−)-verbenone. The lowest response thresholds were to nonanoic acid and (−)-verbenone at 0.01 ng for females and 0.1 ng for males. Other compounds with low response thresholds were nonanal and linalool (0.1 ng for females and males); and (*E*)-2-hexen-1-ol, phenylethyl alcohol, and 1-hexanol (0.1 ng for females and 1 ng for males). In contrast, high concentrations of (*E*)-2-hexenal, salicylaldehyde, methyl salicylate, heptanal, and hexanal (100–1000 ng) were required to elicit measurable electrophysiological responses. Methyl salicylate generated a response in the dose response test although it failed to do so when presented in the angiosperm aerations; concentrations of this compound in the aeration samples were possibly less than the response threshold of the antenna.

Trapping Study Treatment effects were significant for both male (F=7.11; df=7,14; P=0.001) and female (F=10.01, df=7,14; P<0.001) *D. frontalis*. Traps baited with the standard attractant (frontalin and α-pinene) alone caught significantly more beetles of either sex than the unbaited control (Fig. 3). Attractant-baited traps amended with either the bark volatiles or the nonhost volatiles blends caught significantly fewer male beetles than traps baited with the standard attractant alone, and their catches did not differ significantly from the unbaited controls. Attractant-baited traps amended with the nonhost volatiles blend caught significantly fewer female beetles than traps baited with the standard attractant alone, and their catches did not differ significantly from the unbaited controls. In contrast, traps amended with the green leaf volatiles blend, the alcohols blend, the aldehydes blend, or (−)-verbenone did not catch significantly less of either sex than the attractant-only traps.

Discussion

A broad diversity of antennally-active volatiles was identified from the leaves and bark of each angiosperm tree examined (Table 4). Every species produced the green leaf volatiles 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexenal, as well as caryophyllene, α-humulene, limonene, α-pinene, and β-pinene, terpenes often found in conifers. Additional antennally-active volatiles isolated from five or more nonhost species included aromatic compounds (benzaldehyde, benzyl alcohol, and phenylethyl alcohol), terpenes (β-cubebene, linalool), and a green leaf volatile (hexanal). The capacity of *D. frontalis* antennae to detect numerous volatiles present in these diverse angiosperm tree

Table 4 Antennal responses of male and female *Dendroctonus frontalis* to volatile compounds identified from leaf and bark aerations of eight nonhost angiosperm species (*Acer rubrum*, *Carya alba*, *Liquidambar styraciflua*, *Nyssa sylvatica*, *Prunus serotina*, *Quercus flacata*, *Quercus marilandica*, *Quercus nigra*)

Compound	Antennal responses[a]	Tree species[b]							
		A. rubrum	*C. alba*	*L. styraciflua*	*N. sylvatica*	*P. serotina*	*Q. falcata*	*Q. marilandica*	*Q. nigra*
4-allylanisole	M,F			L,B	B				
anisole							L,B		
benzaldehyde	M,F	L	L		L	L,B	L	L	L
benzyl alcohol	M,F				L	L,B	L	L	L
borneol		B	L,B						
camphene		L,B	L,B	L,B	B	L,B	B	B	B
camphor	M,F	B		L,B	B			B	
caryophyllene	M,F	L,B	L,B	L,B	L,B	L,B	L,B	L,B	L,B
β-cubebene	M,F	L	L	L,B	L		L,B	L,B	B
p-cymene		L,B	L	L,B	B	L,B	L		
decanal						L			
α-*p*-dimethylstyrene	M	L		L,B					
eucalyptol	M,F		L,B						
α-fenchene				B	B				
fenchone				B	B				
geraniol	F				L				
guaiacol	M,F				L		L	L	L
hexanal	M,F	L	L,B		L	L,B	L	L	L,B
1-hexanol	M,F	L	L,B	L	L	L,B	L	L,B	L,B
(*E*)-2-hexen-1-ol	M,F	L	L	L	L	L,B	L	L	L
(*Z*)-3-hexen-1-ol	M,F	L	L	L	L	L,B	L	L	L
(*E*)-2-hexenal	M,F	L	L	L	L	L,B	L	L	L
α-humulene	M,F	L,B	L,B	L,B	L,B	B	L,B	L,B	B
isopinocamphone				L,B	B				
limonene	M,F	L,B	L,B	L,B	L,B	L,B	L	L,B	L
linalool	M,F	L	L		L	L	L	L	L
6-methyl-5-hepten-2-one				L					
methyl salicylate		L		L	L	L	L	L	
myrcene	M,F	L,B	L	L,B		L,B			
naphthalene		L			L,B	L,B	L	L	L
nonanal	M,F	L				L			
α-phellandrene						L			
β-phellandrene		L,B		L,B	L	L,B	L	L	L
phenylethyl alcohol	M,F				L	L,B	L	L	L
α-pinene	M,F	L,B	L,B	L,B	L,B	L,B	L	L	L,B
β-pinene	M,F	L,B	L,B	L,B	L,B	L,B	L	L	L
sabinene		L	L	L		B			
styrene		L,B	L	L,B	L	L,B	L,B	L	L,B
terpinen-4-ol	M,F	L	L	L					
α-terpinene	M	L	L	L,B			L		
γ-terpinene			L	L,B				B	B
α-terpineol			L						
terpinolene	M	L	L	L			L		
undecane		L	L		L	L,B	L	L	L
trans-verbenol	F		L						

Table 4 (continued)

Compound	Antennal responses[a]	Tree species[b]							
		A. rubrum	*C. alba*	*L. styraciflua*	*N. sylvatica*	*P. serotina*	*Q. falcata*	*Q. marilandica*	*Q. nigra*
verbenone	M,F				B				

Antennae from five male and five female beetles were exposed to both leaf and bark aeration samples from each tree species (exception: two males for *L. styraciflua* bark and no males for *Q. marilandica* bark). The EAD's were summed by sex, sample type (leaf or bark), and tree species to determine occurrence of positive antennal responses. All sample material collected in Louisiana, USA.

[a] Apparent antennal response by male (M) and/or female (F) *D. frontalis* elicited at the retention time of the indicated compound in traces of one or more of the aeration samples. Blanks indicate no measurable antennal response to a compound in any of the leaf or bark aeration tests.

[b] Compound was present in leaf (L) or bark (B) aeration of the indicated tree species, regardless of whether or not it elicited an antennal response

species indicates that a large number of compounds could function in the discrimination of hosts and nonhosts by *D. frontalis*.

Many of the olfactory stimulants identified in the angiosperm trees have additional possible origins in the habitat of *D. frontalis*, and sensitivity to them may exist that fulfill biological demands other than (or in addition to) host species discrimination. For example, one or more of the nonhost species sampled contained volatiles identified in the resin of host pines of *D. frontalis* (including the aggregation synergist α-pinene) as well as *D. frontalis* pheromone components (*trans*-verbenol and verbenone) (Mirov, 1961;

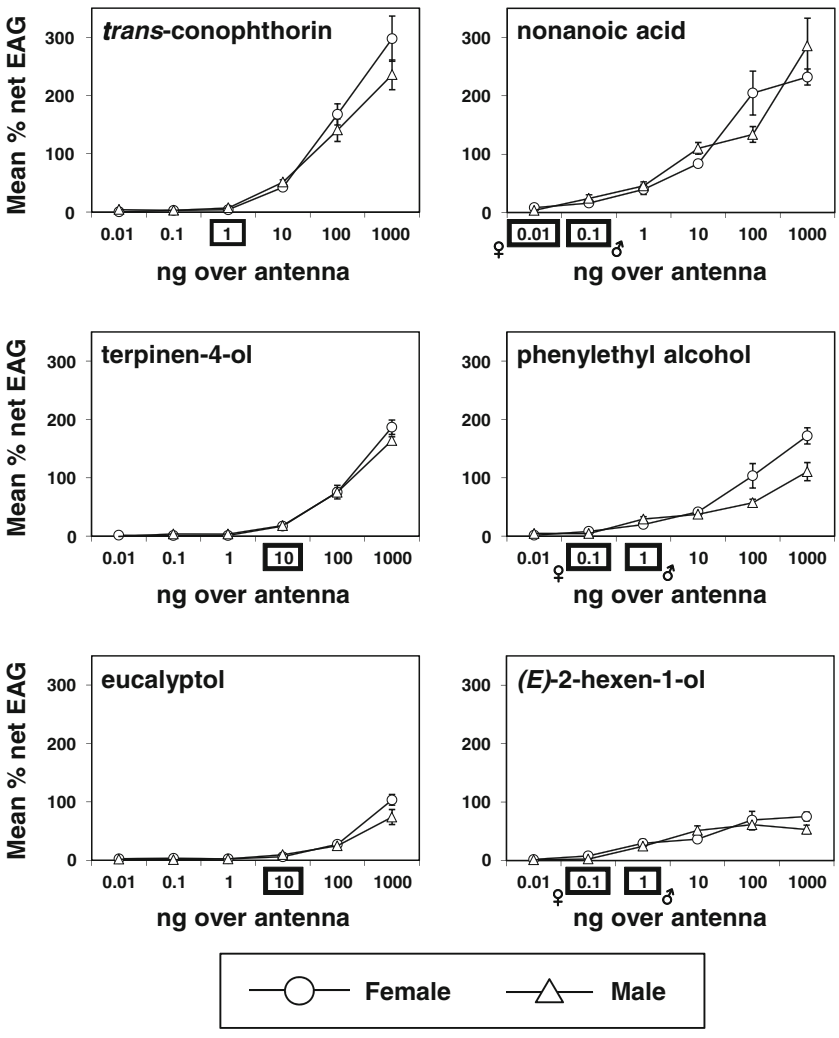
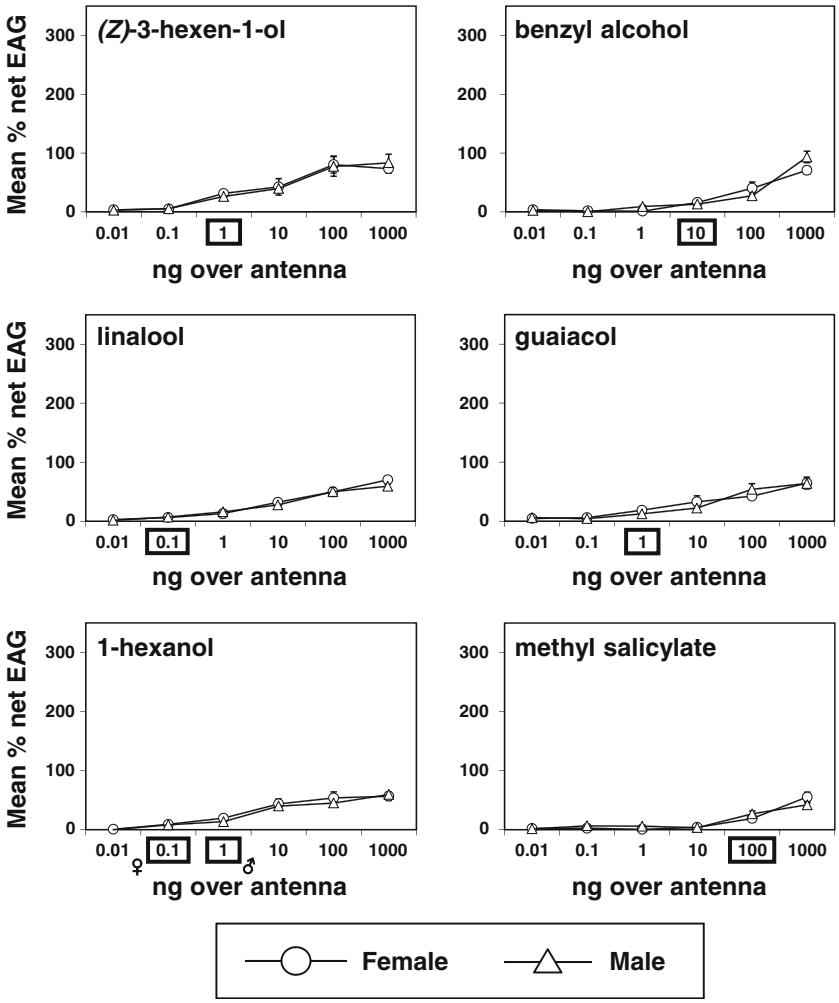


Fig. 1 Mean percent EAD responses (±SE) relative to the internal standard, *cis*-verbenol (4 μg/μl), of female and male *Dendroctonus frontalis* to six dilutions of 18 synthetic nonhost volatiles, nonanoic acid, and (−)-verbenone (N=4 for each sex, compound, and dilution). The response threshold (ng over antenna) for each compound is enclosed by a box. Boxes labeled "♀" and "♂" indicate thresholds for females and males, respectively (absence of a label indicates thresholds were the same for both sexes)

Fig. 1 (continued)



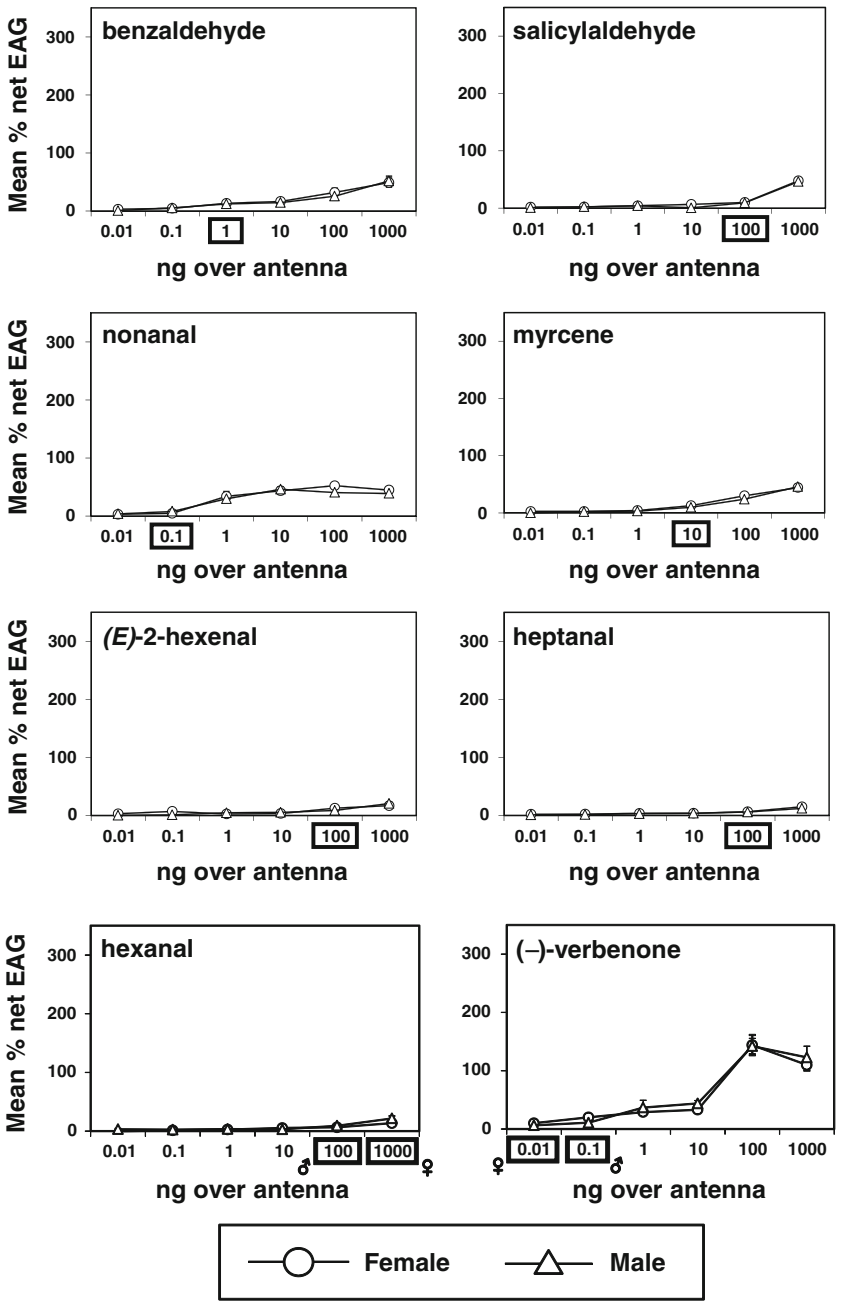
Sullivan, 2011). Nonetheless, the antennal stimulants detected in our GC-EAD analyses presumably represent the full range of olfactory cues available to and thus possibly used by these insects for detecting and rejecting nonhosts, whether by detection of the qualitative presence of particular compounds or perception of particular combinations or ratios. Presumably, compounds not typically associated with conifers are more likely to be involved in rejection of nonhosts by conifer-infesting bark beetles such as *D. frontalis* (Zhang and Schlyter, 2004), and therefore these compounds were the focus of the electrophysiological dose–response and behavioral studies.

Relative olfactory sensitivity as measured by electroantennogram response thresholds is not a reliable predictor of behavioral activity; however, this measure presumably should allow for comparison of the relative distances over which odorants released at the same rate could be perceived by an insect. Thus point sources of compounds with the lowest response thresholds (i.e., nonanoic acid, nonanal, linalool, phenylethyl alcohol, 1-hexanol, and (*E*)-2-hexen-1-ol) apparently have the potential to affect *D. frontalis* behavior over a larger average radius than the others. Except for nonanoic acid, all of these compounds were present in two or more of the angiosperm species tested.

The two most inhibitory nonhost volatile treatments in our trapping study (i.e., the nonhost volatiles blend and the bark volatiles blend) both contained benzaldehyde, benzyl alcohol, guaiacol, heptanal, methyl salicylate, nonanal, and salicylaldehyde (Fig. 3). Nonanal and benzaldehyde were among the compounds that elicited responses at the lowest thresholds with *D. frontalis* antennae. Previous research has shown that a blend containing nonanal and benzaldehyde, along with 1-hexanol, (*Z*)-3-hexen-1-ol, hexanal, and guaiacol, could significantly reduce male *D. frontalis* attraction to frontalin and α-pinene-baited traps (Sullivan et al., 2007a). Thus, nonanal and benzaldehyde in particular warrant further study in future trapping and tree protection experiments.

Some nonhost compounds disrupt bark beetle attraction in an additive and redundant manner, whereas others appear to function synergistically (Huber et al., 2000b; Zhang and

Fig. 1 (continued)



Schlyter, 2004). In our trapping study, the general tendency for test blends with greater numbers of different nonhost volatiles to have a greater inhibitory effect on *D. frontalis* is consistent with additive effects among nonhost components. Zhang and Schlyter (2004) noted that synergism seems to occur among volatiles derived from the bark (e.g., conophthorin, 8-carbon alcohols, and aromatic compounds) and foliage (the green leaf volatiles), whereas volatiles within either category are additive or redundant in activity. They hypothesized that this synergism occurs because nonhost volatiles from either source (i.e., bark or foliage) may be acting on different stages in the host-location process, with nonhost foliage odors likely functioning in host habitat discrimination and nonhost bark volatiles allowing discrimination of the taxonomic suitability of individual trees. However, such synergism was not observed in our trapping study, since the nonhost volatiles blend (i.e., a combination of the leaf volatiles and the bark volatiles blends) did not significantly decrease beetle responses compared to the bark volatiles blend alone. Redundancy of behavioral effects of different nonhost volatiles suggests that differences in the blends produced by individual nonhost species (such as observed in our aerations) may be inconsequential to recognition and rejection by bark beetles.

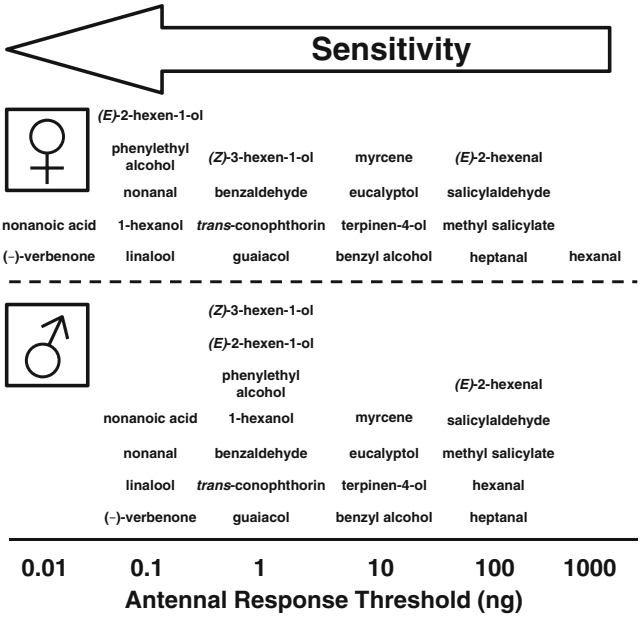


Fig. 2 Ranking of antennal response thresholds (ng over antenna) of female (♀) and male (♂) *Dendroctonus frontalis* to 18 synthetic nonhost volatiles, nonanoic acid, and (−)-verbenone. A lower threshold represents greater olfactory sensitivity to a given compound

Verbenone inhibits *D. frontalis* responses to traps baited with attractants consisting of frontalin and host odors (Payne et al., 1978; Clarke et al., 1999). It has been used with some success in *D. frontalis* spot disruption (Payne et al., 1992; Clarke et al., 1999). The failure of (−)-verbenone to inhibit *D. frontalis* attraction in our trapping experiment may be due to deployment of an insufficient release rate (i.e., 8.8 mg/d). Published reports of inhibition of *D. frontalis* attraction by verbenone have generally involved higher rates (e.g., 12–451 mg/d) (Smith et al., 1993). In the present study, we adjusted the rate of verbenone to be similar to that of the tested nonhost volatiles (i.e., ~10 mg/d) to allow for perhaps more meaningful comparisons in activity. Verbenone is hypothesized to function for *D. frontalis* and many other bark beetle systems as both an antiaggregation pheromone that signals complete colonization of the host bole, and as a microbe-generated indicator of host decay and unsuitability (Byers, 1989; Lindgren and Miller, 2002; Sullivan, 2011). In trapping and tree protection studies, verbenone significantly enhances the capacity of nonhost volatiles to inhibit attraction and attacks by *Dendroctonus* (Borden et al., 2003; Fettig et al., 2009) and *Ips* (Zhang, 2003; Zhang and Schlyter, 2003; Graves et al., 2008; Etxebeste and Pajares, 2011), and it may prove so for *D. frontalis* also.

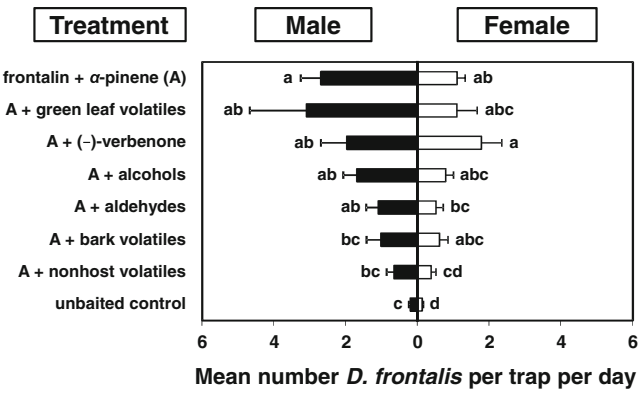


Fig. 3 Mean number of male and female *Dendroctonus frontalis* captured per trap per day (+ SE) in funnel traps baited with attractant (A = frontalin and α-pinene) alone, with (−)-verbenone, or with one of five different combinations of nonhost volatiles: green leaf volatiles = 1-hexanol, (*Z*)-3-hexen-1-ol, hexanal, and (*E*)-2-hexenal; alcohols = 1-hexanol, (*Z*)-3-hexen-1-ol, benzyl alcohol, and guaiacol; aldehydes = hexanal, heptanal, (*E*)-2-hexenal, nonanal, benzaldehyde, and salicylaldehyde; bark volatiles = benzyl alcohol, guaiacol, heptanal, nonanal, benzaldehyde, salicylaldehyde, and methyl salicylate; nonhost volatiles = 1-hexanol, (*Z*)-3-hexen-1-ol, benzyl alcohol, guaiacol, hexanal, heptanal, (*E*)-2-hexenal, nonanal, benzaldehyde, salicylaldehyde, and methyl salicylate. Bars associated with the same letter indicate trap catches within sex which were not significantly different, based on analyses of log transformed catch (Dunnett's test for comparing each baited treatment to the unbaited control and Tukey's HSD for comparing seven baited treatments to each other; $P<0.05$)

trans-Conophthorin elicited strong antennal responses in *D. frontalis* at the highest concentrations tested in the EAD dose response. Although *trans*-conophthorin was not isolated from the sampled nonhosts in the present study, it was included in the EAD dose response tests due to published reports of its potency as an inhibitory nonhost volatile for coniferophagous bark beetles. Conophthorin has been isolated from angiosperm species (e.g., *Betula* spp., *Populus* spp.), and has been shown to elicit antennal responses and/or inhibit semiochemical attraction in numerous scolytid species: *Dendroctonus brevicomis* LeConte (Shepherd et al., 2007), *D. ponderosae* Hopkins (Huber et al., 1999), *D. pseudotsugae* Hopkins (Huber et al., 1999, 2000a; Huber and Borden, 2001), *Dryocoetes confusus* Swaine (Huber et al., 2000a), *Ips perturbatus* (Eichhoff) (Graves et al., 2008), *I. pini* (Say) (Huber et al., 2000a, 2001), *I. sexdentatus* Boern. (Jactel et al., 2001; Etxebeste and Pajares, 2011), *I. typographus* (L.) (Zhang, 2003; Zhang and Schlyter, 2003), and *Trypodendron lineatum* (Olivier) (Borden et al., 2001). Strong antennal sensitivity to *trans*-conophthorin suggests the possibility that behavioral activity also exists with *D. frontalis*. Future trapping experiments should incorporate this nonhost volatile compound individually and in combination with other nonhost volatiles to test this hypothesis.

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