Characterization of an Aggregation Pheromone in *Hylesinus pruinosus* (Coleoptera: Curculionidae: Scolytinae)

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ABSTRACT We conducted laboratory and field bioassays to characterize the pheromone system of an ash bark beetle, *Hylesinus pruinosus* Eichhoff (Coleoptera: Curculionidae: Scolytinae). Solitary females in newly initiated galleries in ash logs produced (+)-*exo*-brevicomin, whereas male beetles paired with females produced (+)-*endo*-brevicomin, lesser quantities of (+)-*exo*-brevicomin, and a third compound that could not be identified. Beetles produced these compounds also after exposure to juvenile hormone III, and they were the sole volatile chemicals isolated from beetles or aerations of infested logs that elicited electrophysiological responses from antennae of either sex. In the field, both sexes were strongly attracted to traps baited solely with either racemic or pure (+)-*endo*-brevicomin. Racemic *exo*-brevicomin was much less attractive to both sexes than racemic *endo*-brevicomin, and it did not increase attraction of *endo*-brevicomin when released in combination. Host odors (volatiles from mechanically damaged ash branches) failed to attract beetles or increase attractiveness of racemic *exo*-brevicomin. Our evidence suggests that male-produced (+)-*endo*-brevicomin is the major component of an aggregation pheromone for *H. pruinosus*, with (+)-*exo*-brevicomin and the unidentified male compound playing an indeterminate role in the chemical ecology of this species. Our data thus show an instance in which the major aggregation pheromone component of a bark beetle is produced by the secondarily arriving sex, a rare occurrence in bark beetles but one which has been reported previously for the Hylesini.

KEY WORDS *endo*-brevicomin, *exo*-brevicomin, bark beetle pheromone, attractant, gas chromatography-electroantennographic detection

Hylesinus pruinosus Eichhoff (Coleoptera: Curculionidae: Scolytinae), an ash bark beetle, is native to the eastern United States and infests trees in the genus *Fraxinus* that have been recently cut or severely weakened by injury, disease, or environmental disturbances such as fire (S. L. Wood 1982, Drooz 1985). It is similar to the smaller, more common *Fraxinus*-infesting species, *Hylesinus aculeatus* Say, in both appearance and biology (S. L. Wood 1982). These beetles carve transverse biramous galleries in their host trees between the bark and wood of the trunk and larger branches (Drooz 1985, Solomon 1995). Unlike tree-killing scolytine taxa, their only economic impact is to manufacturers of rustic ash products on which the bark remains attached (Drooz 1985).

During field studies (Sullivan and Mori 2009) on the chemical ecology of a major bark beetle pest of pines, *Dendroctonus frontalis* Zimmermann, the authors observed large numbers of *H. pruinosus* responding to traps baited with the *D. frontalis* pheromone component (+)-*endo*-brevicomin. Although at least one previous report had suggested that *H. pruinosus* was attracted to isomers of brevicomin (Rabaglia 2002), no studies had been done to determine whether this species produced this compound or used it as a pheromone component. Because we were interested in documenting alternative sources of brevicomin in the habitat of *D. frontalis*, we initiated laboratory and field studies to identify volatiles produced by *H. pruinosus* and determine whether *endo*-brevicomin and perhaps additional compounds were components of an aggregation pheromone for this species.

Materials and Methods

Experimental Insects. Both sexes of *H. pruinosus* were live-trapped in the spring of 2007 and 2008 in the Sandy Creek Wildlife Management Area, Homochitto National Forest in eastern Mississippi. Twelve-unit multiple-funnel traps were baited with (±)-*endo*-brevicomin (Synergy Semiochemicals, Burnaby, British Columbia, Canada; Contech, formerly Phero Tech; Victoria, British Columbia, Canada) at a release rate of

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≈0.2 mg/d. Trap collection cups were fitted with a drain screen at the cup bottom and lined with moistened paper towel and ash twigs to minimize desiccation and injury to captured insects. Beetles were collected every 2–4 d and transported to the laboratory where they were maintained at 4–8°C on moistened paper towel for up to 2 wk before use. Live beetles were sexed by the sounds and abdominal movements associated with male stridulation; the validity of this technique was confirmed through dissection of individuals sorted by this method. Sex of insects either trapped or used in semiochemical isolations was determined or confirmed, respectively, by dissection of genitalia. Voucher specimens of these *H. pruinosus* and those collected in trapping studies (below) are maintained in the collection at USDA Forest Service, Southern Research Station, Pineville, LA.

Semiochemical Isolation. *Method 1: Aerations/Hindgut Extractions of Insects Excised From New Mines.* Logs (≈10 cm diameter by 70 cm) were cut from a healthy white ash (*Fraxinus americana* L.) in the Homochitto National Forest and allowed to dry in the laboratory for 4 d before use. *H. pruinosus* females were confined individually within #00 gelatin capsules over 1-mm-diameter holes (spaced ≈10 cm apart on the bark surface) drilled through the outer bark, and the logs were incubated at room temperature (21–29°C) for 1 d. Five females that had mined into the bark were excised and placed individually into 100-μl conical vials containing ≈3 mg of the adsorbent Super Q (80–100 mesh; Alltech, Deerfield, IL) (Sullivan 2005). Males were added singly to the gelatin capsules covering the entrances of six remaining established females. Male/female pairs were removed from the logs after a further 1 d, and individual beetles were processed using the same method as the unpaired females. Conical vials were incubated at room temperature for 24 ± 3 h, and both the Super-Q adsorbent and the excised hindgut of the beetle from each vial were extracted together in a total of 100 μl redistilled pentane/hexane spiked with 175 ng of the internal standard, heptyl acetate.

Method 2: Aerations of Infested Ash Logs. Volatiles were collected from logs of a green ash (*Fraxinus pennsylvanica* Marshall) artificially infested with both male and female *H. pruinosus.* The tree was purchased from a nursery in Forest Hill, LA and permitted to dry on the floor of a greenhouse for ≈2 wk before sectioning. Individual logs (13–8 cm diameter by 45 cm long) were sampled three successive times: (1) 24 h after initial introduction of 25 females, (2) 16 h after addition of 25 males (2 d after initial female introductions), and (3) ≈17 h after addition of a further 25 females (3 d after initial female introductions). The second introduction of females was made to study possible bigyny in this species and its potential impact on pheromone production.

Beetles were introduced to each log by confining them together inside either a cloth or vented polytetrafluoroethylene (PTFE) bag; visual inspections made at the initiation of each aeration indicated that most beetles introduced the previous day had entered the bark. For volatiles collections, an infested log was enclosed in a PTFE bag (60 by 28 cm), and air inside the bag was sampled at 10 ml/min for 6 h on a PTFE-encased cartridge containing 0.1 g of conditioned Porpak Q adsorbent (50–80 mesh; Alltech). The cartridge was attached to one end of a 1-m-length of corrugated PTFE tubing inserted into the interior of the bag. During aerations, the tubing was threaded through a roll of activated charcoal mesh (Universal Replacement Prefilter; Honeywell, Southborough, MA) around which the mouth of the bag was tightly sealed; this removed contaminating volatiles from outside air that entered the bag. Cartridges were extracted with 1.5 ml redistilled pentane, and the extracts were spiked with 3.5 μg heptyl acetate. The sampling procedure was replicated with four logs, with all procedures performed in the laboratory at room temperature. Portions (0.5 ml) from each replicate extract were pooled and concentrated 20-fold for use in gas chromatography-electroantennographic detection (GC-EAD) studies.

Method 3: Hindgut Extractions of Insects Treated With Juvenile Hormone III. Extracts were made from beetles exposed to juvenile hormone (JH) III (75% purity; Sigma-Aldrich, Milwaukee, WI), an elicitor of pheromone synthesis in some scolytines (Tillman et al. 1999). Acetone (0.5 μl) containing 20 μg JH III was applied to the ventral abdominal surface of five beetles of each sex with a blunt-tipped syringe, and acetone (0.5 μl) alone was administered to five male and four female beetles as controls. Beetles in each sex and treatment were incubated in separate petri dishes lined with moistened paper towel for 1 d at room temperature. Beetle hindguts were excised and extracted individually in 50 μl pentane spiked with 175 ng heptyl acetate. Additionally, hindguts of 10 JH III–exposed males and females were combined within sex and extracted in 0.5 μl pentane. These two extracts were concentrated in air to 0.1 hindgut equivalents/μl for GC-EAD analyses. All solvent extracts were stored at −80°C before analysis.

Chemical Analyses. Olfactory sensitivity of *H. pruinosus* to volatile compounds in both whole log aerations and the extracts of JH III–treated beetles was assayed using GC-EAD apparatus and techniques described in Asaro et al. (2004). A reference electrode ($Ag/AgCl_2$ saline-filled glass) was inserted into the foramen of a beetle's excised head, while saline in the open tip of an identical recording electrode made contact with one side of the intact antennal club (Sullivan 2005). The antennal preparation was positioned in a stream of purified, humidified air (400 ml/min), which received one half the effluent from the GC. Two microliters of sample was injected splitless onto a HP-INNOWax capillary column (60 m by 0.25 mm by 0.25 μm film; Agilent Technologies, Wilmington, DE); helium was the carrier gas, and the temperature program was 35°C (held 1 min), 16°C/min to 80°C, and 8°C/min to 230°C (held 8 min). EAD-active GC peaks were identified and quantified on either a Hewlett-Packard 6890–5973 or G1800C-coupled gas chromatograph-mass spectrometer (GC-MS), using the same

column and temperature program as used in the GC-EAD analyses. Compounds were identified by matches of both retention times and mass spectra to identified standards; they were quantified relative both to the internal standard heptyl acetate and to response curves calculated from analyses of serial dilutions of standards. One EAD-active GC peak could not be identified and was quantified approximately using the standard curve for *endo*-brevicomin. The enantiomeric composition of brevicomin isomers was determined for a subsample of the insect/adsorbent extracts. The subsample included two aerations of logs infested by both sexes and eight extracts from individual beetles (five females and three males, either infesting logs or exposed to JH III) that were found to contain relatively large quantities of brevicomin in our nonchiral analyses. These were reanalyzed by GC-MS using a Beta-Dex 120 cyclodextrin-phase capillary column (60 m by 0.25 mm by 0.25 μm film; Supelco, Bellefonte, PA) with a GC oven program of 40°C (held 1 min), 5°C/min to 70°C, 2°C/min to 155°C, and 25°C/min to 220°C (held 15 min). Standards of brevicomin optical isomers were obtained from Contech.

Trapping Studies. The attractiveness of *H. pruinosus*–produced compounds and host odors were evaluated in three trapping experiments. Twelve-unit multiple-funnel traps were erected 1 m above the ground on metal poles within the same area where the live beetles were collected. For each experiment, traps were established in two to five separate "trap lines," i.e., groups of stationary traps equal in number to the experimental treatments and with each treatment assigned at random to one of the traps. The intertrap spacing was >100 m both within and between trap lines. Trap catches were collected after a period of ≥2 d, and treatments were reassigned randomly among the traps of each trap line (treatment baits were moved without reassignment to any previous trap position) before commencement of another trapping period (up to four). Catch for each successive trapping period at each line of traps was considered a single replicate (N) for calculation of SE bars in figures.

Experiment 1 (22 March to 12 April 2006) used five trap lines (15 traps total) and two consecutive collection periods (14 and 7 d), to compare three bait treatments: (1) (+)-*endo*-brevicomin alone, (2) racemic *endo*-brevicomin alone, or (3) no bait. Experiment 2 (10–24 March 2008) used two trap lines (eight traps total) and four consecutive collection periods (4, 3, 4, and 3 d) to compare four bait treatments: (1) racemic *endo*-brevicomin alone, (2) racemic *exo*-brevicomin alone, (3) both racemic *endo*- and *exo*-brevicomin, or (4) no bait. Experiment 3 (26 March to 7 April 2008) used two trap lines (eight traps total) and four consecutive collection periods (2, 3, 2, and 5 d) to compare four bait treatments: (1) ash volatiles alone, (2) racemic *exo*-brevicomin alone, (3) both ash volatiles and racemic *exo*-brevicomin, or (4) no bait. In experiments 2 and 3, the treatment structure was a 2 by 2 factorial, with factors racemic *endo*-brevicomin and racemic *exo*-brevicomin in experiment 2 and factors ash volatiles and racemic *exo*-brevicomin in experiment 3.

Brevicomin isomers were released from a glass capillary (1.17 mm ID by 32 mm, with one heat-sealed end) secured open-end-up inside an inverted 1-dram glass vial and attached above the fourth funnel of the trap from the bottom (Sullivan et al. 2007). Racemic *endo*-brevicomin (Contech) was 95% chemically pure with <1% *exo*-brevicomin contamination, whereas the (+)-*endo*-brevicomin was synthesized (Sullivan et al. 2007) and was >99% enantiomerically and >95% chemically pure (contaminants had no known behavioral activity with scolytines). Racemic *exo*-brevicomin (Synergy Semiochemicals) was 94% pure with 5% contamination by *endo*-brevicomin. Release rates were determined in a fume hood by measuring volume loss in the capillary for both *endo*-brevicomin (0.2 mg/d at 23 ± 2°C) and *exo*-brevicomin (0.4 mg/d at 25 ± 2°C).

The ash volatiles baits consisted of branches from the crown of the green ash used in semiochemical isolation method 2 (i.e., dried whole ≈2 wk) cut into pieces (10–38 cm long; <4 cm diameter) <1 d before deployment. The outer bark on the largest diameter pieces was scraped away to enhance volatiles release, and each bait consisted of 0.3 kg branches evenly apportioned by diameter and placed into a fine-mesh screen bag. The screen bags were hung on the outside of the trap, and the branches/twigs were replaced with fresh material at the beginning of each trapping period (i.e., every 2–5 d).

For large trap catches, subsamples of 50–100 beetles were sexed, and the sex ratio was multiplied by total catch to estimate numbers of male and female *H. pruinosus.* To meet the homogeneity of variance assumption, trap catches per day were transformed using $\log(x + 0.5c)$, where $c = 1/[\text{max number of days between collections}]$, except for experiment 1, where $\log(x + 1)$ was used. Transformed values were analyzed separately by sex using PROC MIXED to carry out a split-block type of mixed-model analysis of variance (ANOVA) where treatment and collection period were regarded as stripped across each other within replicates. Thus treatment, period, and treatment × period were regarded as fixed effects, and trap line, trap line × treatment, and trap line × period were regarded as random (SAS Institute 2003). The treatment effect was partitioned to reflect the main effects and interaction corresponding to the 2 by 2 factorial structure for experiments 2 and 3. Degrees of freedom were obtained using the Kenward-Roger option. Treatment means were compared using Tukey's mean separation procedure (Tukey's honestly significant difference [HSD], $\alpha = 0.05$).

Results

Chemical Analyses. In GC-EAD analyses, antennae of both male and female *H. pruinosus* responded to three different compounds isolated from either host-infesting or JH III–treated males and females: *exo*-brevicomin, *endo*-brevicomin, and a compound that

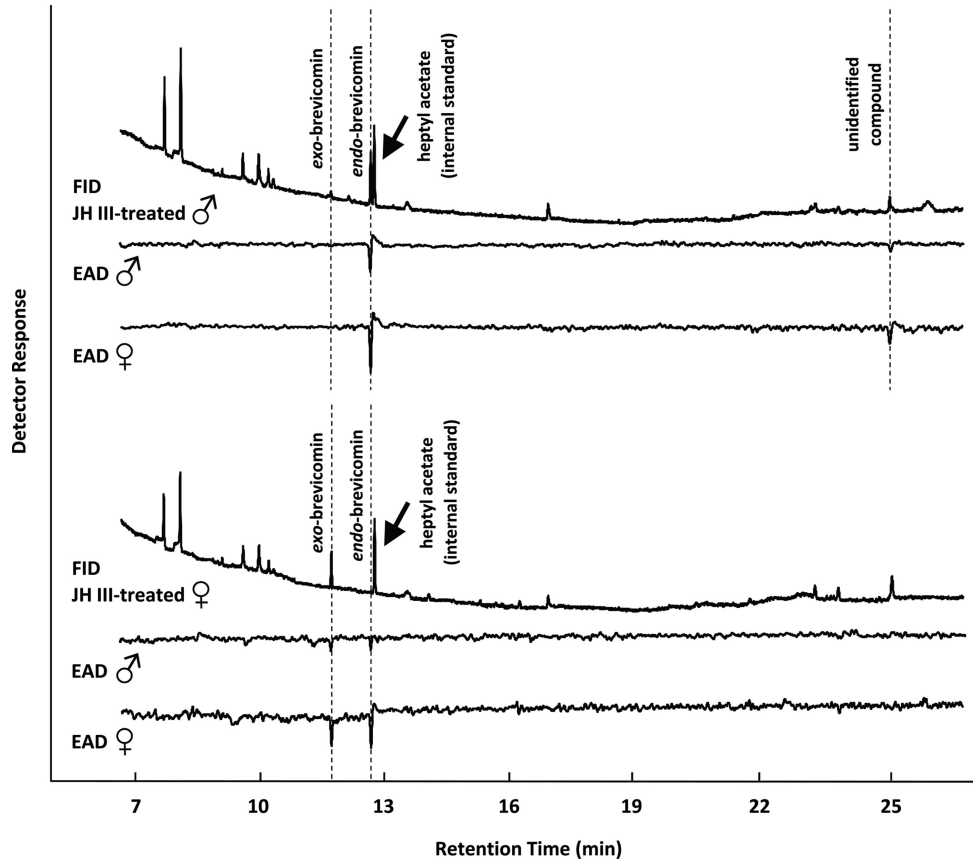

Fig. 1. GC-EAD analyses of either male or female *H. pruinosus* antennae exposed to concentrated hindgut extracts of JH III–exposed beetles. Traces represent output from the GC's flame ionization detector (FID) and electroantennographic detector (EAD). EAD traces for each sex represent summed data from analyses with three different antennae.

could not be identified by GC-MS (Figs. 1 and 2). This unidentified compound was not detected in aerations of beetle-infested logs but was present in samples from individual insects in which *endo*-brevicomin was also present. The molecular formula for the unidentified compound could not be determined because the molecular ion was not evident in its mass spectrum (Fig. 3).

exo-Brevicomin was isolated from all solitary and male-paired females excised from galleries in a host log, with trace amounts detected from some female-paired males (Table 1, method 1). Likewise, it was detected in aerations of whole logs infested with either females alone or both sexes (Table 1, method 2). *endo*-Brevicomin was detected from a majority of paired males excised from infested logs (Table 1, method 1) and could be detected by GC-MS in aerations of whole logs after the addition of males (Table 1, method 2). In contrast, *endo*-brevicomin was never detected by GC-MS from aerations/extracts of solitary mining females (Table 1, methods 1 and 2). All three olfactory stimulants were isolated from three of five JH III–treated males, whereas *exo*-brevicomin was the only olfactory stimulant isolated from a majority of JH III–treated females (Table 1, method 3). In contrast, none of the three olfactory stimulants were detected by GC-MS from any acetone-only treated control insects.

The (+)-enantiomer was the only form of either *exo*- or *endo*-brevicomin detected from *H. pruinosus* by chiral GC-MS. Granting the possibility that the (−)-enantiomer was present but beneath the detection threshold of the instrument, we estimate that *H. pruinosus* produce ≥98% of both (+)-*exo*-brevicomin and (+)-*endo*-brevicomin.

Trapping Studies. In experiment 1, the treatment effect was significant for both sexes (males: $F = 74.46$; df = 2,8; $P < 0.0001$; females: $F = 54.82$; df = 2,8; $P < 0.0001$). Catches of *H. pruinosus* in traps baited with either (±)- or (+)-*endo*-brevicomin were significantly higher than in unbaited traps, but responses did not differ between the two enantiomeric compositions (Tukey's HSD; Fig. 4). In experiment 2, the (±)-*endo*-brevicomin main effect was highly significant for males ($F = 98.22$; df = 1,3; $P = 0.0022$) and females ($F = 96.89$; df = 1,15; $P < 0.0001$), but neither the (±)-*exo*-brevicomin main effect (males: $F = 1.83$; df = 1,3; $P = 0.2690$; females: $F = 0.11$; df = 1,15; $P = 0.7477$) nor its interaction with (±)-*endo*-brevicomin (males: $F = 3.59$; df = 1,3; $P = 0.1544$; females: $F = 2.85$;

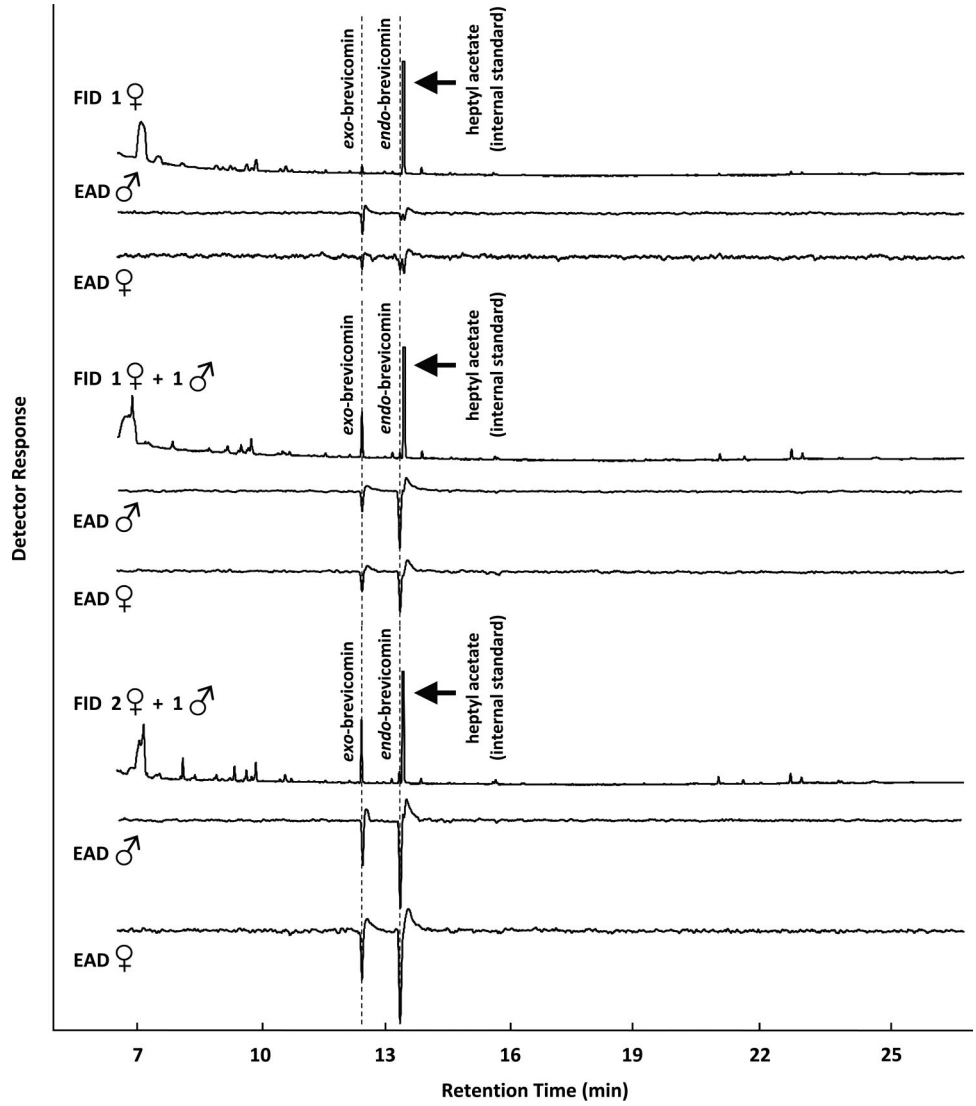

Fig. 2. GC-EAD analyses of either male or female *H. pruinosus* antennae exposed to aerations of ash logs infested sequentially with 25 female, then 25 male, and then a further 25 female *H. pruinosus*. Traces represent output from the GC's flame ionization detector (FID) and electroantennographic detector (EAD). EAD traces for each sex represent summed data from analyses with six different antennae.

df = 1,15; $P = 0.1121$) was significant. (±)-*endo*-Brevicomin either alone or in combination with (±)-*exo*-brevicomin caught significantly greater numbers of both sexes of *H. pruinosus* than either (±)-*exo*-brevicomin alone or unbaited traps (Tukey's HSD; Fig. 5). In experiment 3, the (±)-*exo*-brevicomin main effect was significant for both male ($F = 9.04$; df = 1,15; $P = 0.0089$) and female ($F = 5.57$; df = 1,15; $P = 0.0323$) beetles, but the ash main effect (males: $F = 0.04$; df = 1,15; $P = 0.8517$; females: $F = 0.32$; df = 1,15; $P = 0.5813$) and interaction with (±)-*exo*-brevicomin (males: $F = 0.27$; df = 1,15; $P = 0.6121$; females: $F = 0.74$; df = 1,15; $P = 0.4024$) were not significant. No significant pairwise differences were detected among treatments (Fig. 6); low beetle catches by all treatments may have reduced our ability to detect such differences.

Discussion

Our data implicate male-produced (+)-*endo*-brevicomin as a component of an aggregation pheromone (*sensu* Borden 1982 and D. L. Wood 1982) for *H. pruinosus* because it is released by host-infesting individuals and is highly attractive to both sexes. Only two other olfactory stimulants were produced by attacking *H. pruinosus* [i.e., (+)-*exo*-brevicomin and an unidentified compound]; however, the behavioral activity of these compounds remains unclear. It is noteworthy that the unidentified compound was detected

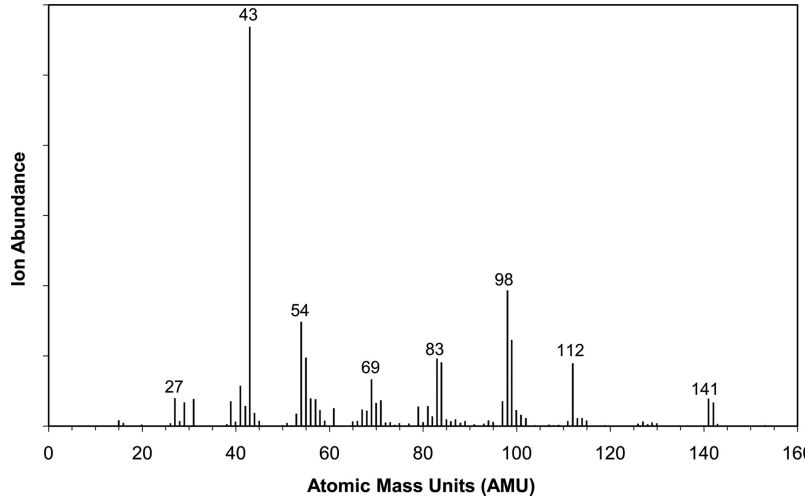

Fig. 3. Mass spectrum of an unidentified olfactory stimulant for *H. pruinosus* isolated from conspecifics.

in samples that included macerated hindgut tissue but not in aerations of whole infested host logs. Hence, it is possible that the unidentified compound is present in the tissues but is not released into the air, or, if released, may have such low volatility (as implied by its relatively long GC retention time) that it can be detected only very close to the source.

Our evidence that (+)-*exo*-brevicomin is produced disproportionately by one sex (i.e., females), is elicited by JH III exposure, and may be somewhat attractive to both sexes (trapping experiment 3; Fig. 6) supports the hypothesis that this compound is a pheromone component for *H. pruinosus.* However, *exo*-brevicomin baits were far less attractive than *endo*-brevicomin baits (trapping experiment 2; Fig. 5), and there was no evidence of a synergistic or inhibitory interaction between *exo*-brevicomin and either *endo*-brevicomin or host odors (trapping experiments 2 and 3; Figs. 5 and 6). Furthermore, the *exo*-brevicomin baits used in our studies were contaminated by 5% *endo*-brevicomin. Hence, it is possible that the response of beetles to the *exo*-brevicomin bait in trapping experiment 3 was an artifact of this contamination. Our ability to draw conclusions concerning the activity and biological role of *exo*-brevicomin may be further complicated by the fact that racemic *exo*-brevicomin baits contained 50% of the apparently unnatural (−)-enantiomer, which conceivably could have interfered with responses to the beetle-produced (+)-enantiomer. Beetle attraction to one enantiomer of a semiochemical is sometimes inhibited by the presence of its antipode (Tumlinson et al. 1977, Birch et al. 1980). However, no

Table 1. Quantities of olfactory stimulants isolated from *H. pruinosus* adults

Isolation method	Mean amount of compound detected		
	exo-brevicomin	*endo*-brevicomin	Unidentified compound
Method 1: aeration/extraction of individual beetles excised from new mines in an ash log	(ng/beetle ± SE)	(ng/beetle ± SE)	(ng/beetle ± SE)
Solitary ♀	12.2 ± 6.6 [5/5][a]	ND [0/5]	ND [0/5]
Paired ♀	42.6 ± 9.7 [6/6]	ND [0/6]	ND [0/6]
Paired ♂	1.1 ± 0.7 [3/6]	13.6 ± 5.2 [5/6]	8.5 ± 2.9 [4/6]
Method 2: aeration of entire ash logs infested with groups of beetles on successive days	(ng/h ± SE)	(ng/h ± SE)	(ng/h ± SE)
25 ♀ (all infested on day 1)	9.2 ± 1.7 [4/4]	ND [0/4]	ND [0/4]
25 ♀ + (25 ♂ added day 2)	47.1 ± 13.1 [4/4]	4.6 ± 1.3 [4/4]	ND [0/4]
25 ♀ + 25 ♂ + (25 ♀ added day 3)	67.1 ± 17.8 [4/4]	12.1 ± 3.1 [4/4]	ND [0/4]
Method 3: Hindgut extraction of beetles treated ≈24 h earlier with JH III	(ng/beetle ± SE)	(ng/beetle ± SE)	(ng/beetle ± SE)
♀	5.9 ± 2.9 [3/5]	0.7 ± 0.7 [1/5]	0.8 ± 0.8 [1/5]
♂	1.9 ± 1.0 [3/5]	14.1 ± 6.9 [3/5]	17.1 ± 8.5 [3/5]

[a] The no. of beetles or bolts from which the compound was detected divided by the total no. of trials (N). The threshold of detection was ≈0.5 ng/beetle or 1 ng/h. Samples for which a compound was not detected were included as zeros in the calculation of means and standard errors.

ND, the compound was not detected by GC-MS from any insect in the treatment; JH III, juvenile hormone III.

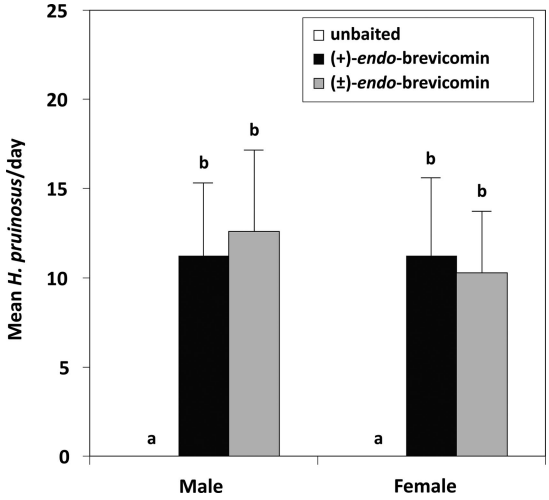

Fig. 4. Mean numbers of male and female *H. pruinosus* captured per trap per day (+SE) in traps baited with racemic *endo*-brevicomin, (+)-*endo*-brevicomin, or nothing. Within sex, bars associated with the same letter were not significantly different (Tukey's HSD on log-transformed catch; $P < 0.05$).

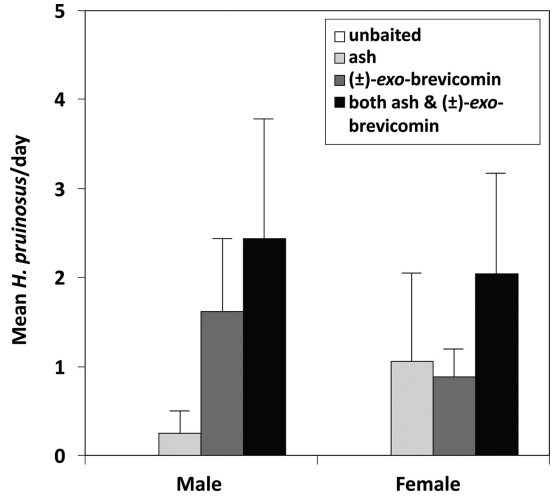

Fig. 6. Mean numbers of male and female *H. pruinosus* captured per trap per day (+SE) in traps baited with host volatiles (ash branches) alone, racemic *exo*-brevicomin alone, both host volatiles and racemic *exo*-brevicomin together, or nothing. Analysis of the data as a 2 by 2 factorial design showed the racemic *exo*-brevicomin main effect to be significant.

interference of this kind was observed in *endo*-brevicomin, because the racemic mixture was equally attractive to *H. pruinosus* as the pure (+)-enantiomer (Fig. 4).

Males appeared to be the major or sole producers of the aggregation pheromone component (+)-*endo*-brevicomin. We failed to detect this compound by flame ionization detector (FID) or GC-MS in females except for a single JH III–treated individual (Table 1).

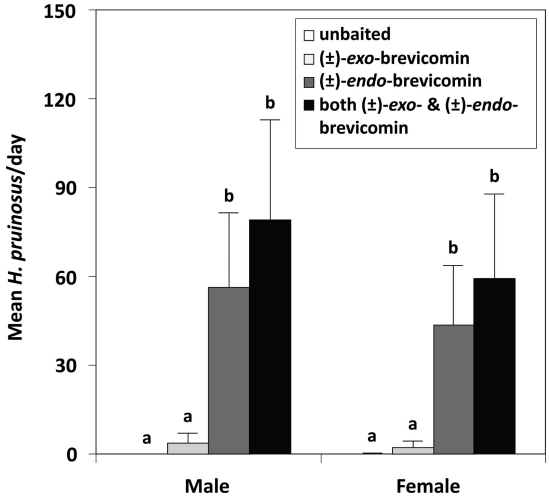

Fig. 5. Mean number of male and female *H. pruinosus* captured per trap per day (+SE) in traps baited with racemic *endo*-brevicomin alone, racemic *exo*-brevicomin alone, both racemic *endo*- and *exo*-brevicomin together, or nothing. Within sex, bars associated with the same letter were not significantly different (Tukey's HSD on log-transformed catch; $P < 0.05$).

However, *H. pruinosus* antennae generated a signal at the retention time of *endo*-brevicomin in EAD analyses of aerations of female-only–infested logs (Fig. 2), suggesting females may produce minute quantities during gallery initiation. Life history studies specifically of *H. pruinosus* have not been published; however, females are the pioneering sex and initiate gallery construction in all *Hylesinus* spp. studied to date (Schönherr 1970, Rudinsky and Vallo 1979, Solomon 1995). Furthermore, our observations of *H. pruinosus* are consistent with female gallery initiation: of 129 gallery systems dissected in our aeration logs, 61 contained a solitary female, whereas only one contained a solitary male. Thus, the secondarily arriving sex in *H. pruinosus* (i.e., males) is apparently the major producer of the aggregation pheromone. This phenomenon, also reported in German populations of *Hylesinus varius* (F.) [= *fraxini* (Panzer)] (Schönherr 1970, Kohnle 1985), is atypical of bark beetles, because the attack-initiating sex is normally the major producer of the aggregation attractant (Kirkendall 1983, Byers 1989).

Other members of the genus *Hylesinus* are known to either produce or respond to *endo*- and *exo*-brevicomin. Male *H. varius* beetles in Germany produce *exo*-brevicomin, which alternately has been found to be attractive (Kohnle 1985, Klimetzek et al. 1986) or inhibitory to both sexes (Francke et al. 1979). Traps baited with either *exo*-brevicomin or *endo*-brevicomin captured *Hylesinus criddlei* (Swaine), *H. aculeatus*, and small numbers of *H. pruinosus* in Maryland (Rabaglia 2002). Additional evidence of semiochemical production and communication in the genus *Hylesinus* includes male-produced attractive and inhibitory compounds in German populations of *H. varius*

(Schönherr 1970, Francke et al. 1979, Kohnle 1985). Ash logs infested with female *H. varius* attracted conspecifics in Slovakia (Rudinsky and Vallo 1979). Male and female *Hylesinus californicus* (Swaine) were attracted to female-infested *Fraxinus latifolia* Benth. logs in Oregon (Rudinsky and Vernoff 1979).

In addition to *Hylesinus*, numerous scolytine bark beetles use *exo*-brevicomin, *endo*-brevicomin, or both for intraspecific communication. *exo*-Brevicomin has been isolated from *Dendroctonus adjunctus* Blandford, *Dendroctonus brevicomis* LeConte, *Dendroctonus jeffreyi* Hopkins, *Dendroctonus ponderosae* Hopkins, *Dendroctonus terebrans* (Olivier), *Dryocoetes affaber* (Mannerheim), *Dryocoetes autographus* (Ratzeburg), and *Dryocoetes confusus* Swaine (Libbey et al. 1974, Rudinsky et al. 1974, Hughes et al. 1976, Kohnle and Vité 1984, Borden et al. 1987, Phillips et al. 1989, Camacho et al. 1994, Paine et al. 1999). Studies have identified *endo*-brevicomin from *D. brevicomis*, *D. frontalis*, *D. ponderosae*, *D. affaber*, *D. autographus*, and *D. confusus* (Libbey et al. 1974, Rudinsky et al. 1974, Kohnle and Vité 1984, Borden et al. 1987, Smith et al. 1993, Camacho et al. 1994). Male-produced *exo*-brevicomin is attractive to *D. adjunctus* and *D. jeffreyi* conspecifics, inhibits the attractiveness of frontalin for *D. terebrans*, and is multifunctional for *D. ponderosae* (Hughes et al. 1976, Borden et al. 1987, Phillips et al. 1989, Paine et al. 1999). *exo*-Brevicomin, released by female *D. brevicomis*, is an essential component of this species' aggregation pheromone blend (Silverstein et al. 1968). Male *D. frontalis* release (+)-*endo*-brevicomin, which strongly increases attraction of conspecifics to female-produced frontalin and host odors (Sullivan et al. 2007). In *Dryocoetes* spp. both brevicomins are produced by males and may be attractive or inhibitory to both sexes depending on concentration, chirality, and/or blend ratio (Kohnle and Vité 1984; Borden et al. 1987; Camacho et al. 1993, 1994).

Ash bark beetles are known to attack only severely weakened or dead hosts; hence, the aggregation pheromone of *H. pruinosus* ostensibly plays no role in organizing mass attacks for overcoming resistance of vigorous trees, as commonly occurs in aggressive bark beetle species (Vité and Francke 1976, D.L. Wood 1982, Byers 1989). Bigyny (one male simultaneously paired with two females in a gallery) has been reported in the genus *Hylesinus* and a few other bark beetle taxa (Kirkendall 1983). If bigynous, male *H. pruinosus* may release *endo*-brevicomin to attract a second female to their gallery after pairing with the gallery-initiating female. However, we were unable to locate or induce attacks by *H. pruinosus* in the field to verify the natural mating system, whereas dissections of the ash logs infested for our aerations showed that only 4 of 129 gallery systems were occupied by more than one female despite the 2:1 ratio of introduced females to males.

It is possible that *endo*-brevicomin from paired males is attractive to both sexes merely because it signals the presence of suitable breeding material and potential mates; males may produce it because it increases their odds of inseminating multiple females either in a single gallery or, conceivably, several different ones. Furthermore, occurrence of polygamy and the precise role of the sexes in pheromone production may be more fluid within species of *Hylesinus* than other, better-studied bark beetle genera. Løyning and Kirkendall (1999) reported that isolated populations of *H. varius* were either monogynous or bigynous, with either males or females producing attractant pheromones.

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