Olfactory Responses of the Hemlock Woolly Adelgid Predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), to Natural and Synthetic Conifer Volatiles

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Olfactory Responses of the Hemlock Woolly Adelgid Predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), to Natural and Synthetic Conifer Volatiles[1]

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Abstract   *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is a specialist predator of the hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), native to the Pacific Northwest. It has been introduced into the eastern United States for biological control of exotic hemlock woolly adelgid populations that threaten native hemlock. The possible role of olfactory cues in host finding by this predator has received little study. We used gas chromatography–electroantennographic detection (GC-EAD) to test adult *L. nigrinus* olfactory sensitivity to volatiles from foliage of both adelgid-infested and uninfested eastern hemlock [*Tsuga canadensis* (L.) Carrière] and western hemlock [*Tsuga heterophylla* (Raf.) Sarg.], and two hemlock woolly adelgid nonhost species (eastern white pine, *Pinus strobus* L., and western white pine, *Pinus monticola* Douglas ex D. Don). Adelgid infestation did not alter *L. nigrinus* EAD response profiles to volatiles from either hemlock species. In total, antennal preparations detected only two compounds in samples of foliage volatiles: myrcene in all four tree species and nonanal in eastern hemlock alone. However, in GC-EAD tests with synthetic blends of common conifer volatiles presented at higher concentrations than in our foliage samples, we additionally recorded responses to (−)-limonene, terpinolene, *alpha-p*-dimethylstyrene, linalool, (−)-bornyl acetate, 4-allylanisole, and *alpha*-humulene. The apparent absence of olfactory stimulants specific to adelgid-infested foliage is consistent with published ambulatory olfactometer tests in which *L. nigrinus* adults were not more attracted to infested than uninfested foliage. Myrcene and nonanal should be further explored as compounds produced by hemlock woolly adelgid host trees that may influence *L. nigrinus* prey-finding efficiency.

Key Words   *Adelges tsugae*, Adelgidae, biological control, predation, GC-EAD

The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), was inadvertently introduced from Japan into the eastern United States, where it was discovered in the early 1950s (Havill et al. 2006). It has since caused extensive mortality of the native eastern and Carolina hemlocks, *Tsuga canadensis* (L.) Carrière and *T. caroliniana* Engelmann, respectively, apparently due to absence of

natural enemies and lack of evolved defenses to this pest (McClure 1987, Montgomery and Lyon 1996, Wallace and Hain 2000). Hemlocks are important components of forest ecosystems, which enhance water quality and provide habitat for numerous wildlife species (Evans et al. 1996, Vose et al. 2013). The hemlock woolly adelgid uses its stylet mouthparts to feed on nutrients stored in the host's xylem ray parenchyma cells (Oten et al. 2012, Young et al. 1995), and heavy infestations cause damage and tree death in as little as 4 yr (McClure 1991). In the eastern United States, the life cycle of the hemlock woolly adelgid is characterized by two sessile parthenogenic generations, sistens and progrediens, on hemlocks and a winged sexupara generation that does not successfully reproduce (Havill et al. 2014, McClure 1989). The sistens generation aestivates over the summer as first-instar nymphs, followed by development during the fall and winter, while the shorter-lived progrediens generation matures in the spring and early summer.

Biological control is one of the primary strategies being utilized to attempt to mitigate the devastating impacts of the hemlock woolly adelgid (Onken and Reardon 2011). One agent being used in classical biological control efforts is *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a small (<3 mm) beetle often found in close association with the hemlock woolly adelgid on western hemlock, *Tsuga heterophylla* (Raf.) Sarg., in the Pacific Northwest (Kohler et al. 2008, Leschen 2011, Mausel et al. 2011a). This specialist predator of all adelgid life stages can complete its development only by feeding on the hemlock woolly adelgid (Zilahi-Balogh et al. 2002). More than 200,000 beetles have been released in the eastern United States, and the predator has since become successfully established in several locations (Havill et al. 2014, Mausel et al. 2010). The life cycle of the univoltine *L. nigrinus* is synchronous with the hemlock woolly adelgid, with newly eclosed adults undergoing aestival diapause over the summer (simultaneously with the aestivation of first-instar adelgid sistens) and emerging in the fall to feed on sistens immatures (Zilahi-Balogh et al. 2003a, 2003b). Throughout the fall and winter, *L. nigrinus* continues to feed on the sessile adelgids at the bases of needles, laying eggs in the woolly sistens ovisacs. The larvae of *L. nigrinus* feed on sistens adults and progrediens eggs during the spring, followed by pupation and summer diapause in the soil (Havill et al. 2014, Zilahi-Balogh et al. 2002).

The process of prey location by *L. nigrinus* is largely unknown, and only a few behavioral studies have so far been conducted (Broeckling and Salom 2002, Flowers et al. 2007, Mausel et al. 2011b, Wallin et al. 2011). Short-range responses by *L. nigrinus* to odors from both host and nonhost trees for the hemlock woolly adelgid have been demonstrated, and long-range detection of olfactory stimuli has been proposed (Broeckling 2002, Wallin et al. 2011). However, the chemical composition of semiochemicals involved in host finding has not been characterized. Attraction to compounds associated specifically with the hemlock woolly adelgid, infested tree tissue, or the adelgid's preferred host species presumably could aid *L. nigrinus* and other natural enemies in locating prey or their prey's habitat. Furthermore, changes in the degree of association of these compounds with the prey (as might occur if the prey invaded a new habitat or developed a new host plant association) or presence of nonhost-associated sources of these cues in the introduced habitat could influence the efficacy of *L. nigrinus* as a biological control agent (Vet and Dicke 1992).

Coupled gas chromatography–electroantennographic detection (GC-EAD) is a method for distinguishing and isolating olfactory stimulants present in complex odor blends in natural or artificial substrates (Arn et al. 1975, Bjostad 1998). The isolated olfactory stimulants then can be identified by coupled gas chromatography–mass spectrometry and other analytical techniques. Such identified olfactory stimulants may be purchased or synthesized and investigated in bioassays for possible behavioral activity relevant to the insect's biology or management. The objectives of this study were to identify olfactory stimulants of *L. nigrinus* that distinguish the volatile profiles of (a) hemlock woolly adelgid–infested hemlock foliage from uninfested foliage, and (b) foliage of hemlock woolly adelgid host tree species from nonhost tree species.

Materials and Methods

Collection of insects and volatile samples. Adult *L. nigrinus* were collected with beat sheets from western hemlock infested with hemlock woolly adelgid on 4–9 November 2010 in the greater Seattle, WA, area. Beetles were shipped overnight to Asheville, NC, and maintained on adelgid sistens–infested branch tips of eastern hemlock, which were collected near Asheville, NC. The cut ends of the branches were placed in 30-ml plastic cups containing hydrated floral foam (Oasis Instant Deluxe; Smithers-Oasis Company, Kent, OH) and maintained in an incubator (Model I-36LL; Percival Scientific Inc., Perry, IA) at 6°C/4°C (day/night) and a photoperiod of 12 h:12 h (L:D).

Organic volatiles from living branches of adelgid hosts (two species: eastern and western hemlock) and nonhosts (two species: eastern white pine, *Pinus strobus* L., and western white pine, *Pinus monticola* Douglas ex D. Don) were sampled *in situ* using methods similar to those described in Shepherd et al. (2010). Samples were collected from the following trees: five infested and five uninfested eastern hemlock, 27–28 October 2010 in Kentucky Ridge State Forest near Pineville, KY (11–20°C); six infested and six uninfested western hemlock, 4–9 November 2010 in the greater Seattle, WA, area (8–15°C); four eastern white pine, 26 October 2010 in Bent Creek Experimental Forest, Asheville, NC (22°C); and four western white pine, 4–9 November 2010 in the greater Seattle, WA, area (8–15°C). A single branch was aerated from each sampled tree. Adelgid density on host trees was determined by counting the number of woolly masses on the outer 30 cm of the aerated branch. Mean $\pm$ SE numbers of woolly masses per infested branch were 211 ± 55 on eastern hemlock and 55 ± 12 on western hemlock. Uninfested branches of host trees had no woolly masses. The two *Pinus* species are not hemlock woolly adelgid hosts and thus did not have any adelgids present on their branches.

The end of each sampled branch was enclosed in a polytetrafluoroethylene (PTFE) bag (60 × 28 cm) with the bag's mouth clamped tightly around a 5 × 15-cm strip of activated charcoal mesh (Universal Replacement Prefilter; Honeywell, Southborough, MA) wrapped multiple times around the branch base to form a cylinder. This cylinder removed organic volatiles from air drawn into the bag interior during aeration thereby ensuring that all sampled volatiles were from the sampled branch. A solvent-cleaned and activated PTFE cartridge containing 0.1 g of Porapak Q® adsorbent (50/80 mesh; Alltech, Deerfield, IL) was connected to the

end of a 1-m length of corrugated PTFE tubing and inserted into the bag through the charcoal mesh. The open end of the cartridge was positioned at the opposite end of the bag from the mouth to maximize flow of sampled air across the enclosed foliage. The exterior opening of the tubing was attached to a portable vacuum pump that drew air through the cartridge at 50 ml/min for 3 h. Following the sampling, the cartridge ends were sealed with laboratory film for transport to the laboratory, where the cartridges were stored in a freezer for no longer than 7 d prior to extraction with 1.2 ml of pentane. Cartridge extracts from trees of each sampled species/infestation status were pooled (six total extracts) and concentrated 10-fold via evaporation in a heated sand bath and stored in a $-80°C$ freezer until analysis. Heptyl acetate (1.77 µl; Aldrich Chemical Co., Milwaukee, WI) was added to each sample as an internal standard.

GC-EAD analyses. Coupled GC-EAD was used to record electrophysiological responses of *L. nigrinus* antennae to the six pooled foliage samples (representing each tree species–infestation combination) and a blend of synthetic conifer oleoresin volatiles (10 µg/µl in hexane). Apparatus and methods were similar to those in Asaro et al. (2004). Synthetic volatiles included were (−)-*alpha*-pinene (Acros Organics, Geel, Belgium); 1,4-cineol, *alpha*-humulene (Fluka Chemie AG, Buchs, Switzerland); tricyclene, (−)-*beta*-pinene, (+)-sabinene, 3-carene, myrcene, *alpha*-phellandrene, *alpha*-terpinene, (−)-limonene, eucalyptol, *gamma*-terpinene, *p*-cymene, terpinolene, *alpha-p*-dimethylstyrene, (−)-bornyl acetate, *beta*-caryophyllene, and 4-allylanisole (Aldrich Chemical Co., Milwaukee, WI). Between five and seven beetles were tested with each of the six pooled samples. For each assay, a glass micropipette reference electrode (Ag/AgCl; filled with Beadle–Ephrussi saline and 0.5% polyvinylpyrrolidone) was inserted into the foramen of the excised head of the beetle. The tip of an identically prepared recording electrode was cut so the opening matched the diameter of the antennal club; it was then placed against the tip of the antennal club so the saline contacted the club surface. The antennal preparation was secured within a continuous airstream (400 ml/min; humidified and purified with activated charcoal) into which the GC effluent was released. Beetles were sexed prior to the assay as described by Shepherd et al. (2014).

An Agilent 5890 GC (with flame ionization detector [FID]) with an HP INNOWax column (60 m × 0.25 mm × 0.25-µm film; Agilent Technologies, Wilmington, DE) and the temperature program 40°C held for 1 min, increased 16°C/min to 80°C, then increased 7°C/min to 230°C (held for 10 min) were used for all analyses. For each assay, 1 µl of sample was injected into the GC (splitless for the aeration samples; split 1/20 for the synthetic mixture) with an injector temperature of 200°C and helium as the carrier gas. Half of the effluent from the GC was diverted to the airstream over the antenna and half to the FID. The identities of compounds detected by the EAD or FID were investigated by analysis of samples on a coupled gas chromatograph–mass spectrometer (GC-MS; Agilent model 6890-5973) using the same column and instrument settings as the GC-EAD. Olfactory stimulants were identified by matching retention times (both in the FID and mass selective detector runs) and mass spectra to those of identified standards. A "peak" (i.e., a deflection exceeding the average background noise level) on the EAD trace was considered a genuine olfactory response if it occurred at the same retention time repeatedly (i.e., four out of five, four out of six, or five out of seven antennal preparations). Nonanal

Table 1. Identified volatile compounds from aerations of eastern hemlock (infested and uninfested), western hemlock (infested and uninfested), eastern white pine, and western white pine foliage.

Label	Compound	Source Tree*
1	Tricyclene	EH
2	*alpha*-Pinene	EH, WH, EWP, WWP
3	Camphene	EH, EWP, WWP
4	*beta*-Pinene	EH, WH, EWP, WWP
5	Myrcene	EH, WH, EWP, WWP
6	*alpha*-Phellandrene	EH, WH, EWP, WWP
7	Limonene	EH, WH, EWP, WWP
8	*beta*-Phellandrene	EH, WH, EWP, WWP
9	*gamma*-Terpinene	EH, EWP, WWP
10	*p*-Cymene	EH
11	Terpinolene	EH, EWP, WWP
12	(Heptyl acetate)	Internal standard
13	Nonanal	EH
14	Bornyl acetate	EH
15	Sabinene	WWP
16	3-Carene	WWP
17	*alpha*-Terpinene	EWP, WWP

* EH, eastern hemlock; WH, western hemlock; EWP, eastern white pine; WWP, western white pine.

was present in very low concentrations in the eastern hemlock samples, so we confirmed its olfactory activity by testing it (GC-EAD analysis of a 1:1 nonanal:myrcene blend) on three *L. nigrinus* antennae at concentrations of 0.1 μl/ml and 10 μl/ml in hexane.

Results

Sixteen compounds were identified in the aeration samples from the two hemlock (hemlock woolly adelgid host) and two pine (nonhost) species (Table 1). No qualitative differences in foliage volatile profiles or EAD responses were observed between adelgid-infested and uninfested eastern hemlock, or between adelgid-infested and uninfested western hemlock (Figs. 1, 2). The only volatiles in samples of tested hemlock woolly adelgid host species that elicited antennal responses were myrcene and nonanal in eastern hemlock and myrcene in western hemlock (Figs. 1, 2). In GC-EAD tests with a blend of myrcene and nonanal injected
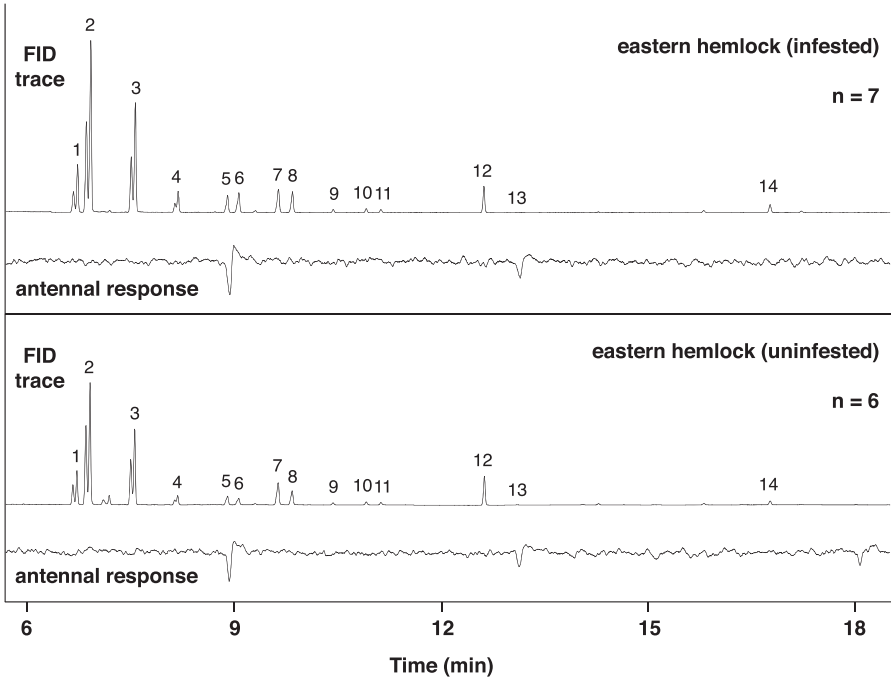
Fig. 1. Electrophysiological responses of *Laricobius nigrinus* antennae to volatiles collected from aerations of eastern hemlock foliage either infested with hemlock woolly adelgids or uninfested. Individual volatiles were simultaneously introduced to the antennal preparation and recorded using a flame ionization detector (FID). The "antennal response" represents the summed electroantennographic detection (EAD) traces for each treatment. We observed responses to myrcene (5) and nonanal (13). All identified volatiles are designated by number (see Table 1).

at 0.1 **µ**l/ml and 10 **µ**l/ml concentrations (Fig. 3), antennal responses were produced by nonanal at both concentrations but to myrcene only at the higher concentration. Myrcene was the only compound that elicited antennal responses in foliage samples of the two species of pine (Fig. 4). The EAD responses to both myrcene and nonanal stood conspicuously above the baseline noise level, suggesting substantially stronger olfactory responses to these two compounds than other components in the tree odor blends. No conspicuous differences were observed between the electrophysiological responses of male and female beetles to any of the tested samples.

Beetle antennae responded to seven compounds included in the synthetic conifer oleoresin volatile blend and one contaminant compound (linalool) that was present in relatively small concentration (Fig. 5). Myrcene, (−)-limonene, terpinolene, *alpha-p*-dimethylstyrene, linalool, (−)-bornyl acetate, 4-allylanisole, and *alpha*-humulene were all antennally active. We did not record responses to
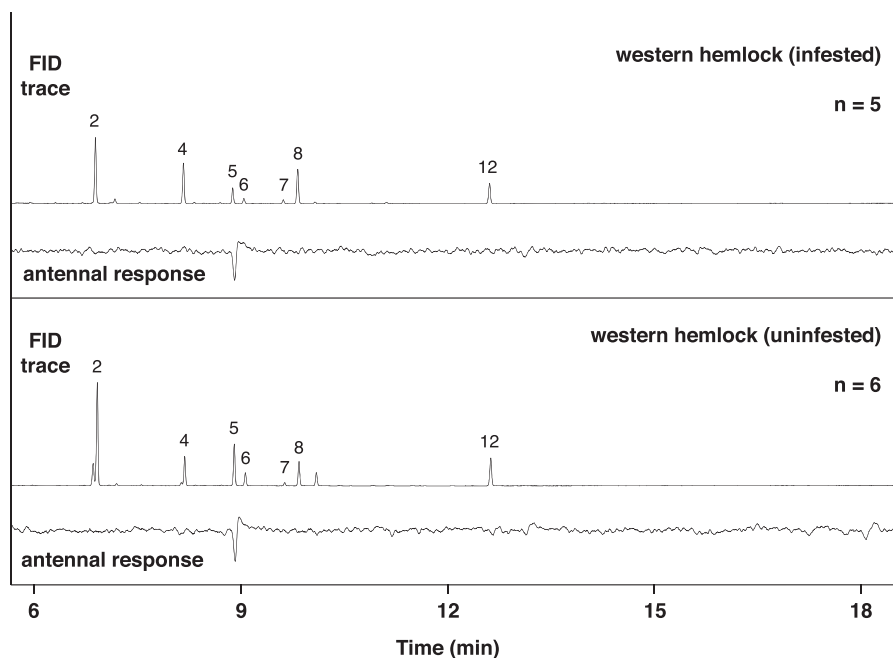
Fig. 2. Electrophysiological responses of *Laricobius nigrinus* antennae to volatiles collected from aerations of western hemlock foliage either infested with hemlock woolly adelgids or uninfested. Individual volatiles were simultaneously introduced to the antennal preparation and recorded using a flame ionization detector (FID). The "antennal response" represents the summed electroantennographic detection (EAD) traces for each treatment. We observed responses only to myrcene (5). All identified volatiles are designated by number (see Table 1).

tricyclene, (−)-*alpha*-pinene, (−)-camphene, (−)-*beta*-pinene, (+)-sabinene, 3-carene, *alpha*-phellandrene, *alpha*-terpinene, 1,4-cineol, eucalyptol, *gamma*-terpinene, *p*-cymene, and *beta*-caryophyllene at the tested concentration.

Discussion

Natural enemies can locate hosts by responding to odors arising from the host itself, odors induced in the host's food plant by host feeding, or the constitutive odors of the food plant (Cortesero et al. 2000, Dickens 1999, Mumm et al. 2008). Odor cues more closely correlated with the location of the host should allow more efficient host location. These relatively "reliable" cues (including odors arising from the host itself or generated by the host's food plant specifically in response to host feeding) provide more exact targeting of the location of the host than less precisely
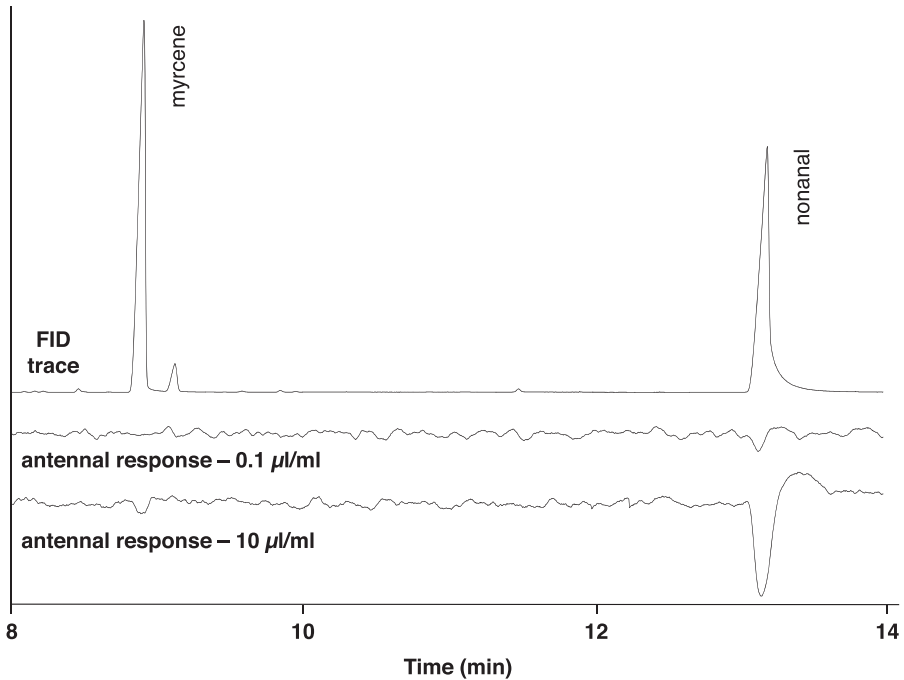
Fig. 3. Example of electrophysiological responses of a single *Laricobius nigrinus* antenna to two concentrations of myrcene and nonanal (0.1 μl/ml and 10 μl/ml in hexane). Individual volatiles were simultaneously introduced to the antennal preparation and recorded using a flame ionization detector (FID).

host-associated but more "detectable" cues (such as the constitutive odors of the host's food plant) (Vet and Dicke 1992, Vet et al. 1991).

Our GC-EAD analyses failed to detect olfactory stimulants for *L. nigrinus* that distinguished adelgid-infested from uninfested hemlock of either species. Furthermore, adelgid-infested and uninfested eastern hemlock volatile profiles in our analyses resembled those reported in Broeckling and Salom (2003), who likewise found no qualitative differences in volatiles produced by adelgid-infested and uninfested eastern hemlock. Our results indicate that *L. nigrinus* may not sense any volatile compounds associated specifically with hemlock woolly adelgid prey or adelgid-infested foliage that might serve as host-finding cues. This result is consistent with olfactometer studies that found no significant difference in attraction by walking *L. nigrinus* to odors of adelgid-infested and uninfested hemlock foliage, and no attraction to isolated, live hemlock woolly adelgids (Wallin et al. 2011). Attraction by *L. nigrinus* to foliage of hemlock woolly adelgid host tree species regardless of the presence of prey implies that constitutive (rather than induced) host plant volatiles are the only compounds that may play a role in *L. nigrinus* host location. Although not observing qualitative differences in the odor profiles of hemlock woolly adelgid–infested and uninfested foliage, Broeckling and Salom
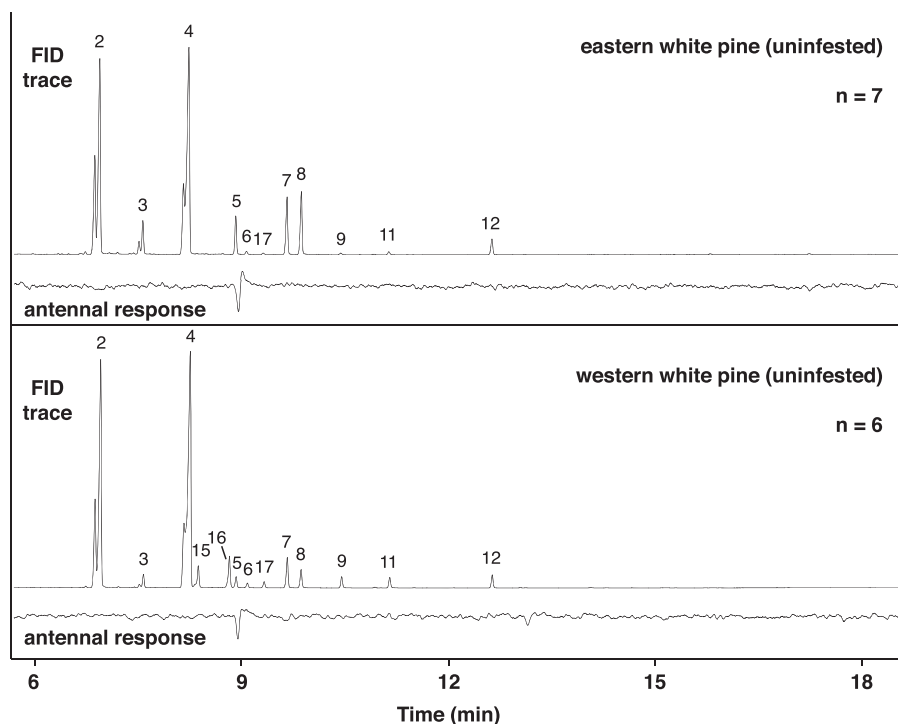
Fig. 4. **Electrophysiological responses of *Laricobius nigrinus* antennae to volatiles collected from aerations of uninfested eastern and western white pine foliage. Individual volatiles were simultaneously introduced to the antennal preparation and recorded using a flame ionization detector (FID). The ''antennal response'' represents the summed electroantennographic detection (EAD) traces for each treatment. We observed responses only to myrcene (5) in both samples. All identified volatiles are designated by number (see Table 1).**

(2003) found that adelgid-infested foliage released an approximately fourfold higher rate of the odors associated with uninfested foliage and observed a small change in the relative proportion of *alpha*-pinene present. It is possible that an increase in the release rate of constitutive odors by the food plant of the hemlock woolly adelgid as a result of infestation may help *L. nigrinus* locate infested trees or localized concentrations of adelgids. However, such a concentration increase could also be stimulated by physical damage to the host possibly unrelated to herbivory and thus would provide a very nonspecific and relatively unreliable cue (Vet et al. 1991).

Additionally, we failed to detect olfactory stimulants for *L. nigrinus* that specifically distinguished hemlock woolly adelgid host tree species from the investigated nonhosts. *Laricobius nigrinus* antennal preparations detected only one olfactory stimulant in volatiles from the hemlock woolly adelgid host western hemlock and the nonhosts eastern and western white pine: the common conifer
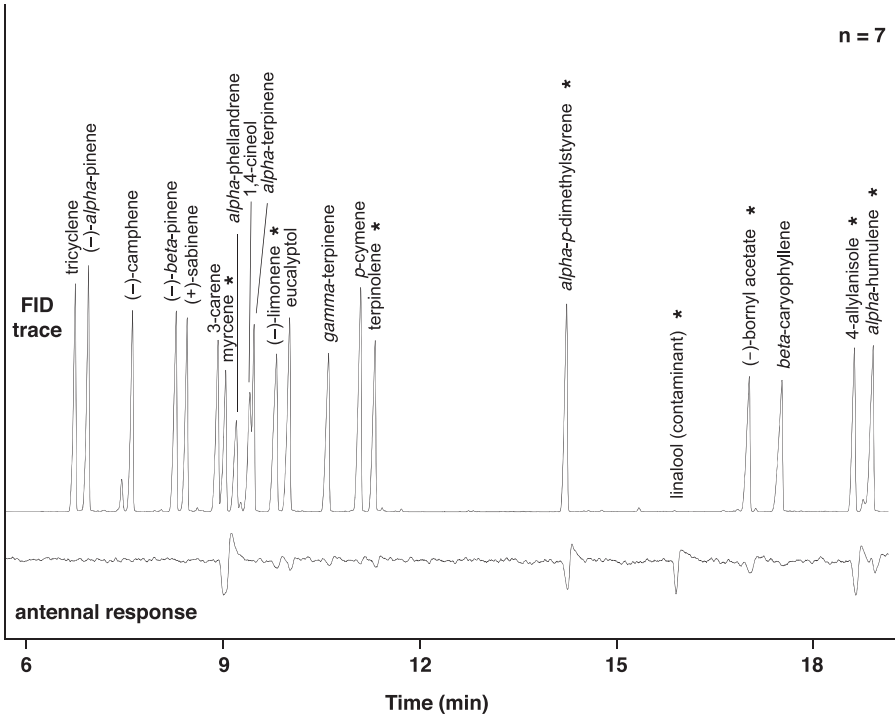
Fig. 5. Electrophysiological responses of *Laricobius nigrinus* antennae to synthetic volatiles commonly found in conifer resins. Individual volatiles were simultaneously introduced to the antennal preparation and recorded using a flame ionization detector (FID). The "antennal response" represents the summed electroantennographic detection (EAD) traces for each treatment. Compounds that elicited antennal responses are marked with an asterisk (*).

monoterpene, myrcene. Behavioral studies likewise imply that *L. nigrinus* does not consistently differentiate odors of hemlock woolly adelgid host and nonhost tree species. Although odors of foliage of hemlock woolly adelgid nonhosts Douglas-fir [*Pseudotsuga menziesii* var. *menziesii* (Mirbel) Franco] and ponderosa pine (*Pinus ponderosa* Douglas ex Lawson) were repellant to *L. nigrinus* in olfactometer assays, odors of nonhosts western white pine and white spruce [*Picea glauca* (Moench) Voss] equaled or exceeded the attraction to hosts eastern and western hemlock (Wallin et al. 2011). In the eastern range of the hemlock woolly adelgid, failure to discriminate host trees and nonhosts could increase the chances for *L. nigrinus* to encounter and hybridize with *Laricobius rubidus* LeConte, a congener that primarily feeds on pine adelgids on eastern white pine (Havill et al. 2012).

Although *L. nigrinus* antennae registered responses to only two compounds in aerations of host and nonhost foliage (i.e., myrcene and nonanal), GC-EAD trials with a synthetic mixture of common conifer volatiles indicated the existence of

additional olfactory stimulants (Fig. 5). Three of these compounds (limonene, terpinolene, and bornyl acetate) were detected by GC-MS in aeration samples of one or more of the sampled tree species, but they did not stimulate antennal responses presumably because their concentrations in the foliage samples were too low. (It should be noted that the concentrations of volatiles collected in the samples were likely low due to relatively low ambient air temperatures, and this could have influenced the relative concentrations among sample types.) Thus (not surprisingly), the antennae of *L. nigrinus* are apparently sensitive to compounds produced by our sampled foliage in addition to myrcene and nonanal, and this implies that more compounds than these two could potentially be involved in host location. However, when exposed to odors in the natural proportions with which they arise from host foliage, *L. nigrinus* is able to detect these two compounds more readily (and presumably at greater distances from the source) than other olfactory stimulants potentially present in the sampled foliage. Our data suggest a particularly high degree of sensitivity to nonanal, as it generated a response at relatively low concentrations (and lower concentrations than generated a response for myrcene). Thus, both nonanal and myrcene should be investigated for a possible role in host-seeking behavior.

The *L. nigrinus* introduced into the eastern United States for biological control on eastern and Carolina hemlock were originally derived from populations in northwestern North America that were consuming hemlock woolly adelgids on other host tree species. This switch in host tree species by the hemlock woolly adelgid could presumably alter host-finding efficiency by *L. nigrinus* released in the eastern United States because it would change the odor cues associated with the adelgid's host from those with which their host-finding behaviors would have evolved. *Laricobius nigrinus* were more attracted to foliage of western than eastern hemlock in olfactometer bioassays (Wallin et al. 2011), and therefore it is perhaps significant that *L. nigrinus* antennae responded to a compound (nonanal) in eastern hemlock foliage that was evidently absent in western hemlock. The implication is that nonanal may play a role in this discrimination.

Acknowledgments

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