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Behavioral Responses of *Laricobius* spp. and Hybrids (Coleoptera: Derodontidae) to Hemlock Woolly Adelgid and Adelgid Host Tree Odors in an Olfactometer

ARIELLE L. ARSENAULT,[1] NATHAN P. HAVILL,[2] ALBERT E. MAYFIELD,[3] and KIMBERLY F. WALLIN[1,2,4]

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ABSTRACT The predatory species *Laricobius nigrinus* (Fender) and *Laricobius osakensis* (Shiyake and Montgomery) (Coleoptera: Derodontidae) have been released for biological control of hemlock woolly adelgid (*Adelges tsugae*; Hemiptera: Adelgidae) in eastern North America. *L. osakensis* is native to Japan, whereas *L. nigrinus* is endemic to the Pacific Northwest of the United States and Canada. After release, *L. nigrinus* was found to hybridize with the native eastern species, *Laricobius rubidus* (LeConte). The purpose of this study is to observe prey location behaviors of these three *Laricobius* species and *L. nigrinus* × *L. rubidus* (Ln × Lr) hybrids. Olfactometer bioassays were used to test response to host odors of adelgid-infested eastern hemlock, uninfested eastern hemlock, and uninfested eastern white pine. Predators reacted in the olfactometer more quickly when adelgid-infested foliage was included as a choice. *L. nigrinus* preferred infested eastern hemlock over uninfested eastern white pine, and *L. rubidus* preferred uninfested eastern white pine over uninfested eastern hemlock. *Laricobius* hybrids did not show a preference for foliage types known to be primary adelgid hosts (eastern hemlock and eastern white pine). Unequal preference by species of *Laricobius* for host trees of different adelgid prey could therefore be maintaining *Laricobius* species barriers despite hybridization. *L. osakensis* for this study were reared in the laboratory, whereas other species in this study were collected from the field, yet still were attracted to infested and uninfested eastern hemlock. This species also responded most quickly in the olfactometer, which is encouraging for successful biological control with this species.

KEY WORDS biological control, host location behavior, *Laricobius* spp., hybridization, *Tsuga canadensis*

Eastern hemlock (Tsuga canadensis (L.) Carrière) and Carolina hemlock (Tsuga caroliniana Engelmann) in the eastern United States are suffering high rates of mortality (Ellison et al. 2005) due to hemlock woolly adelgid (Adelges tsugae Annand (Hemiptera: Adelgidae)), an invasive insect introduced from Japan (Havill et al. 2006). Several predators have been released, and others are being considered for release, as part of a classical biological control program to exert top-down control on hemlock woolly adelgid populations (Flowers et al. 2007, Kohler et al. 2008, Onken and Reardon 2011, Jones et al. 2014). There are separate hemlock woolly adelgid lineages native to Japan, China, Taiwan, and western North America (Havill et al. 2006), so these regions have all been explored for natural enemies. Biological control efforts began in 1992 and initially focused on several lady beetle (Coleoptera: Coccinellidae) predators from Japan and China which were discovered during early surveys for natural enemies in Asia and performed well in initial evaluations (Cheah et al. 2004, Onken and Reardon 2011). However, the current focus of the hemlock woolly adelgid biological control program has shifted to other predatory species, largely because the lady beetle species proved difficult to rear, did not become established, or exhibited low recovery in postrelease monitoring studies (Onken and Reardon 2011, Havill et al. 2014, Jones et al. 2014). Up to 50 species of generalist and specialist predators have been found associated with hemlock woolly adelgid in its native ranges in surveys (Kohler et al. 2008).

Laricobius species (Coleoptera: Derodontidae) are adelgid specialists, unlike other members of the family Derodontidae, which are fungal feeders (Lechen 2011). Their phenologies and life histories are highly synchronized with their prey (Salom et al. 2005, Zilahi-Balogh et al. 2006). Explorations for biological control agents in the native ranges of hemlock woolly adelgid led to the location and assessment of several *Laricobius* species in Asia and western North America. They were quickly recognized as candidates for importation as biological control agents due to their long coevolutionary history with adelgids and their ability to achieve high

[1] The Rubenstein School of Environment and Natural Resources, The University of Vermont, 81 Carrigan Dr., Burlington, VT 05405.
[2] U.S. Department of Agriculture, Forest Service, Northern Research Station, 51 Mill Pond Rd., Hamden, CT 06513.
[3] U.S. Department of Agriculture, Forest Service, Southern Research Station, 200 W.T. Weaver Blvd., Asheville, NC 28804.
[4] Corresponding author, e-mail: kwallin@uvm.edu.

population densities on adelgid-infested conifers (Franz 1958; Zilahi-Balogh et al. 2003; Salom et al. 2005; Kohler et al. 2008; Montgomery et al. 2011a, b).

Laricobius nigrinus (Fender) is native to the northwestern United States and Canada, and can be found associated with hemlock woolly adelgid on western hemlock (*Tsuga heterophylla*) (Zilahi-Balogh et al. 2003, Kohler et al. 2008, Mausel et al. 2010, Grubin et al. 2011). As shown by studies in its native range, in the laboratory, and following release in the eastern United States, *L. nigrinus* completes its development on hemlock woolly adelgid and is highly host-specific (Mausel et al. 2012). Since 2003, over 100,000 individuals have been released and the species has successfully established at numerous sites across the invasive range of hemlock woolly adelgid, which spans 18 states from Georgia to Maine, United States (Mausel et al. 2012).

Laricobius rubidus (LeConte) is endemic to the eastern United States (Lechen 2011). The primary prey for this species is pine bark adelgid (*Pineus strobi* (Hartig)) on eastern white pine (*Pinus strobus* L.), but it has also been found on eastern hemlock feeding on hemlock woolly adelgid (Zilahi-Balogh et al. 2006). *L. rubidus* and *L. nigrinus* are recently diverged sister species (Montgomery et al. 2011b, Havill et al. 2012). In postrelease monitoring studies, mating between these species was observed in the field, and individuals with traits intermediate between them have been collected (Mausel et al. 2008). Mating between *L. nigrinus* and *L. rubidus* was subsequently found to result in hybridization, which was an unexpected consequence of releasing *L. nigrinus* in the eastern United States (Davis et al. 2011, Havill et al. 2012). Behavioral differences between these *Laricobius* hybrids and their parental species have yet to be thoroughly investigated.

Introductions of nonnative species can have impacts on their native relatives through hybridization and introgression (i.e. gene flow; Mallet 1998, Mooney and Cleland 2001). When related species are brought into contact, hybridization can proceed quickly, but this initial gene flow often creates an unstable hybrid zone, where some of the parental populations overlap and interspecies crossing occurs. Hybrid zones are resolved in one of three ways: evolution of mating barriers, fusion of the two taxa into a single species, or the extirpation of one of the parental species (Remington 1968; Harrison 1983, 1986; Wainger and Mazzotta 2011). Hybridization of biological control agents can have unpredictable effects, such as a loss of host specificity (Stouthamer et al. 2000, Hora et al. 2005) or increased fecundity of the biological control agent (Szűcs et al. 2012). There is evidence that hybridization can sometimes reduce fitness from generation to generation, causing a phenomenon known as "hybrid breakdown" (Burton 1990, Dopman et al. 2009). Hybridization could reduce the behavioral predictability of organisms, which would subsequently impair the efficacy of the biological control program, so it is important to understand the behavior of the hybrid individuals, in addition to that of the parental species.

Insect behavior is frequently driven by volatile cues, which can originate from various trophic levels in the environment (Lucas 2001, de Bruyne and Baker 2008, Wallin 2012). Insects can use volatile cues to identify and locate suitable food items and habitats, avoid predation, and find mates (Wallin et al. 2011, Keesey et al. 2012). Insects can use volatile cues from plants, conspecifics, and other insect species to attract natural enemies (Dicke and Sabelis 1988, Turlings et al. 1990). Cues from host foliage are accessible from great distances and are readily detectable in the environment, whereas the small size of prey often means that they emit less detectable, but more reliable cues; prey-specific volatile cues are an indication of their availability at the site (Vet et al. 1991). The differences in detectability versus reliability can be problematic for insects because they may be drawn to easily detectable plant volatiles when prey is not present, or cannot detect the more reliable cues from the prey itself. One way that predators might overcome this "reliability-detectability problem" is to use herbivore-induced volatile cues that can be sensed over a long range while also bearing specific indication of herbivore feeding (Vet et al. 1991, Havill and Raffa 2000, Keesey et al. 2012).

L. nigrinus underwent considerable prerelease testing for host specificity and host location (Zilahi-Balogh et al. 2003, Flowers et al. 2007, Mausel et al. 2010, Wallin et al. 2011), but the behavior of Ln × Lr hybrids had not been tested. Differences in location behavior between *L. nigrinus* and *L. rubidus* and their hybrids could be a factor in determining hybrid fitness, and could also affect the efficacy of the biological control program. Several studies conducted at *L. nigrinus* release sites in the eastern United States show consistent hybridization on hemlock woolly adelgid-infested eastern hemlock (Havill et al. 2012, Jones et al. 2014, Mayfield et al. 2014, Fischer et al. 2015). In addition, *L. nigrinus* and *L. rubidus* seem to be maintaining their parental lineages, with *L. nigrinus* dominant on eastern hemlock and *L. rubidus* dominant on eastern white pine, perhaps due to their different host preferences (Fischer et al. 2015). Differences in location behavior between the species could explain this pattern. Evaluation of the host location behavior of these and other biological control agents can enhance our confidence in a predator's ability to locate target prey. This, in turn, will enable resource managers to focus efforts and resources more effectively, discontinue research on potential agents that do not meet the criteria for host location, and thus improve the success of the control program (Wallin 2012).

Laricobius osakensis (Shiyake and Montgomery) is a recently described species that is endemic to Japan where it preys on hemlock woolly adelgid that infests southern Japanese hemlock (*Tsuga seiboldii* (Carriere)) and northern Japanese hemlock (*Tsuga diversifolia* (Maximowicz) Masters) (Lechen 2011, Montgomery et al. 2011a). It was discovered in 2005, reared and evaluated in quarantine for several generations (Lamb et al. 2012), and was first released in 2014 (Fischer et al. 2015). *L. osakensis* completes its life cycle on hemlock woolly adelgid, reduces hemlock woolly adelgid densities on hemlocks in their native range, withstands low temperatures, and does not develop on

non-adelgid hosts (Vieira et al. 2011, Lamb et al. 2012). However, little is known about its host location behavior.

Multiple-choice olfactometer bioassays can test preferences among a variety of stimuli. The first objective of this study was to evaluate and compare the responses of field-collected *L. nigrinus*, *L. rubidus*, and *L. nigrinus* × *L. rubidus* to host odors, including eastern hemlock (the main eastern host of *L. nigrinus* prey), eastern hemlock infested with hemlock woolly adelgid, and eastern white pine (the main eastern host of *L. rubidus* prey) to determine to what extent the behavior of hybrid individuals differs from the parental species. The second objective was to evaluate the responses of laboratory-reared *L. osakensis* to odors produced by host plants of adelgids, including eastern hemlock, eastern white pine, and eastern hemlock infested with hemlock woolly adelgid.

Materials and Methods

Insect and Foliage Collection. Bioassays were conducted to test the ambulatory responses of adult beetles to prey and foliage. Bioassays were conducted from 2011–2013. We tested adult beetles collected near Asheville and Banner Elk, North Carolina, in spring and fall 2011, and fall 2012. Beetles were shipped on ice and tested within 72 h of arrival. Based on a previous study that used molecular methods to identify beetles from this region (Havill et al. 2012), we expected these to be a mix of *L. nigrinus*, *L. rubidus*, and hybrids. The beetles were stored in 95% ethanol after bioassays and stored at −20°C for identification, using genetic analysis described below.

In 2012 we also tested adult *L. osakensis* individuals that were laboratory reared to the F_2 generation at the Beneficial Insects Rearing Facility at Virginia Polytechnic Institute. Two shipments of 50 individuals each, packed on ice without a food source, were shipped overnight to the Forest Service laboratory in South Burlington, VT, in December 2012. Each beetle was tested once. Lab-reared *L. osakensis* individuals were lent for the purpose of these bioassays and were shipped as they became available. At the completion of the bioassays, they were returned to the Rearing Facility within 12 h so they could be reintroduced into the colony.

Hemlock woolly adelgid-infested hemlock foliage was shipped from the same location from which the beetles were collected for each bioassay. Foliage from uninfested eastern hemlock and eastern white pine was collected in South Burlington, VT, and treated the same as shipped foliage. Foliage pieces were ~6 cm long and were from the terminal end of a branch. Infested foliage contained hemlock woolly adelgid at an approximate density of two ovisacs per cm. This foliage was clipped and the cut end wrapped in damp paper towels and parafilm. It was then sealed tightly in plastic bags, and stored no longer than 48 h at 2–3°C until used in the assay. Carolina hemlock is less prevalent in the environment and was not available for this study.

Olfactometer Methods. The response of individual beetles to foliage was measured as described in Arsenault et al. (2015) and Wallin et al. (2011) in a 30 by 30 by 3 cm^3 four-chambered olfactometer arena (Analytical Research Systems, #OLFM-4-C-2440PE, Gainesville, FL). The arena consisted of a base with air output, a walking chamber with four air inputs, and a 9-mm circular central opening to introduce insects and attach a vacuum source. Odor sources were placed in glass chambers attached to the arms of the arena. Four flow meters (Brooks Instrument, Hatfield, PA) controlled airflow at a rate of 0.12 Mpa into the glass chambers that contained either a test material, or a blank control; these carried volatiles into the olfactometer. For experiments that required fewer than four arms, the airflow was turned off in the arms that were not in use. Volatiles were removed from the arena through the vacuum in the center, which maintained steady air flow.

Beetles were starved 24–25 h prior to bioassays to increase their responsiveness to stimuli. Experiments were conducted generally between 08:00 and 20:00 the next day. For each experiment, an individual was placed into the center of the assay arena. Four fields of equal size (~75 cm^2 each) in front of each odor source arm, and a 9-cm central field could be seen through the walls of the olfactometer. Each source chamber contained a different prey host material and was positioned randomly prior to the bioassay for each individual. Host foliage was replaced every hour to ensure that changes in chemical composition over time did not confound the results, and glass chambers were rinsed with ethanol and deionized water each time foliage was replaced. The placement of the glass chambers was randomized on each run, but the foliage type within each chamber remained the same throughout a set of experiments. The walking area was cleaned with ethanol and deionized water between each trial.

Each beetle had the choice of leaving the central field to cross into one of the four delineated fields. The maximum time a beetle was allowed to walk in the arena without choosing a field was 10 min, after which the beetle was removed. When the beetle remained in one of the delineated fields for 60 s, the final position at the end of the behavioral assay was recorded, as well as the time required for the beetle to choose a field. When the beetle attempted to crawl into an odor source inlet arm, that treatment was considered its final choice, and the beetle was removed from the arena. If a beetle remained in the central field for 10 min without choosing a field or an arm, the behavior was recorded as "no choice." Because of these criteria, between 5–10 individuals could be assayed during each respective hour before foliage was replaced. All individuals were included in analyses, as failure to choose can also be informative in the implementation of an effective biological control program. Since the methodology for live sexing of *Laricobius* was not published until after these bioassays took place, and *Laricobius* do not orient using sex pheromones (Shepherd et al. 2011), beetles were not sexed as part of this study.

Genetic Analysis. *L. rubidus* and *L. nigrinus* cannot be distinguished from their hybrids by morphology; therefore, the beetles collected in North Carolina were

identified after completion of bioassays through molecular methods in order to sort behavioral responses by beetle species. Species was determined using the methods described in Havill et al. (2012). Briefly, abdomens of Laricobius were removed under a dissecting microscope, and the remainder of the insect was preserved for future analyses. DNA was extracted from the abdomen using the Promega IQ DNA protocol. Six microsatellite loci were scored for each individual and the genotypes were analyzed using STRUCTURE 2.3.2 (Pritchard and Wen 2007) and NEWHYBRIDS 1.1 (Anderson and Thompson 2002) software, which compared them to a catalogue of known individuals for each species (Havill et al. 2012).

Bioassays. Three-way-choice bioassays were completed in the spring of 2011 and four-way-choice bioassays were completed in fall of 2011 and fall of 2012. Three-way-choice bioassays included uninfested foliage treatments from hosts that beetles could encounter in the field as part of a biological control program—eastern hemlock, eastern white pine, and a blank control. The four-way-choice bioassays included these same treatments plus eastern hemlock infested with hemlock woolly adelgid. The number of individual beetles tested in each experiment was based on the availability of field-collected specimens.

Mean response times and choices for each species were pooled across all three-way trials and all four-way trials. These data were used to compare the time it took for each species to respond to stimuli and the relative occurrence of each choice, plus whether the addition of hemlock woolly adelgid-infested foliage had an effect on response time.

Statistical Analysis. The proportions of *L. nigrinus*, *L. rubidus*, and hybrids were compared for each collection date and source tree species using chi-square analysis to test the difference between observed and expected (1:1:1) values.

One-way ANOVAs were performed to test whether there were differences in response times based on host plant origin and among different assay replicates. Individuals that remained in the center field for 600 s were removed without making a choice.

For three- and four-way-choice tests, the beetles' final positions in the olfactometer were analyzed using separate Cochran Q tests (Zar 1999) for each species. This nonparametric test was selected since outcomes were binary—when a beetle chose one field, it, by definition, did not choose the others, in a randomized block design. Since beetles were initially placed in the center field, remaining there for the duration of the assay is a qualitatively different behavior than choosing one of the stimulus fields. For this reason, analyses were conducted with and without beetles that stayed in the center field. If a significant overall treatment effect was found for a choice assay ($P < 0.05$), then multiple pair-wise Cochran Q test comparisons were completed among stimulus fields.

For three- and four-way-choice tests, comparisons of response times were completed using two-way analysis of variance (ANOVA) with beetle species and stimulus choice as independent factors. Tukey's HSD post hoc analyses were used to compare within-factor differences as well as the interaction between factors. Beetles that remained in the center field were not included in these analyses because all individuals were removed at 600 s, therefore all values are identical. All statistical analyses were carried out using SPSS v.20 (IBM Corp.).

Results

Proportion of Wild-Caught Beetles. The percent of Ln × Lr hybrid individuals collected from eastern hemlock and eastern white pine ranged from 8.0 to 28.3% on eastern hemlock, and from 13.3 to 18.5% on eastern white pine (Table 1). On average, hybrids comprised of 16.0 and 15.9% of collections from eastern hemlock and eastern white pine, respectively. The relative proportions of *L. nigrinus*, *L. rubidus*, and Ln × Lr *rubidus* were not significantly different across years ($F = 0.87$, $P = 0.48$). *L. nigrinus* was more abundant on eastern hemlock than *L. rubidus* (79.8 vs. 4.2%). The opposite was the case on eastern white pine where *L. rubidus* was more abundant than *L. nigrinus* (83.1 vs. 1.0%).

Three- and Four-Way-Choice Bioassays. The tree species from which *Laricobius* spp. were collected did not affect the amount of time it took for individuals to respond to volatiles in the olfactometer ($F = 1.23$, $P = 0.26$, Table 2). Since the tree species did not affect

Table 1. Proportion of *L. nigrinus*, *L. rubidus*, and *L. nigrinus* × *L. rubidus* collected from eastern hemlock and eastern white pine

Details of insect collection						
Season	Eastern hemlock			Eastern white pine		
Species	*L. nigrinus* ($n = 254$)	*L. rubidus* ($n = 14$)	*Ln × Lr* ($n = 61$)	*L. nigrinus* ($n = 1$)	*L. rubidus* ($n = 243$)	*Ln × Lr* ($n = 48$)
Spring 2011	79.4%	8.8%	11.8%	1.9%	79.6%	18.5%
Fall 2011	67.9%	3.8%	28.3%	—Not collected—		
Fall 2012	92.0%	0.0%	8.00%	0.0%	86.6%	13.3%
Average	79.8%	4.2%	16.0%	1.0%	83.1%	15.9%

Insects from eastern hemlock were collected near Banner Elk, NC, and those from eastern white pine were collected near Asheville, NC. Beetle identity was determined with genetic analysis using microsatellites.

Table 2. Mean time (± SE) *Laricobius* spp. adults took to choose a stimulus odor field, by host plant origin and collection date

Comparison factor	*N*	Mean ± SE (s)	*F* ratio	*P* value
Host plant origin			1.23	0.267
Eastern hemlock	393	319.8 ± 14.3		
Eastern white pine	253	299.5 ± 11.5		
Collection date			34.28	<0.001*
Spring 2011 (3-way choice)	156	431.9 ± 17.3*		
Fall 2011 (4-way choice)	160	247.4 ± 17.1		
Fall 2012 (4-way choice)	156	281.7 ± 11.8		

Comparisons were completed using a one-way ANOVA for each category. These analyses include individuals that remained in the center field. Asterisks indicate significant differences in preference when $P < 0.05$.

the response times, it was possible to pool analyses across host origins. Individuals assayed in three-way choice tests in the spring of 2011 (which did not include hemlock woolly adelgid-infested hemlock as a choice) responded on average 168 s slower than those assayed in four-way choice tests in the fall of 2011 and 2012 (which included hemlock woolly adelgid-infested foliage; $F = 34.28$, $P < 0.001$, Table 2). In this analysis, center fields were included to give weight to those individuals who did not respond to volatiles and/or make choices.

Response to Three-Way Bioassay by Time and Proportion. In three-way choice bioassays, which contained only uninfested host foliage, all beetle species were significantly more likely to remain in the center field than to choose a stimulus field (Table 3). Also, when the center field was removed from the analysis, there was no preference for any stimulus field by any *Laricobius* species in an analysis of choice distribution as a Cochran Q statistic (Table 3, Supp Tables 1, 2, 3F [online only]), nor was there significant effect of *Laricobius* species ($F = 1.614$, $P = 0.207$), choice ($F = 2.439$, $P = 0.095$), or the interaction of *Laricobius* species and choice on time required to make a choice ($F = 1.355$, $P = 0.259$, Table 4).

Response to Four-Way Choice by Time. Two-factor ANOVA of times required to choose a stimulus indicated differences among *Laricobius* species in four-way choice tests pooled across all years ($F = 12.114$, $df = 3$, $P > 0.001$); however, their responses did not differ by the stimulus field that was chosen ($F = 0.061$, $df = 3$, $P = 0.980$; Table 5). There was also not an interaction between *Laricobius* species and stimulus ($F = 1.313$, $P = 0.229$ [Table 5]). *Laricobius nigrinus* required more time to choose a stimulus field on average than any other *Laricobius* species tested in these bioassays (vs. *L. osakensis*: 91.5 s longer, $P < 0.001$, vs. *L. rubidus*: 44.5 s longer, $P = 0.001$, vs. Ln × Lr hybrids: 48.2 s longer, $P = 0.074$), whereas no difference was observed comparing *L. osakensis* vs. *L. rubidus* (30.2 s difference, $P = 0.122$), *L. osakensis* vs. Ln × Lr hybrids (26.4 s difference, $P = 0.582$), or *L. rubidus* vs. Ln × Lr hybrids (3.8 s difference, $P = 0.999$; Table 5).

Response to Four-Way Choice by Proportion. All *Laricobius* species in four-way choice tests demonstrated significant ambulatory behavior and chose stimulus fields, with the exception of Ln × Lr hybrids, where the result was marginally significant (Table 6). *L. nigrinus* preferred infested eastern hemlock over other species of trees in pair-wise comparisons (Table 6, Supp Table 4 [online only]), and also preferred eastern hemlock to the blank field. *L. rubidus* remained in the center field most often and significantly more often than most stimulus fields, but did not do so significantly more often than it chose eastern white pine. *L. rubidus* preferred eastern white pine to eastern hemlock but not to eastern hemlock with hemlock woolly adelgid (Table 6, Supp Table 5 [online only]). Ln × Lr hybrids made choices more readily than spending time in the center field, but this was marginally insignificant (Table 6, Supp Table 6 [online only]). Analysis of hybrid behavior with the removal of the center field demonstrated no overall treatment effect. *L. osakensis* chose the fields containing infested and uninfested hemlock more than the blank field and the center field. There were no significant differences between any of the stimulus fields containing host foliage (Table 6 and Supp Table 7 [online only]).

Discussion

L. osakensis was reared in the laboratory with little to no exposure to field environmental cues, and still chose host foliage readily (Table 6). This species also responded most quickly in the olfactometer, which is encouraging for the biological control program. Experimentation in its native range and in the laboratory

Table 3. Percentage of wild-caught *Laricobius* sp. choosing each stimulus field in three-way choice bioassays

Stimulus field	*L. nigrinus*	*L. rubidus*	Ln × Lr hybrids
E. hemlock	15.3% (14)[a]	18.5% (10)[a]	8.6% (23)[a]
E. white pine	16.3% (15)[a]	14.8% (8)[a]	13.0% (3)[a]
Blank	17.3% (16)[a]	9.2% (4)[a]	13.0% (4)[a]
Center	51.0% (50)[b]	53.7% (29)[b]	65.0% (15)[b]
Overall treatment effect, with center field	$\chi^2 = 36.469$, $P < 0.001$*	$\chi^2 = 27.231$, $P < 0.001$*	$\chi^2 = 19.957$, $P < 0.001$*
Overall treatment effect, without center field	$\chi^2 = 0.125$, $P = 0.939$	$\chi^2 = 1.652$, $P = 0.413$	$\chi^2 = 0.250$, $P = 0.882$

Overall treatment effect was calculated using Cochran Q statistic with and without the center field. Sample sizes can be found in parentheses. For significant tests, pair-wise post hoc analyses were completed. Cells marked with an asterisk (*) demonstrate significance ($P < 0.05$) of an overall treatment effect, and superscripts demonstrate within-species significance by stimulus field.

Table 4. Mean response times of each *Laricobius* species to each stimulus field in three-way comparisons

Stimulus field	*L. nigrinus*	*L. rubidus*	Ln × Lr hybrids	Means by stimulus
E. hemlock	263.8 ± 43.8 (14)	276.9 ± 37.8 (10)	411.7 ± 104.3 (3)	317.5 ± 29.2
E. white pine	201.2 ± 26.8 (15)	284.4 ± 43.1 (8)	304.3 ± 112.8 (3)	263.3 ± 24.2
Blank	254.3 ± 31.9 (16)	152.0 ± 32.5 (4)	245.5 ± 67.8 (4)	217.3 ± 25.0
Means by species	239.8 ± 19.8	256.9 ± 25.5	313.0 ± 52.1	

Response time is shown in seconds ± SE. Sample size for each species and each stimulus treatment is in parentheses. Comparisons were analyzed using a two-factor ANOVA. There is no significant difference by stimulus, species, or species by stimulus interaction.

Table 5. Mean response times of each *Laricobius* species to each stimulus field in four-way comparisons pooled for all years

Stimulus field	*L. nigrinus*	*L. rubidus*	*L. osakensis*	*L. nigrinus* × *L. rubidus*	Means by stimulus
E. hemlock w/ hemlock woolly adelgid	144.4 ± 15.3 (75)	142.1 ± 17.9 (32)	85.5 ± 13.4 (28)	167.8 ± 42.8 (13)	128.68 ± [c]
E. hemlock	146.6 ± 20.4 (52)	92.45 ± 5.8 (24)	114.5 ± 20.6 (22)	139.3 ± 28.1 (6)	130.71 ± [c]
E. white pine	156.9 ± 28.3 (39)	123.8 ± 9.8 (47)	90.7 ± 16.2 (17)	183.4 ± 41.2 (15)	129.18 ± [c]
Blank	210.4 ± 30.4 (28)	120.0 ± 12.5 (31)	53.7 ± 15.1 (11)	161.3 ± 43.3 (13)	133.90 ± [c]
Means by species	166.16 ± 10.4[a]	121.68 ± 6.3[b]	91.51 ± 8.7[b]	117.93 ± 20.3[b]	

Response time is shown in seconds ± SE. Sample size for each species and each stimulus treatment is in parentheses. Comparisons were analyzed using a two-factor ANOVA. Superscripts indicate significant difference between groups.

E. hemlock with hemlock woolly adelgid signifies hemlock woolly adelgid-infested eastern hemlock foliage.

Table 6. Proportion of *Laricobius* species choosing each stimulus field, in four-way comparisons

Stimulus field	*L. nigrinus* ($n = 229$)	*L. rubidus* ($n = 200$)	Ln × Lr hybrids ($n = 51$)	*L. osakensis* ($n = 89$)
E. hemlock with hemlock woolly adelgid	32.8% (75)[a]	16.0% (32)[a,b,c]	21.4% (13)[a,b]	31.5% (28)[a]
E. hemlock	22.7% (52)[b]	12.0% (24)[a]	11.8% (6)[b,c]	24.7% (22)[a]
E. white pine	17.0% (39)[b,c]	23.5% (47)[b,c,d]	29.4% (15)[a]	19.1% (17)[a,b]
Blank	12.2% (28)[c,d]	15.5% (31)[a,b,c]	21.4% (13)[a,b]	12.4% (11)[b,c]
Center	15.2% (35)[b,c]	33.0% (66)[d]	7.8% (4)[c]	11.2% (10)[b,c]
Overall treatment effect with center field	$\chi^2 = 22.904$, $P < 0.001$	$\chi^2 = 28.150$, $P < 0.001$	$\chi^2 = 9.294$, $P = 0.054$	$\chi^2 = 13.023$, $P = 0.011$
Overall treatment effect, without center field	$\chi^2 = 17.451$, $P = 0.001$	$\chi^2 = 8.388$, $P = 0.039$	$\chi^2 = 3.979$, $P = 0.264$	$\chi^2 = 8.051$, $P = 0.045$

Overall treatment effect was calculated using Cochran Q statistic with and without the center field. For significant tests, pair-wise post hoc analyses were completed. Superscripts demonstrate within-assay significance by stimulus field.

E. hemlock with hemlock woolly adelgid signifies hemlock woolly adelgid-infested eastern hemlock foliage.

shows that *L. osakensis* is highly synchronous with hemlock woolly adelgid, is a voracious hemlock woolly adelgid predator, and can only develop to adulthood on this pest (Lamb et al. 2012). After considering these factors, as well as the host location behavior documented here, we believe that *L. osakensis* is a good candidate agent for biological control of hemlock woolly adelgid, and will contribute to the control program when released.

Fluctuations in hybridization rates are not uncommon shortly after different species come into contact (Howard 1993). The proportion of hybrids in the North American *Laricobius* samples was variable from year to year. There was some indication in early monitoring that hybridization rates were increasing over time (Havill et al. 2012), but several recent studies showed variation from year to year, with rates settling to 11–13% on average (Havill et al. 2012, Jones et al. 2014, Mayfield et al. 2014, Fischer et al. 2015). For the two years of our study the rates ranged from 8–28% with an average of 16% (Table 1) consistent with the other studies. The accumulating evidence suggests that habitat features may be the strongest factor for species prevalence and hybridization rates. For example, we found that pure parental species were most abundant on the expected host trees: *L. nigrinus* were most abundant on eastern hemlock and *L. rubidus* were most abundant on eastern white pine. The proportion of hybrids in a particular environment may expand or contract when compared to the parental species due to localized environmental changes or conditions (Howard 1993). This does not necessarily mean that hybrids are more or less viable or fecund than the parental species, but rather, may relate to parental traits, such as host preference, relative to specific locations.

Three-way bioassays contained uninfested hemlock and white pine foliage, while four-way bioassays also contained hemlock foliage with hemlock woolly adelgid. *Laricobius* spp. spent less time in the center field in the assays with hemlock woolly adelgid included, and responded more quickly overall as a result (Tables 3, 4, 5, 6). One explanation for this might be that volatiles from hemlock woolly adelgid-infested foliage are a driver for *Laricobius* response in the olfactometer. Recent studies have demonstrated a quantifiable change in volatiles released by eastern hemlock with and without hemlock woolly adelgid infestation. Nonterpenoid compounds such as benzyl alcohol, which are known plant defenses, do seem to be induced by hemlock woolly adelgid feeding (Gomez et al. 2012, Pezet et al. 2013). Many predators use induced odors from the host plant to locate their prey, rather than odors from the prey itself (Lima and Dill 1990, Dicke 1999, Cortesero et al. 2000, Gingras et al. 2002). These results suggest that *Laricobius* species are responding to plants that are being damaged by hemlock woolly adelgid, thereby increasing the reliability of prey location. Alternatively, beetles may have responded more quickly in the fall 2011 and 2012 assays because fall is early in the period of adult *Laricobius* activity, whereas spring represents the end of adult life span and beetles may have largely finished seeking hosts and/or mates and therefore been less responsive. However, the collection that occurred during spring of 2011 comprised of a large sample of all three field-collected species, especially *L. rubidus*, so we found it important to include this collection in our data set.

Each North American *Laricobius* species preferred the host tree species and by extension, the adelgid host, that was expected based on their host preferences and high host specificity, and their distribution on host trees in the eastern United States. *L. nigrinus* preferred infested hemlock over all other choices in pair-wise comparisons, whereas there was no difference in

preference between eastern hemlock and eastern white pine (Supp Table 4 [online only]). *L. rubidus* chose the field containing eastern white pine more than other host fields, and significantly more than the field containing eastern hemlock, but not more than the field containing eastern hemlock infested with hemlock woolly adelgid (Supp Table 5 [online only]). Due to the dominant distribution of each species on different host trees, the analysis included *L. nigrinus* collected mostly from eastern hemlock and *L. rubidus* mostly from eastern white pine. Therefore, their behavior could be affected by previous exposure to host volatiles, in addition to genetically determined behavior.

Our data do not indicate a difference in behavior among *Laricobius* hybrids and their parent species (Tables 5 and 6). In pairwise comparisons, hybrids and parental species did not differ in the time required to choose each stimulus field. Hybrids responded well in the olfactometer and remained in the center field less often than they chose a stimulus field. However, they did not show preference for foliage from one host over another. This may indicate that hybrids are less discriminating than the parental species, or that different individuals respond to odors from the preferred hosts of either parental species.

Potential effects of hybridization on predator–prey interactions should be considered in biological control programs. Hybridization could be beneficial or detrimental. For example, host specificity can be lost (Hora et al. 2005) and hybridization can produce both host-specific and nonspecific genotypes (Szűcs et al. 2011). Reciprocal crossings can produce generations with higher fecundity and fitness than either parental lineage (Szűcs et al. 2012) and may displace the native species (Yara et al. 2010). If populations on different hosts are not reproductively isolated, which is the case with the North American *Laricobius* species, it is important to continue to monitor the effects of hybridization on predator fitness, host specificity, and impact on prey.

The observation that despite hybridization, pure *L. nigrinus* and *L. rubidus* are predominant on eastern hemlock and eastern white pine, respectively, suggests a role of prey preference in maintaining species barriers. Future studies could include eastern white pine infested with pine bark adelgid, which might demonstrate whether slow response and remaining in the center field is a characteristic of *L. rubidus*, or whether they did not respond as well because their primary prey was not available. It could also help to parse out whether hybrids are indiscriminant or behave intermediately.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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