

## Biological Control - Parasitoids and Predators

# Subterranean Survivorship and Seasonal Emergence of *Laricobius* spp. (Coleoptera: Derodontidae), Biological Control Agents for the Hemlock Woolly Adelgid

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### Abstract

Following the adventive arrival, subsequent spread, and ensuing impact of *Adelges tsugae* Annand (Hemiptera: Adelgidae), the hemlock woolly adelgid (HWA) in the eastern United States, a robust initiative was launched with the goal of decreasing ecosystem impacts from the loss of eastern hemlock (Pinales: Pinaceae). This initiative includes the use of biological control agents, including *Laricobius* spp. (Insecta: Coleoptera). Laboratory production of these agents is limited by subterranean mortality and early emergence. Therefore, the subterranean survivorship and timing of emergence of a mixture of *Laricobius* spp. was investigated. PVC traps internally lined with a sticky card and covered with a mesh screen were inserted into the soil to measure the percent emergence of adults based on the number of larvae placed within. The number of emerged adults in the field and laboratory-reared larval treatments was adjusted based on emergence numbers in the control and used as the response variable. Independent variables included in the final model were: treatment (field-collected vs. laboratory-reared), organic layer depth (cm), soil pH, and April-to-December mean soil moisture. No differences were found in survivorship between field-collected and laboratory-reared treatments. As pH and organic layer increased survivorship decreased, significantly. Although the majority of emergence occurred in the fall, emergence also occurred in spring and summer. The occurrence of spring and summer emergence and low survivorship ( $17.1 \pm 0.4\%$ ) in the field across all treatments suggests that these are characteristics of *Laricobius* spp. field biology in their introduced range and not artifacts of the laboratory rearing process.

**Key words:** predator, IPM, invasive species

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an exotic invasive forest and urban pest of eastern hemlock, *Tsuga canadensis* L. (Pinales: Pinaceae), and Carolina hemlock, *Tsuga caroliniana* Engelman (Pinales: Pinaceae), that causes significant tree mortality. HWA was accidentally introduced from Japan to the eastern United States prior to the 1950s, presumably on ornamental nursery stock (Gouger 1971, Stoetzel 2002). HWA is native to Japan, mainland China, Taiwan, and the western United States (Havill et al. 2016). Since the 1950s, HWA has continued to spread throughout most of eastern hemlock's range and is now established in at least 22 states and in Nova Scotia, Canada

(Havill et al. 2016, Kantola et al. 2019, Virginia Tech 2019). The spread of HWA is projected to occur concurrently with increases in global temperatures associated with climate change (Kantola et al. 2019).

HWA has a complex life cycle that depends on the availability of its Japanese primary host, tiger-tail spruce, *Picea torano* Voss (Siebold ex K. Koch) (Pinales: Pinaceae) to maintain sexual generations, and a secondary host hemlock (*Tsuga* spp.), (McClure 1989, Havill et al. 2016). However, within its introduced range in eastern North America, a suitable primary host is not present, resulting in an anholocyclic life cycle exclusively on hemlock with two generations

per year. Typically, the sistens (overwintering) generation occurs from June to late March and the progrediens (spring) generation occurs from late March to June (McClure 1989, 1996, Gray and Salom 1996). The sistens generation is the longest of the two generations due to its summer aestivation (McClure 1989, Gray and Salom 1996). As nymphs develop, they produce a woolly flocculence that ultimately surrounds the adult. Throughout the oviposition period, eggs are deposited beneath the flocculence (which serves as an ovisac) in a tight bundle using a chorionic connection that tethers the posterior end of each egg together (Fernald and Cooley 1898, Gillette 1907).

HWA feeds by inserting its stylet into parenchyma cells of either needles, twigs, or branches and extracting plant nutrients (Young et al. 1995, Oten et al. 2014). The progression of infestation by HWA on eastern and Carolina hemlocks starts with the death of the needles and leads to branch dieback. If infestations are heavy enough, persistent over multiple years, and are timed with deleterious environmental effects (i.e., drought), entire populations of infested hemlocks can perish (Abella 2018).

There are negative ecological, economical, and sociological effects from the decline of hemlock on the landscape caused by HWA, with costs borne primarily by local municipalities and homeowners (Stadler et al. 2006, Li et al. 2014, Lovett et al. 2016). Ecological effects following the decline of eastern and Carolina hemlock stands include but are not limited to; altered soil composition, altered nutrient cycling, and changes in the diversity of fauna and flora within both associated terrestrial and aquatic ecosystems (Eschtruth et al. 2006; Ellison et al. 2005; Siderhurst et al. 2010). Currently, the most effective and widely used approach for treating HWA infestations is through an integrated pest management strategy, combining chemical and biological control tactics (Mayfield et al. 2020). Chemical treatments, primarily through the use of neonicotinoid insecticides, have played a major role in decreasing individual tree mortality. Although chemical treatments are known to be effective, cost, accessibility, and unwanted environmental effects limit the scale and specific areas at which they can be applied (Benton and Cowles 2016). The use of *Laricobius* spp., as biological control agents, has been one of the principal focuses of the HWA biological control effort.

*Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) have been used as classical biological control agents against HWA, in the eastern United States, for the past 9 and 17 years, respectively (Mayfield et al. 2020, Foley et al. 2021). *Laricobius rubidus* LeConte (Coleoptera: Derodontidae) is the only *Laricobius* species endemic to eastern North America. *L. rubidus*' primary and preferred host is the pine bark adelgid (PBA), *Pineus strobi* Hartig (Hemiptera: Adelgidae) which occurs naturally on eastern white pine, *Pinus strobus* L. (Pinales: Pinaceae) (Zilahi-Balogh et al. 2005, Fischer et al. 2015). Both eastern white pine and eastern hemlock and their respective Adelgid species often occur sympatrically. Because *L. rubidus* can be found in association with *L. nigrinus* at low proportions on HWA infested hemlock in the field, as it is collected for food, they are often unintentionally introduced into the laboratory colony (Fischer et al. 2015, Foley et al. 2021).

*Laricobius* spp. have two distinct life phases: arboreal and subterranean. In the arboreal phase, adult *Laricobius* spp. consume HWA sistens nymphs during the fall and winter. In late winter and early spring they oviposit into the HWA ovisac, and the larvae begin feeding on HWA progrediens eggs in the spring (Zilahi-Balogh et al. 2002, Lamb et al. 2005, Vieira et al. 2011). Through larval predation and movement, *L. nigrinus* causes significant disturbance to the overwintering generation's ovisacs (Jubb et al. 2020). However, there

is a density-dependent response within the subsequent HWA spring generation that likely compensates for HWA mortality caused by *L. nigrinus* (Crandall et al. 2020).

In the subterranean phase, *L. nigrinus* and *L. osakensis* burrow into the soil, pupate, and are presumed to enter a state of dormancy (aestivation), whereby they remain underground throughout summer (Zilahi-Balogh et al. 2002). Following the subterranean dormant period, *L. nigrinus* and *L. osakensis* adults emerge from the soil and reassociate with their arboreal habitat. This has been documented to occur as early as the beginning of October for both *L. nigrinus* and *L. osakensis*, based on field data (Wiggins et al. 2016, Virginia Tech 2019). However, in laboratories where *Laricobius* spp. are reared, adult emergence has consistently been noted to occur as early as mid-June for both *L. nigrinus* and *L. osakensis* (Foley et al. 2021).

Deployment of *L. nigrinus* and *L. osakensis* for biological control of HWA has occurred through laboratory mass production efforts by universities and governmental agencies (Foley et al. 2021), and through field collection and redistribution of *L. nigrinus* (McDonald et al. 2011). *Laricobius nigrinus* has been released throughout most of the HWA-infested range of eastern hemlock and continues to spread from those original release sites to new locations (Toland et al. 2018, Foley et al. 2019, Jubb et al. 2021, Virginia Tech 2019). The mass-production and redistribution efforts for *L. nigrinus* over the past 17 years has played a pivotal role in establishing this species in the eastern United States.

Historically, the mass production of these agents at Virginia Tech's insectary and other rearing facilities have been consistently limited by two factors: high colony mortality (63%) during the subterranean phase, and early emergence of adult beetles before suitable prey (HWA nymphs that have broken aestivation) are available in the fall (Foley et al. 2021). It is unknown if the low subterranean survivorship and early emergence seen in the mass rearing laboratories are artifacts of the rearing process, or if they are characteristic of their field biology in the introduced range. The objectives of this study were to determine the subterranean mortality and timing of emergence of *Laricobius* spp. collected in the field vs. reared in the laboratory, how mortality and emergence are affected by abiotic factors, and how subterranean survivorship in the field compares to historical data collected during laboratory mass-production of these agents.

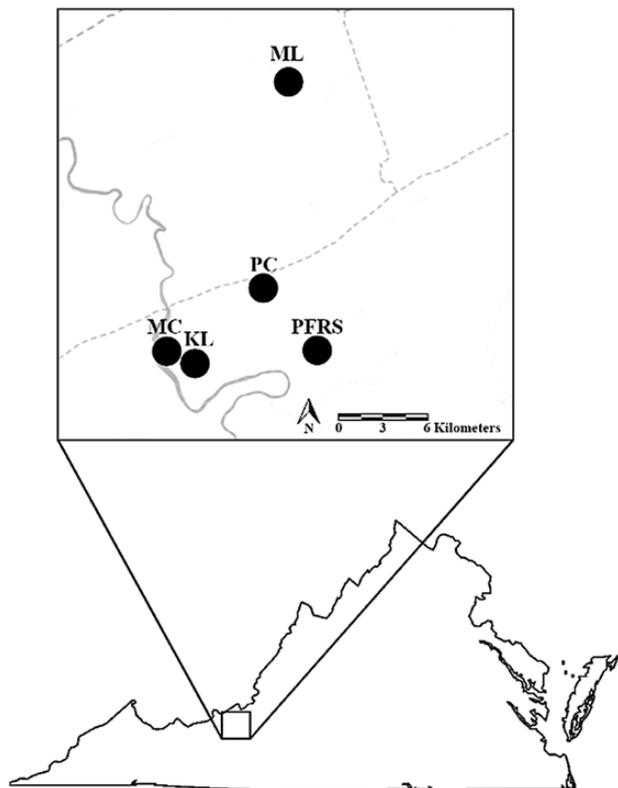
## Material and Methods

*Laricobius nigrinus* and *L. osakensis* are ecologically and functionally similar in that they are both host-specific to HWA and have a similar phenology (Zilahi-Balogh et al. 2002, 2003, Vieira et al. 2011). *Laricobius* spp. larvae used in this experiment were either field-collected or lab-reared. Field-collected larvae were from collections made on HWA-infested eastern hemlock within the urban environment of Blacksburg, VA, (37.2137° N, -0.4090° W) in 2019 and 2020. Branch clippings containing 3<sup>rd</sup> and 4<sup>th</sup> instar field-larvae were collected, brought back the Prices Fork Research Station at Virginia Tech, and placed in Berlese funnels next to an open window to simulate outside temperatures. Laboratory-reared larvae were obtained from *L. osakensis* mass-rearing colonies at Virginia Tech in 2019 and 2020. Rearing followed the standard *Laricobius* spp. protocol outlined by Foley et al. (2021), at a constant temperature of 13° ± 2°C and 12:12 (L:D) throughout larval development in Berlese funnels. Species determination based on egg and larval morphology is not possible among the three *Laricobius* species present in the eastern United States. However, using the presence and shape of the pronotal tooth and coloration of the beetle, adults

can be used to differentiate among the known *Laricobius* spp. in the eastern United States (Leschen 2011). For this reason, posthoc determination of the proportions of each *Laricobius* spp. present within the field-collected and laboratory-reared cohorts were determined using a subset of the larvae, reared to adults, not directly used for this experiment.

Field-collected and laboratory-reared funnels were checked daily for the presence of prepupae. When prepupal larvae were present in the bottom of the funnels, they were collected using a fine tipped paintbrush, grouped in sets of ten, placed in a petri dish (15 x 1.5 cm, Fisherbrand) lined with a moist filter paper, and assigned a field site. Five locations in southwest Virginia (Fig. 1, Table 1) representing a diversity of soil conditions and containing eastern hemlock and were chosen. The same locations were used in both years of the study; however, the exact location of each plot within site varied from year to year.

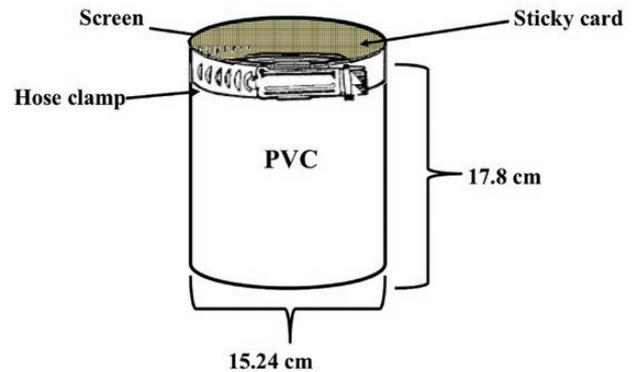
Within 2 hr after larval collection, larvae were transferred to the field, placed into their respective trap on top of the soil, and allowed to burrow into the soil. For each site there were three treatments: 1) field-collected, 2) laboratory-reared, and 3) a control (no larvae). Laboratory-reared larvae were not tested at Poverty Creek in 2019. The numbers of replicates per site in 2019 for the field-collected larvae treatment were 10, 6, 20, 10, and 9 at Kentland, McCoy, Mountain Lake, Poverty Creek, and Price's Fork Research Station, respectively. The numbers of replicates per site in 2019 for the laboratory-reared larvae treatment were: 10, 9, 20, and 10 at Kentland, McCoy, Mountain Lake, and Price's Fork Research Station, respectively. The number of replicates at all sites tested in 2020 was 10



**Fig. 1.** Locations of five study sites (black dots) used to assess the subterranean survivorship of *Laricobius* spp. from 2019–2020 comparing to collection sources (laboratory-reared vs. field-collected). KL, Kentland Farms; MC, McCoy; ML, Mountain Lake; PC, Poverty Creek, and PFRS, Price's Fork Research Station.

**Table 1.** *Laricobius* spp. subterranean survivorship monitoring sites in western Virginia with five sites tested over two years

Site	Plot	Year	GPS	
			Latitude	Longitude
Price's Fork Research Station	PFRS-1	2019	37.212410	-80.489480
	PFRS-2	2020	37.212401	-80.489538
Kentland Farms	KL-1	2019	37.20776	-80.590034
	KL-2	2020	37.207727	-80.589328
McCoy	MC-1	2019	37.215049	-80.602392
	MC-2	2020	37.213485	-80.602058
Poverty Creek	PC-1	2019	37.253039	-80.534788
	PC-2	2020	37.253034	-80.534786
Mountain Lake	ML-1	2019	37.377594	-80.517432
	ML-2	2019	37.377521	-80.517431
	ML-1	2020	37.378025	-80.51651
	ML-2	2020	37.378022	-80.51652



**Fig. 2.** *Laricobius* spp. subterranean emergence trap. Individual traps were schedule 40 PVC, internally lined with a 7.62 cm wide sticky card, topped with a fine mesh screen, and secured with a hose clamp.

with the exception of Mountain Lake, where the total number of replicates were 20.

Traps consisted of a PVC pipe, 17.8 cm tall and 15.2 cm in diameter, with a wall thickness of 2 cm (schedule 40). The upper half of each trap was internally lined with a 7.6 cm sticky card (Olson Products Inc., Medina, OH). The top opening of the pipe was fitted with a 0.46 x 0.46 mm fine mesh screen (NBC Meshtec Americas Inc., Batavia, IL) and secured with a hose clamp (Fig. 2). The sticky cards allowed for the capture of any emerging adults throughout the season. The bottom of the trap was inserted 1.25–5.10 cm into the soil, depending on terrain composition. In 2019, field-collected and laboratory-reared prepupae were placed into their traps, at each site, within a 9 d window (9–18 April) and 3 d window (26–29 April), respectively. In 2020, both *L. nigrinus* field-collected and laboratory-reared prepupae were placed into their traps, at each site, within a 7 d window (7–14 April). For any given site and year, the treatments (field-collected, laboratory-reared, and control) were applied evenly whereby each site had all the larvae placed into their respective traps within one field day. Traps were monitored from April until the following December on a bi-weekly to monthly schedule.

The average depth of three soil samples using a soil push sampler (JMC N-3 handle with 1.9 cm diameter) was used to measure the organic layer depth for each site. Organic layer depth was visually measured to the nearest cm from the top of the unincorporated

leaf litter to the bottom of the fully incorporated organic layer. Soil samples were brought to the Virginia Tech Soil Testing Laboratory (Blacksburg, VA) where soil pH was determined. Soil moisture was measured using a HydroSense II portable system (Campbell, Logan, UT). Temperature data used for this analysis were collected using BioSIM/11. BioSIM simulates air temperature for the differences between weather stations using site location characteristics (i.e., latitude, longitude, and elevation) (Régnière et al. 2014).

### Statistical and Data Analyses

Post-hoc species determination revealed that *L. nigrinus*, *L. osakensis*, and *L. rubidus* LeConte (Coleoptera: Derodontidae) were present in the laboratory-reared colony at proportions of 67, 32, and 1%, respectively, in 2019 ( $n = 4,402$ ), and 43, 50, and 7%, respectively, in 2020 ( $n = 13,298$ ). The proportions of *Laricobius* spp. present from field collections showed a proportion of *L. nigrinus* and *L. rubidus* at 90 and 10%, respectively, in 2020 ( $n = 438$ ). Foley et al. (2019), using the same collection location as this study (Blacksburg, VA), documented the proportion of *Laricobius* spp. adults present to be 98% *L. nigrinus* and 2% *L. rubidus* ( $n = 154$ ).

*Laricobius* spp. subterranean survivorship data were collected over 2 yr, (2019–2020). At each site where emergence occurred within the controls, the number of beetles per year, rep, and site were used as an adjustment factor for the other two treatments (field-collected vs. laboratory-reared). The average adult emergence adjustment factor was then subtracted from the number of adults that emerged from each treatment (field-collected vs. laboratory-reared) and replicate. In addition, an offset of zero was used for treatment replicates with no adult *Laricobius* emergence in order to avoid negative emergence values. The subterranean survivorship was then determined by dividing the adjusted total number of emerged adults per replicate by the initial number of larvae placed into the trap and then used as the dependent variable.

All statistical analyses were conducted using R (version 4.0.2; R Core Team 2021); the package *tidyverse* was used for data organization and the packages *car*, *lme4*, and *multcomp* were used for analysis. A  $P \leq 0.10$  was used as the cutoff for model inclusion, and  $P \leq 0.05$  was used as the cutoff for independent variable significance. A set of generalized linear models using the adjusted percent emergence as the response variable was constructed using a log link function, considering the predictor variables: site, treatment (field-collected vs. laboratory-reared), trial year (2019 and 2020), organic layer depth (cm), soil pH, April-to-December mean soil moisture, and April-to-December mean air temperature ( $^{\circ}\text{C}$ ). Site was excluded from the model in favor of site-level variables (e.g., pH, organic layer depth, temperature, and moisture); since some site-level variables were not measured across time, it was not possible to include site in the model. Cross-validation was performed in order to remove predictors found to be insignificant. In addition, two of the potential predictors, organic layer depth and April-to-December mean air temperature, were found to be highly negatively correlated ( $r = -0.73$ ,  $P < 0.001$ ); thus, we could only use one of them. We decided to use organic matter depth as it had a slightly lower variance inflation factor and was more biologically relevant to this study. Variable-level model significance was accessed by analysis of deviance. Analysis of deviance allows for the model comparisons; whereby the full model, which includes all independent variables, is tested against null models; without one or more of the independent variables using a chi-square test. In addition to the asymptotic  $z$ -tests for significance, the partial deviance tests (the GLM analog to the partial F test) were used to evaluate the significance of each term.

The timing of the subterranean emergence of *Laricobius* species treatments were partitioned based on seasons. HWA and *Laricobius* spp. are seasonally dependent, whereby, 1st instar HWA nymphs and *Laricobius* spp. adults enter dormancy in late spring, remain dormant all summer, and by late fall break their dormancy. The spring season spanned from the first month the prepupae went into the soil (April) until the end of June. The summer season spanned from the beginning of July to the end of September. The fall season spanned from October to the end of December. Emergence occurred in spring at three of the five sites (60%) across all treatments. Emergence during summer and fall occurred at every site across all treatments. The number and proportion of total emergence for each treatment by season are reported.

The mean historical subterranean survivorship of laboratory-reared *Laricobius* spp. is  $37.5 \pm 13.6\%$  (Foley et al. 2021) and was used to compare the subterranean survivorship in this study using one sample test of proportions, with the alternative hypothesis that field subterranean survivorship is less than that of laboratory subterranean survivorship.

### Results

Through significance tests ( $P < 0.10$ ) and violations of multicollinearity, trial year, site, and April-to-December mean air temperature were removed from the final model. The final model, which predicted percent adjusted adult emergence, included treatment, soil pH, organic layer depth, and April-to-December mean soil moisture was significant via deviance test, compared to the null model (Table 2). There was no detectable difference in subterranean survivorship between *Laricobius* spp. that were field-collected to those that were laboratory-reared (Table 2). The mean pH was 5.2 and ranged from 4.1 to 6.4 at sites McCoy and Price's Fork Research Station, respectively. The organic layer depth mean and standard deviation was  $3.54 \pm 3.06$  cm, respectively. The organic layer depth ranged from 0.77 cm at Poverty Creek to 9.33 cm at Mountain Lake. As the organic layer depth increased, survivorship decreased significantly (Table 2). The April-to-December mean moisture was marginally significant ( $P = 0.074$ ) with values ranging from 13.5% at McCoy to 32.7% at Mountain Lake. As soil moisture increased, survivorship increased (Table 2).

The timing of seasonal emergence was not limited to fall, regardless of collection/rearing technique or the adjustment factor (Table 3). Emergence within the control treatment occurred at two of the five sites (McCoy and Price's Fork Research Station) across both

**Table 2.** Results of the Poisson distributed generalized linear model (GLM) with a log-link function investigating the adjusted subterranean survivorship of *Laricobius* spp

Parameters	df <sup>a</sup>	Estimate (SE) <sup>b</sup>	Pr(> z ) <sup>c</sup>
Intercept (field-collected)	-	-0.4824 (0.385)	0.210
Treatment	1	-0.0704(0.103)	0.493
pH	1	-0.2523(0.087)	0.004*
Organic layer	1	-0.1286 (0.029)	<0.001*
Moisture <sup>d</sup>	1	0.0207(0.012)	0.074

<sup>a</sup>df: degrees of freedom.

<sup>b</sup>SE, Standard error.

<sup>c</sup>Pr(>|z|) represents p-value significance.

<sup>d</sup>Moisture: April-to-December average.

\*Significant ( $P < 0.05$ ).

**Table 3.** *Laricobius* spp. emergence partitioned based on season and treatment

Treatment	Spring				Summer		Fall				Total	Total*
	(4/1–6/30)				(7/1–9/30)		(10/1–12/31)					
	n	%	n*	%*	n	%	n	%	n*	%*		
Field-collected	15	6.6	9	4.1	15	6.6	198	87	189	85	228	222
Laboratory-reared	12	6.2	6	3.2	29	14.9	153	79	149	79	194	188
Control	14	58	-	-	0	0	10	42	-	-	24	-
Total	41	9.2	15	3.8	44	9.9	361	81	338	85	446	397

\*Adjustment factor was determined by taking the average number of *Laricobius* spp. to emerge per year, site, and rep within the control treatment and subtracted by the number of adults to have emerged within each traps for each other treatment (field-collected and laboratory-reared). No emergence occurred in summer and therefore no adjustment was necessary.

years. No emergence occurred in control traps in summer and therefore no adjustment factor for this time period was necessary (Table 3). Regardless of the adjustment factor, most of the emergence occurred in fall (Table 3). In spring, the total percent emergence was reduced by more than half when adjusted for emergence within the controls (Table 3).

The control-adjusted combined average field subterranean survivorship for laboratory-reared *Laricobius* spp. was  $18.0 \pm 1.1\%$  and for field-collected *Laricobius* spp. was  $16.2 \pm 1.1\%$ , with a mean of  $17.1 \pm 0.8\%$ . This was significantly less than the historical laboratory subterranean survivorship of  $37.5\% \pm 13.6\%$  (Foley et al. 2021) (One-sample proportion,  $X^2 = 395.2$ ,  $df = 1$ ,  $P < 0.001$ ).

## Discussion

Analysis of the subterranean survivorship and timing of emergence by species were confounded by the presence of unknown *Laricobius* larvae species within both the field-collected and laboratory-reared colonies. Attempts were made to identify which *Laricobius* spp. were present during emergence, however, removal of the adults from the sticky card resulted in their complete maceration, rendering morphological identification very difficult. No attempts were made to molecularly identify *Laricobius* spp. following their emergence. *L. nigrinus* and *L. osakensis* are ecologically and functionally similar in their predatory host preference and phenological synchrony. Therefore, analyses were made on the basis of rearing technique (laboratory-reared vs. field-collected) rather than directly on species. The subterranean survivorship in the lab-reared treatment, which consisted mostly of *L. nigrinus* and *L. osakensis*, and some *L. rubidus*, was not statistically different from the survivorship in the field-collected treatments, which consisted of mostly *L. nigrinus* with some *L. rubidus*. Therefore, it is likely that the observed patterns in this study are from the introduced classical biological control agents.

While subterranean mortality was expected to be relatively high (~60%), the amount of mortality observed ( $82.9 \pm 0.8\%$ ) in this field study was higher than anticipated and was significantly and substantially less than that of the historical production of these agents in the laboratory (Foley et al. 2021). Results from this experiment show that abiotic influences in a field setting are critical factors to the subterranean survivorship of *Laricobius* spp. It is unclear at which subterranean life stage (prepupa, pupa, or adult) the majority of mortality is occurring. These data also reveal that emergence of *Laricobius* predators of HWA is not limited to the fall season (Table 3).

In this study, as pH increased subterranean survivorship decreased (Table 2). Conifers, including *Tsuga* spp. are well adapted to (and create through litter deposition) low pH conditions and grow best when the pH is below neutral (<7) (Mladenoff 1987, Finzi et al.

1998). *Laricobius* beetles have adapted to feed on conifer-feeding adelgids, and thus may be well adapted for aestivation in acidic soil conditions. Also, soil pH has a strong influence on soil microbial activity, including fungal communities (Matthies et al. 1997, Nilsson et al. 2007, Lauber et al. 2008, Rousk et al. 2009). The secretion of honeydew by HWA and subsequent colonization of that honeydew by sooty mold fungi may have served as an ecological linkage from fungivory to predatory feeding behaviors in the evolution of *Laricobius* (Leschen 2000). Although it is assumed that *Laricobius* spp. have evolved to fully replace fungi with insects in their diet, there is a possibility that these species consume fungi as a supplement to their adelgid diet. The importance of fungi as a nutrient source could be particularly important during the subterranean life cycle of these species when metabolic activity is presumably lowest, their host is unavailable, and hyphae are abundant. Although speculative, if *Laricobius* spp. do in fact consume fungi, and the availability and composition of their dietary fungal community decreases as pH increases, this could partially explain our results.

Jones et al. (2014) reported no detectable effect of organic layer depth on *L. nigrinus* subterranean survivorship. In our study, the depth of organic layer was a significant variable, and as organic layer increased, subterranean survivorship decreased. The burrowing depth and distance that larvae travel in order to pupate is unknown. It is also unknown whether adults remain active in the soil column following eclosion. A potential explanation for the relationship between organic layer and *Laricobius* spp. subterranean survivorship is that as organic layer depth increases the depth at which the larvae burrow also increases to a point where they become unable to re-emerge from the soil. Furthermore, it is possible that with increases in the amount of organic matter, measured by depth, there could be an increase in the number of soil-inhabiting predators (Brady and Weil 2010). Thus, it is possible that the relationship observed here between increased organic layer depth and decreased *Laricobius* spp. survival could be partially attributed to the presence of subterranean predators.

Soil moisture was recorded each time emergence was quantified in order to capture the temporal variability of this dynamic soil property. Although April-to-December mean moisture was a marginally significant variable in the model, moisture levels outside of the 20 to 40% range have been documented as a significant mortality factor in the subterranean survivorship of *Laricobius* within the laboratory (Lamb et al. 2007). Additionally, Lamb et al. (2007) reported that moisture levels at 30 or 45% were shown to cause a significant increase in early emergence, relative to moisture of  $\geq 60\%$ . While the April-to-December mean moisture across all sites in this study was 21.4%, the mean minimum was 2.1%, and therefore, the presence of such dry soil likely offers a partial explanation to our results.

Another potential explanation for the relatively high rate of subterranean mortality observed here and in other studies (Zilahi-Balogh et al. 2003, Salom et al. 2012, Jones et al. 2014, Foley et al. 2021), is the degree and duration of handling the larvae. Zilahi-Balogh et al. (2003) postulated that inadequate moisture and excessive handling time might contribute to the high rate of *Laricobius* spp. larva mortality observed in the laboratory. Naturally, *Laricobius* spp. prepupae drop directly from the hemlock branch onto the soil surface and then burrow into the soil to begin the pupation and aestivation process. However, as in this study and in the mass-rearing laboratory, as larvae drop from branches into their funnels, a person must visually determine which larval life stage is present (prepupa or otherwise) and must group prepupae into cohorts that will be eventually placed in their respective subterranean container. Although efforts were taken to get the prepupa into the traps as quickly as possible and to reduce overall handling time of each larva, it likely played a role in their overall survivorship. Future studies should aim at further reducing or eliminating the handling time of larvae when determining *Laricobius* spp. subterranean survivorship in the field or otherwise.

In an attempt to quantify the subterranean survivorship of *L. nigrinus* by letting the larvae drop naturally from the host into the soil and relying on visual detection rather than an active trapping method to record the number of adults, Jones et al. (2014) reported *L. nigrinus* subterranean survivorship within a field setting to be 0.28%. *Laricobius* spp. adults are known to drop from a surface, whether from tree branches or the siding of a trap, when disturbed. Therefore, it is possible that the percent survivorship assessed by Jones et al. (2014) was underestimated by missing observations of adults that may have dropped back to the soil upon inspection. The smaller trap design presented here, which was internally lined with a sticky card, allowed for the capture of any emerging adults within the trap without having the adults drop back down to the soil and likely further contributed to the 150-fold increase ( $17.1 \pm 0.4\%$ ) in survivorship compared to Jones et al. (2014). Regardless of differences in the subterranean survivorship reported, what is clear is that populations of *Laricobius* spp. experience high rates mortality during this life phase as part of their natural biology.

Historically, use of a beat sheet to collect adults within the canopy of hemlocks has been the main technique for determining the timing of emergence of HWA adult predators. The few studies that have characterized *L. nigrinus*, *L. osakensis*, and *L. rubidus* seasonal emergence relied on the beetles traveling up the side of a trap into a collection vial mounted on top (Wiggins et al. 2016), or through visual inspections (Jones et al. 2014). In this study, the placement of the traps in the ground throughout the entire subterranean season (spring through fall), the use of sticky cards, and routine monitoring, allowed for seasonal emergence data to be captured without having the beetles dropping back down to the ground upon inspection.

It is unclear what *Laricobius* spp. are doing following their spring and summer emergence. *Laricobius erichsonii* Rosenhauer (Coleoptera: Derodontidae), a classical biological control agent used against the invasive balsam woolly adelgid, *Adelges piceae* (Ratzeburg), has been documented to emerge from the soil following pupation, as teneral adults (Lawrence and Hlavac 1979). Upon reemergence from the soil, *L. erichsonii* associates with their adelgid prey in their arboreal habitat, only to drop back to the soil for the remainder of their prey's dormant period (Lawrence and Hlavac 1979). It is not currently known what the seasonal timing of emergence is for the *Laricobius* spp. observed in our study in their respective native ranges. However, early emergence, whether observed here and throughout the past 17 yr of rearing these species in the laboratory, is most likely a natural part of their biology and the fact

that emergence occurred from the control is further support of this hypothesis. It should be noted that the numbers of adults emerging in the spring were similar among each treatment including the control (Table 3). Since we could not confirm species identification following their emergence, we do not know if any of those beetles were ones placed in the soil or were ones already present in the soil. This suggests that investigations that enable the molecular identification of emerging species are warranted.

These results suggest that the significant mortality that occurred during laboratory rearing (Foley et al. 2021) was not merely an artifact of the rearing procedures and laboratory conditions to which the beetles were exposed (Salom et al. 2012). The average historical laboratory survivorship of these species ( $37.5 \pm 13.6\%$ ) was more than double the average of this field study ( $17.1 \pm 0.8\%$ ). Although conclusions from this study are based on rearing/collection technique rather than species, *L. nigrinus* had the highest proportional presence within the field-collected colonies. Therefore, it is likely that the results from the field-collected cohorts are suggestive of the natural biology of this species. However, the same overarching conclusions cannot be made for what was presumed to be *L. osakensis* laboratory-reared colonies, due to the almost even mixture of *L. nigrinus* to *L. osakensis* and to a lesser degree *L. rubidus*. Even though there was no detectable statistical difference between the treatment types, it is possible there was a species-specific interaction based on rearing technique and subterranean competition that was not explored in this study. In the future, further efforts should be made to standardize the species composition.

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