EVALUATION OF *LARICOBIUS NIGRINUS* (COLEOPTERA: DERODONTIDAE) OVIPOSITION BEHAVIOR AND THE EFFICACY OF EGG-STAGE RELEASES AS A BIOLOGICAL CONTROL METHOD OF HEMLOCK WOOLLY ADELGID (*ADELGES TSUGAE;* HEMIPTERA: ADELGIDAE) IN WESTERN NORTH CAROLINA

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Biology.

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April 2021

ACKNOWLEDGMENTS

I would like to thank my committee members for all of their assistance and edits, this process is difficult even in the best of times, so I am incredibly grateful for everyone's flexibility with virtual meetings and socially distant field visits while navigating a global pandemic. In particular, I would like to thank Dr. Angela Mech for her excel wizardry as well as her eternal patience, optimism, and guidance throughout this project. Thank you to Dr. James Costa for helping with site setups, insightful edits, and challenging me to grow as a writer. Thank you to Dr. Jane Dell for constant kindness, thoughtful observations, and for providing a lab space for this research. To my reader, Dr. Luiz Silveira, thank you for your enthusiasm to assist and provide constructive feedback on my thesis.

I am grateful to the North Carolina Policy Collaboratory (UNC) and the Highlands Biological Station (WCU) for funding my research, and to our collaborators at the Beneficial Insect Lab of the North Carolina Department of Agriculture and Consumer Services, especially Dr. Steven Turner for sharing his knowledge of rearing protocols and his contribution of beetles throughout this study. Big thanks to Wiley and Clare Ellis and Carson Ellis for graciously providing study sites for *Laricobius* releases. Additional thanks to Dr. Peter Bates, Gabby Williams, Sierra Croney, and Leslie Costa for volunteering their time to help with implementation of this research.

Furthermore, I want to thank Regan Daniels, Amanda-Jean Blackburn, and Carson Ellis for joining me in fieldwork, providing support and friendship, and for making my time at WCU a memorable experience. I'm eternally grateful to my parents, Edwin Gonzalez and Adriana Hernandez, for instilling me with a lifelong love of learning. Finally, I want to thank my husband, Jack Schulte, for his continuous encouragement and love.

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LIST OF ABBREVIATIONS

HWA- Hemlock Woolly Adelgid

NCDA&CS - North Carolina Department of Agriculture and Consumer Services

WCU - Western Carolina University

ABSTRACT

EVALUATION OF *LARICOBIUS NIGRINUS* (COLEOPTERA: DERODONTIDAE) OVIPOSITION BEHAVIOR AND THE EFFICACY OF EGG-STAGE RELEASES AS A BIOLOGICAL CONTROL METHOD OF HEMLOCK WOOLLY ADELGID (*ADELGES TSUGAE;* HEMIPTERA: ADELGIDAE) IN WESTERN NORTH CAROLINA

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The hemlock woolly adelgid (HWA), *Adelges tsugae* (Hemiptera: Adelgidae), is an invasive and devastating pest of native hemlock trees in eastern North America. Since HWA's introduction to western North Carolina in the early 2000s, state and federal agencies have been attempting to preserve eastern and Carolina hemlock in the region through both chemical treatments and biological control. Chemical control in a forest ecosystem is not as viable an option due to high costs and environmental impacts, making biological control using natural enemies an important management goal. Currently, the most commonly released predatory beetle to combat HWA in eastern North America is *Laricobius nigrinus* (Coleoptera: Derodontidae). However, laboratory rearing of *L. nigrinus* has been constrained by high mortality rates (65-75%). This mortality rate can be partially explained by the life cycle of *L. nigrinus*, which in nature pupates in the soil and undergoes a dormant period during the summer similar to HWA. The struggle with low emergence rates may suggest that the beetles may be especially sensitive to environmental factors during their pupation period. It is important that adult predator beetles emerge as HWA is emerging in the field, otherwise early emergence can result in high mortality due to lack of available food. To try and overcome these low lab-reared survival rates and reduce the risk of unsynchronized emergences, the efficacy of releasing lab-oviposited *L. nigrinus* eggs in the field instead of lab-reared adults was evaluated. In addition, the oviposition rate of *L. nigrinus* was tested based on different female beetle densities to help develop an efficient lab oviposition protocol. Trends suggested a negative density-dependent relationship indicating that the number of mated females should be limited per oviposition cage to ensure the highest number of eggs laid. In the fall and winter of 2020-2021, a recovery experiment was conducted using soil emergence tents in Jackson County, North Carolina, by releasing approximately 230 *L. nigrinus* eggs. The soil emergence tents recovered five times as many adult *L. nigrinus* underneath trees where *L. nigrinus* eggs were released when compared to control trees that had no egg releases, providing strong evidence that this deployment method is valid. Together these studies suggest that altering current oviposition protocols and releasing *L. nigrinus* in the egg stage rather than as adults could be less labor intensive and a more cost-effective approach to HWA biological control – qualities needed in the struggle to manage HWA populations. Finally, recommendations for new protocols are provided for lab managers and forest health professionals that rear and release *L. nigrinus* populations.

CHAPTER 1: INTRODUCTION

Eastern hemlock (*Tsuga canadensis*) is an evergreen tree found in both pure and mixed stands of temperate forests in eastern North America with a range that extends from Canada to Georgia and over to Wisconsin (Godman and Lancaster, 1990). Eastern hemlock is also considered a foundation species because of its role in microclimate amelioration, soil ecology, nutrient cycling, watershed stabilization, and impact of plant species composition in forests (McWilliams and Schmidt 2000). Their evergreen foliage and high crown-bulk density creates a cool, moist microclimate with slow rates of nitrogen cycling year-round. The influence of eastern hemlock is not just on terrestrial ecosystems, aquatic ecosystems also benefit because hemlocks can regulate stream temperature, stream flow, water chemistry, and light availability (Abella 2014). Eastern hemlocks play an important role in the forests in which they are found, and no other known tree species can replace their function. However, eastern hemlock as well as Carolina hemlock (*T. caroliniana*) have experienced great decline and mortality as a result of the introduction of a single non-native, invasive species.

The hemlock woolly adelgid (HWA; *Adelges tsugae*) is an invasive and devastating pest of hemlock trees in eastern North America. Native to Asia and the Pacific Northwest region of North America, HWA was first reported in the eastern United State in 1951 in Richmond, Virginia following it introduction from Japan (Gouger 1971). A member of the hemipteran family Adelgidae, HWA has a complicated life cycle with both asexual and sexual generations depending on the availability of tigertail spruce (*Picea torano*) and hemlock (*Tsuga* sp.), their primary and secondary host trees. In eastern North America, in the absence of *P. torano*, the population is limited to asexual reproduction on hemlock trees (Havill et al. 2014). HWA's ability to continuously reproduce asexually has contributed to the success of its invasion throughout the eastern United States (McClure 1989) (Fig. 1).

There are two wingless generations of HWA in a year, the sistens and progediens. Sistens hatch as early as late spring in warmer climates and aestivate throughout the summer as first-instar nymphs. The nymphs awake from their dormancy in the fall to feed, excrete their woolly wax, and then start oviposition in late winter; adult females are hidden inside their ovisacs. In late winter/early spring, the progredien eggs will hatch and nymphs can grow to maturity by late spring. The progediens then lay eggs that will become the next sistens generation (McClure 1989, Gray and Salom 1996).

HWA feeds on hemlock trees by inserting their long, piercing-sucking mouthparts into the plant tissue at the base of a needle, where they remain immobile for the rest of their life cycle while feeding on the nutrients from the xylem tissue. Some research suggests that while the insects are feeding, they may inject their saliva into the tree to help them break down the nutrients before sucking them back up (Oten et al. 2014). After 2-4 years of feeding by HWA, hemlock trees tend to experience a loss of foliage and dieback that eventually results in mortality, especially when combined with other stressors. Hemlocks of all age classes are susceptible to HWA attack, and may succumb in as little as five years (Havill et al. 2014). Today, non-native HWA can be found on hemlock trees across eastern U.S., ranging from Georgia to Maine. Unlike western hemlock (*T. heterophylla*) and other coevolved Asian hemlock species, the eastern hemlock species show no natural defense or resistance to HWA, and HWA has no natural predators native to eastern North America (McClure 1987).

Since HWA arrived in western North Carolina in the early 2000s, two main management practices have been developed in an effort to preserve hemlock species. The first is treatment with systemic pesticides. This short-term solution can be successful on a small scale, but is not as feasible across large forest landscapes; it would be both far too expensive and would have significant non-target impacts in the ecosystem (Mayfield et al. 2020). The second management approach is through biological control, which offers a long-term management strategy by using natural predators of HWA to keep the population in check. It is important to consider the evolutionary history of hemlocks, adelgids, and their natural enemies in attempting to develop an effective biological control program in order to find a predator with both high specificity and a higher chance of success (Havill et al. 2014).

Biological control is a major component of management programs implemented against HWA in forest ecosystems. Over the past 25 years, several candidate predators have been tested, including *Sasajiscymnus tsugae*, *Scymnus sinuanodulus*, *S. ningshanensis* (Coleoptera: Coccinellidae), *Leucopis* spp. (Diptera: Chamaemyiidae), *Laricobius nigrinus*, and *L. osakensis.* In addition, eastern North America has a native species of *Laricobius*, *L. rubidus*. While it has been observed to feed on HWA, it primarily feeds on pine bark adelgid (*Pineus strobi*) and cannot manage the HWA population alone (Havill et al. 2014). Currently, *L. nigrinus* is the most commonly released predator, as other predators have either been unsuccessful at establishing a population, shown difficulties in mass rearing, or are still being studied. (Havill et al. 2014). *Laricobius nigrinus* is originally from the Pacific northwest region of North America, where it feeds on native HWA populations. Their lifecycle is synchronous with HWA's (Fig. 1), with *L. nigrinus* females laying their eggs directly in HWA ovisacs so that larvae can feed immediately after hatching. It is important to note that *L. nigrinus* has only one generation per year, compared to two for HWA, but the adults feed on HWA sistens in the fall and winter, and the larvae feed on progredien eggs and early nymphs in the spring (Cheah et al. 2004).

The interest in *L. nigrinus* as biological control of HWA stems from its ability to be reared in large numbers in a laboratory setting (Salom et al. 2012). The rearing process typically begins when HWA and *L. nigrinus* both start to lay eggs around late winter after selecting wild-caught beetles to act as founders of the lab colony. Adult *L. nigrinus* are randomly placed in containers with branches that are heavily infested with HWA to serve as a food source as they mate and lay eggs. After approximately one week, the adults are removed from the containers and the branches are moved to new larval rearing chambers that are designed to catch any larvae as they drop to aestivate and ultimately pupate. Any collected larvae are moved to well ventilated summer aestivation boxes filled with moist soil. Throughout this process, temperature in the rearing lab must be monitored to mimic the conditions of their natural habitat and season. In early October, the aestivation boxes are exposed to lower temperatures (12-15°C) and after about two weeks adults will start to emerge until late December (Lamb et al. 2005). Only after all of these steps are adult *L. nigrinus* ready for field releases.

To improve the current biological control program against HWA, it is imperative to increase the number of *Laricobius* beetles released. It was hypothesized that the more *L. nigrinus* females present in a rearing cage, the more eggs will be laid, to a certain point. The effect of intraspecific competition on *L. nigrinus* oviposition behavior is unknown, but results could allow us to better understand the biology of this biological control beetle, as well as determine the best inoculation density needed to attain the highest number of individuals for releases. Although some studies have stated that female *L. nigrinus* only lay a single egg per adelgid ovisac or only count the number of *L. nigrinus* eggs per branch (e,g., Zilahi-Balogh et al. 2003, Lamb et al. 2011), preliminary observations suggested that this may not be the case. Inoculated branches were examined in March 2019 in a pilot study and determined that, of the 12 hemlock branches studied, over 33% of the HWA ovisacs had more than one *L. nigrinus* egg in it (A. Mech, *personal communication*). Those numbers reflect cages with ~10 mating *L. nigrinus* pairs each, so it was impossible to tell if the observed distribution of eggs within ovisacs was the result of some females laying more than one egg in an ovisac or if multiple females are laying eggs in the same ovisac.

The success rate in rearing *L. nigrinus* in a laboratory setting has been modest, demonstrating a need for a more efficient protocol to aid in conservation efforts. Although scientists have been rearing *L. nigrinus* in labs for over a decade, the number of adults that emerge from pupation compared to the number of larvae that dropped into the soil is relatively poor, on the order of 25-35% (Steven Turner, *personal communication*). The total number of beetles produced each season is closely related to the quality and availability of food (Salom et al. 2012), requiring a consistent supply of fresh HWA ovisacs as artificial diets have been found to result in higher mortality during development. However, the differences between larval cohorts are a significant factor in the variation of emergence rates, indicating that there may be additional unknown factors in a lab setting that affect mortality rates (Salom et al. 2012). This led us to wonder if *L. nigrinus* larvae and/or pupae would do better in a natural setting rather than an artificial setting, which could be tested by deploying them during their egg stage instead of as adults.

Releasing *Laricobius* eggs in the field allows the beetles to feed, grow, aestivate, and undergo pupation under natural conditions. During the fall and winter of 2019-2020, a pilot study in southern Jackson Co., NC evaluated different recovery methods and yielded promising results of *L. nigrinus* adults collected through the use of soil emergence tents (BioQuip, Rancho Dominguez, CA) following the release of eggs. The pilot study was, accordingly, followed up with an expanded field experiment utilizing soil emergence tents in 2020-2021 to collect and determine establishment of *Laricobius*. This study aimed to gain a better understanding of *L. nigrinus* oviposition behavior to ensure optimal rearing protocols and to test an alternative release approach for *L. nigrinus*, by deploying eggs directly onto HWA-infested trees in the field.

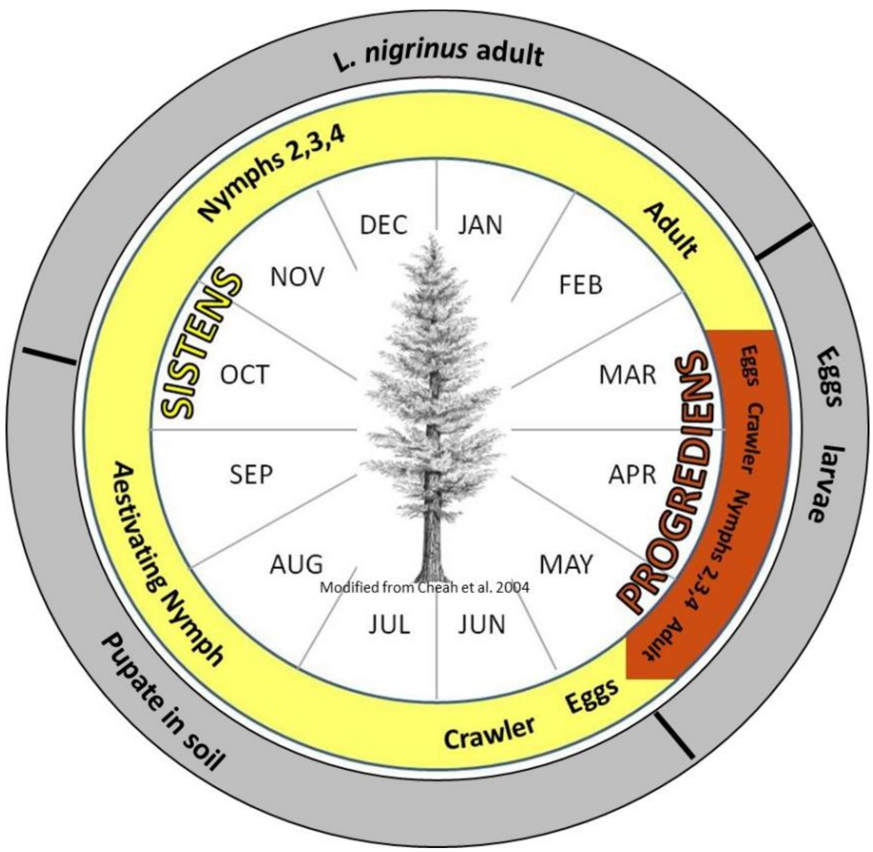

Figure 1. Annual lifecycle of hemlock wooly adelgid (sistens in yellow and progrediens in orange) and *Laricobius nigrinus* (in gray) on hemlock in North America. Modified from figure created by Cheah et al. (2004)

CHAPTER 2: MATERIALS AND METHODS

Section 2.1: *Laricobius nigrinus* oviposition behavior

A controlled experiment was designed to investigate the oviposition behavior of female *L. nigrinus* when placed in cages with varying densities of other females. Eastern hemlock branches were collected from trees near Fisher Creek in Pinnacle Park located in Jackson County, NC (35°25'27.6"N 83°11'24.9"W). Approximately five trees with moderate to high HWA densities based on visual estimates were selected from this site in order to reduce potential variation of HWA development stages. Because recently hatched *L. nigrinus* larvae require HWA eggs as a food source, selected branches had to have a high density of fresh HWA ovisacs, indicating live, egg-laying HWA. Collections occurred during the first week of March, before the peak of natural *L. nigrinus* egg-laying to try and ensure that any oviposition found was the result of lab-reared females rather than from any potential field populations.

After the collections, branches were shipped overnight to the North Carolina Department of Agriculture and Consumer Services (NCDA&CS) Beneficial Insect Laboratory; during transit they were wrapped in wet paper towels to avoid desiccation and placed in a Styrofoam cooler to avoid high-temperature exposures. Small mesh cages (n = 16) were set up in a randomized block design, where the treatment was the number of lab-reared mating *L. nigrinus* pairs (1, 2, 4, or 8 pairs), which was randomly assigned per cage and repeated four times. Each cage was also filled with three hemlock twigs infested with HWA as a food source and site for oviposition. Twigs were clipped (5-12 cm) and randomly assigned a cage with three separated twigs per wet foam brick per cage (n = 48 twigs). Each cage was then given its treatment number of *L. nigrinus* mating pairs and left for 18 days. This allowed enough time for mating and oviposition while ending the experiment before *L. nigrinus* egg hatch. Cages were set up in a temperature and humidity-controlled indoor room at the Beneficial Insect Lab.

After 18 days, adult *L. nigrinus* were removed from the cages, twigs were placed in marked bags, and then overnighted to Western Carolina University (WCU) for examination. All 48 twigs were placed in a refrigerator until they could be assessed; all twigs were evaluated within five days of delivery. For each twig, the number of HWA ovisacs were counted and then each was destructively sampled to look for the presence and number of *L. nigrinus* eggs. *Laricobius* eggs are identified by their oval shape (approximately 0.37-0.50 mm in size) and their bright yellow color compared to the smaller, reddish-brown HWA eggs found in the ovisac (Zilahi-Balogh et al. 2006) (Fig. 2). Based on this information, two variables were calculated at the twig level, the number and proportion of HWA ovisacs with at least one *L. nigrinus* egg, and the average number of *L. nigrinus* eggs per oviposited HWA ovisac, with the average number of eggs laid per female *L. nigrinus* being calculated at the cage level.

Simple linear regression was used to determine the relationship between 1) the average number of *L. nigrinus* eggs per oviposited HWA ovisac per twig and the number of mated females in the same cage, 2) the average number of *L. nigrinus* eggs laid per female and the number of females in the cage, and 3) the number of HWA ovisacs with *L. nigrinus* eggs and the total number of HWA ovisacs available. Logistic regression was used to determine if the proportion of HWA ovisacs with *L. nigrinus* eggs laid is correlated to the number of mating pairs. All calculations were conducted in Excel and R (R Core Team, 2020).

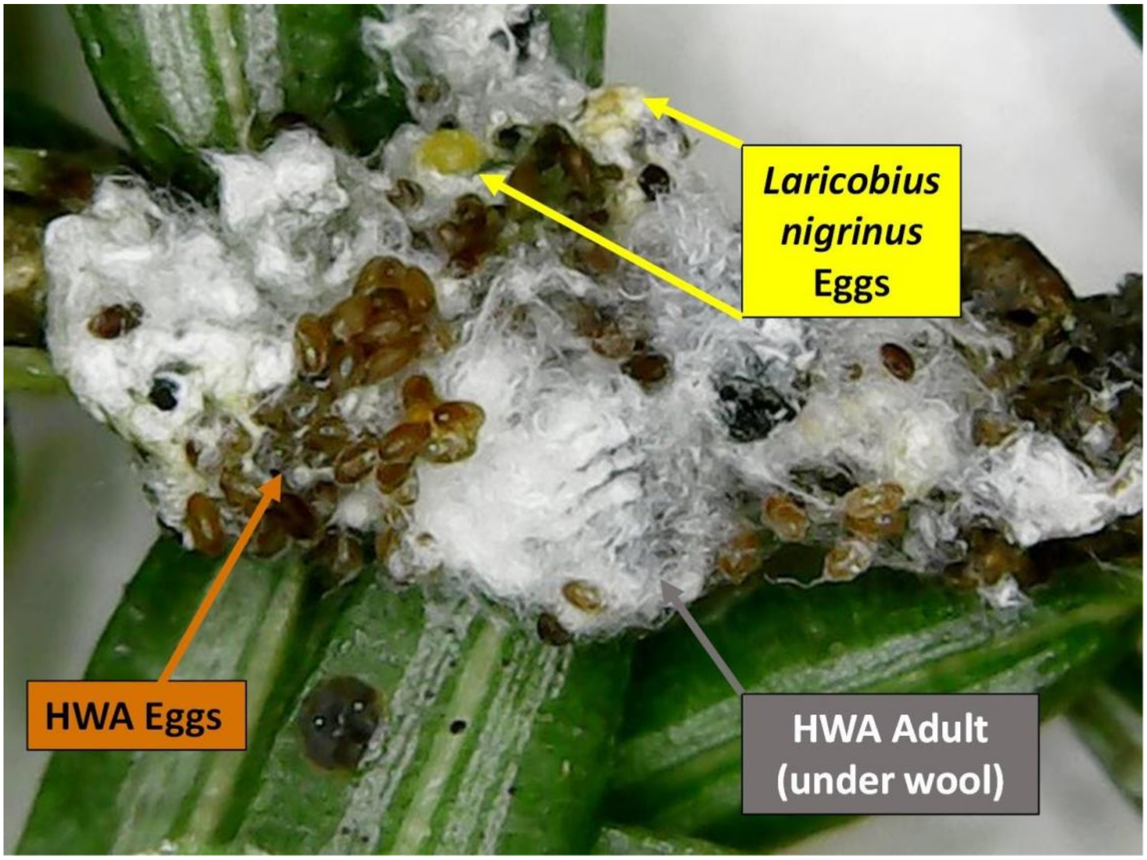

Figure 2. Sample HWA ovisac showcasing the difference between HWA eggs (orange/brown) and *Laricobius* eggs (yellow).

Section 2.2: Efficacy of *Laricobius nigrinus* egg-stage releases

Site Selection

The study site was located at a private property in Cashiers, NC (35°06'48.0"N 83°05'19.5"W) that had at least six hemlocks (three control trees and three release trees) with a fairly abundant HWA population and allowed for adequate spacing (at least 250 m) between release trees and control trees. The trees also needed to be small enough (approximately 5m or less) that branches could be reached in order to attach HWA ovisacs with *L. nigrinus eggs*. Due to the fact the *L. nigrinus* has been released in North Carolina since the early 2000s, there could already be an established population of *L. nigrinus* in the general area, therefore control trees were included to estimate a baseline population.

Experimental Prep

In early February 2020, each release tree was examined to determine the number of available HWA ovisacs on the branches. It was assumed that each *L. nigrinus* would need around 20 HWA ovisacs to ensure sufficient food availability in order for the larva to have the best chance of enough to survive to adulthood (Steven Turner, *personal communication*). A hemlock branch was flipped over and the number of ovisacs were counted on that branch and then divided by 20. The resulting number was marked on flagging tape and indicated the number of *L. nigrinus* eggs that could be released on that specific branch.

Oviposition

HWA infested branches were collected from trees near Fisher Creek in Pinnacle Park (Sylva, NC) and were shipped overnight to the NCDA&CS Beneficial Insect Laboratory for inoculation of *L. nigrinus* eggs. Samples were wrapped in wet paper towels and kept in a Styrofoam cooler during transit. Once the shipment arrived, the branches were put into cages with *L. nigrinus* mating pairs for inoculation. After 18 days, adult *L. nigrinus* were removed from the cages, the samples were placed in marked bags, and then overnighted to WCU for examination of ovisacs.

Attachment

A subsample of 12 branches were removed upon arrival from the Beneficial Insect Lab and each ovisac present was dissected to determine the average number of *L. nigrinus* eggs per ovisac. In the spring of 2020, there was found to be roughly one *L. nigrinus* for every seven HWA ovisacs. Based on this estimate, inoculated branches were trimmed to have the appropriate number of *L. nigrinus* eggs required to match the number that the branch could support, as labeled by the number on the flagging tape. For example, if a field site branch had 100 HWA ovisacs, it could support five *L. nigrinus* eggs. That branch would then be marked with tape labeled "5" and when it came time to attach the inoculated branches, it would receive a branch with 35 HWA ovisacs because we assumed that there is one *L. nigrinus* egg for every seven HWA ovisacs. Once the branches were trimmed to the appropriate size for each tree, the inoculated twigs were physically attached to the experimental release trees with pipe cleaners. Approximately 231 *L. nigrinus* eggs were released in March, with each of the three release trees receiving between 76 and 78 eggs. After releasing the eggs, the trees were left alone over the remainder of the spring and summer as the larvae hatched, fed on HWA, and eventually pupated in the soil.

Collecting

A pilot study conducted in the previous 2019 season compared the efficiency of different *L. nigrinus* adult collection methods between sticky traps, beat sheeting, and soil emergence tents. The study found that 88% of the total *Laricobius* recovered were collected through soil emergence tents, leading us to utilize only emergence tents in the 2020 season. In early September 2020, a total of 16 3.6 ft^3 soil emergence tents (BioQuip model #2885) were placed under both release and control hemlock trees, with each tree having 2-3 emergence tents directly below the branches where *L. nigrinus* eggs had been attached earlier that year, with the number of tents per tree dependent on the size of the tree. Soil emergence traps resemble a floorless tent and funnel insects that come out of the soil into a collection bottle at the top of the trap, allowing for simple and quick collections (Fig. 3). Traps were collected weekly from mid-September through mid-December, then every two weeks from mid-December through the end of January 2021 after two consecutive collection trips found no *L. nigrinus* individuals.

For this study, the collection bottles were filled with water, and once collected, the containers were put in a freezer for 48 hours and then put in the refrigerator to thaw for 48 hours in order to ensure that there are no surviving specimens. *Laricobius* beetles were separated from any bycatch and then all beetles were examined under a dissecting scope to identify to the species level based on the coloration of their elytra. *L. nigrinus* can be easily recognized as their elytra are black in color, whereas *L. rubidus* has a distinct copper-colored streak. After identification, all *Laricobius* beetles were stored in labeled vials filled with ethanol for long-term storage.

To determine how many of the *L. nigrinus* caught under release trees were due to egg releases and not a previously established population, it was important to find a variable that could explain the variability in recovery based on the size of the tree. Because the trees were different sizes and could therefore harbor different densities of field *Laricobius* populations, measurements for three different tree variables (height, volume, and number of branches directly over the tent) were taken to evaluate which would be the best for standardization.

Analyses

Linear regression was used to determine the relationship between *L. nigrinus* catches and the different tree characteristics to determine which had the strongest correlation and could be used to standardize the catch data. A non-parametric t-test (Mann-Whitney) compared the average number of *L. nigrinus* between control and release trees. All calculations were completed in Excel and R (R Core Team 2020).

Figure 3. Two soil emergence tents used to recover adult *Laricobius nigrinus*, placed underneath a hemlock tree where eggs had been previously deployed.

CHAPTER 3: RESULTS

Section 3.1: *Laricobius nigrinus* oviposition behavior

A total of 791 HWA ovisacs were dissected on the 48 hemlock twigs in order to find the number of *L. nigrinus* eggs per ovisac per mating pair density. One of the 16 cages (with four mating pairs) had no oviposition from *Laricobius* females during the 18 days this experiment was conducted and was therefore excluded from data analysis; it is unclear what factors led to this outlier. *Laricobius* eggs were discovered in 22% of all available HWA ovisacs, with a sum of 275 *Laricobius* eggs found.

In the cages where only one female was present, the average number of *Laricobius* eggs per oviposited ovisac on each twig was 1.30 (± 0.08 SE) eggs, indicating that individual females can lay more than one egg per HWA ovisac (Table 1). All four single females had at least one instance where they laid more than one egg per HWA ovisac, with the maximum number of *L. nigrinus* eggs per ovisac to be four. However, the number of *Laricobius* eggs laid per HWA ovisac did not increase with the number of mating pairs in the cage ($p = 0.59$, d.f = 14, $R^2 = 0.02$; Fig. 4). In fact, the average number of *Laricobius* eggs laid per HWA ovisac by females was practically the same whether there was one female or eight in the cage (Table 1). Interestingly, the average number of eggs laid per female *Laricobius* was found to decrease as the number of females per cage increased, indicating potential negative density-dependent feedback ($p = 0.08$, d.f. = 3, $R^2 = 0.69$) (Fig. 5). In addition, the proportion of HWA ovisacs with *Laricobius* eggs also did not significantly increase as the number of mating pairs increased ($p = 0.24$, d.f. = 14) (Fig. 6; Table 1). Lastly, there was a marginally significant positive linear relationship between the number of HWA ovisacs available and the number of *Laricobius* eggs laid; the more HWA ovisacs available, the more HWA ovisacs that had at least one *Laricobius* egg, regardless of the number of females that it shared a cage with (p = 0.10, R^2 = 0.20) (Fig. 7).

Table 1. Summary statistics regarding *Laricobius nigrinus* oviposition behavior under lab conditions based on the number of mating pairs included in experimental cages.

No. of Females	Average number of *Laricobius* eggs ovisac^{-1} twig^{-1} cage^{-1} (± SE)	Average proportion of ovisacs twig cage^{-1} (± SE)	Average number of *Laricobius* eggs female^{-1} cage^{-1} (± SE)
1	1.30 (± 0.08)	0.18 (± 0.05)	1.87 (± 0.97)
2	1.74 (± 0.20)	0.29 (± 0.03)	2.46 (± 0.42)
4	1.61 (± 0.08)	0.29 (± 0.05)	1.22 (± 0.26)
8	1.35 (± 0.19)	0.17 (± 0.03)	0.27 (± 0.07)

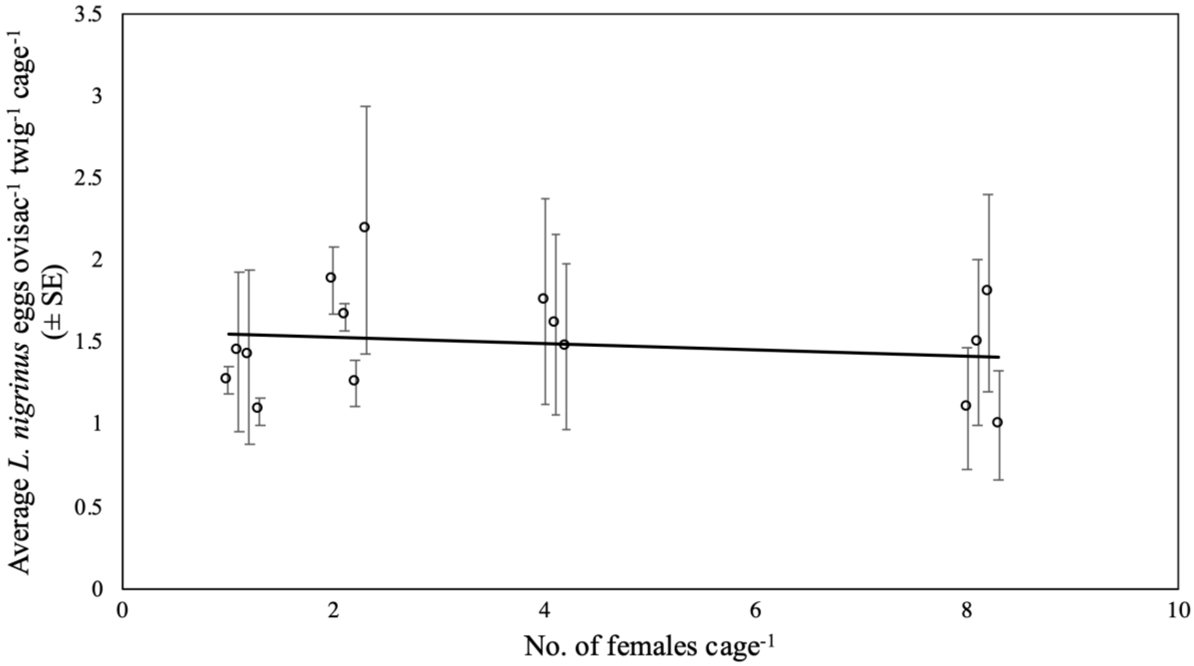

Figure 4. Relationship between the average (± SE) number of *Laricobius nigrinus* eggs laid per oviposited hemlock woolly adelgid ovisac per twig per cage and the number of female *L. nigrinus* in that cage (p = 0.59, d.f = 14, R^2 = 0.02).

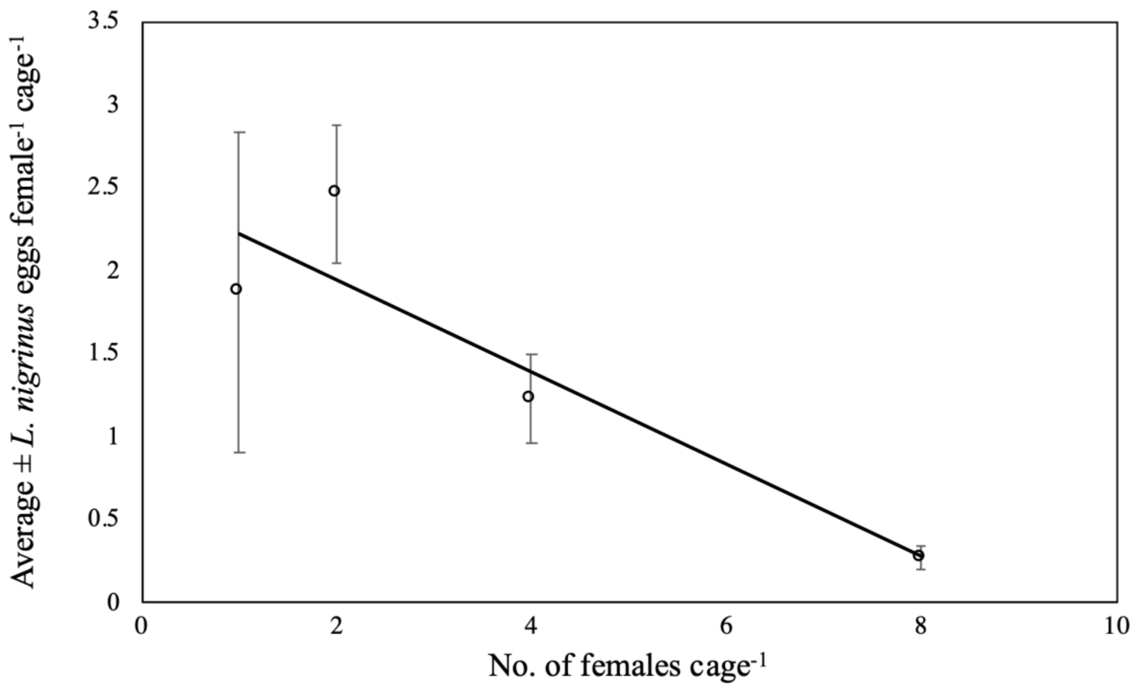

Figure 5. Negative relationship between the average (±SE) number of *Laricobius nigrinus* eggs laid per female and the number of female *L. nigrinus* in that cage (p = 0.08, d.f. = 3, R^2 = 0.69).

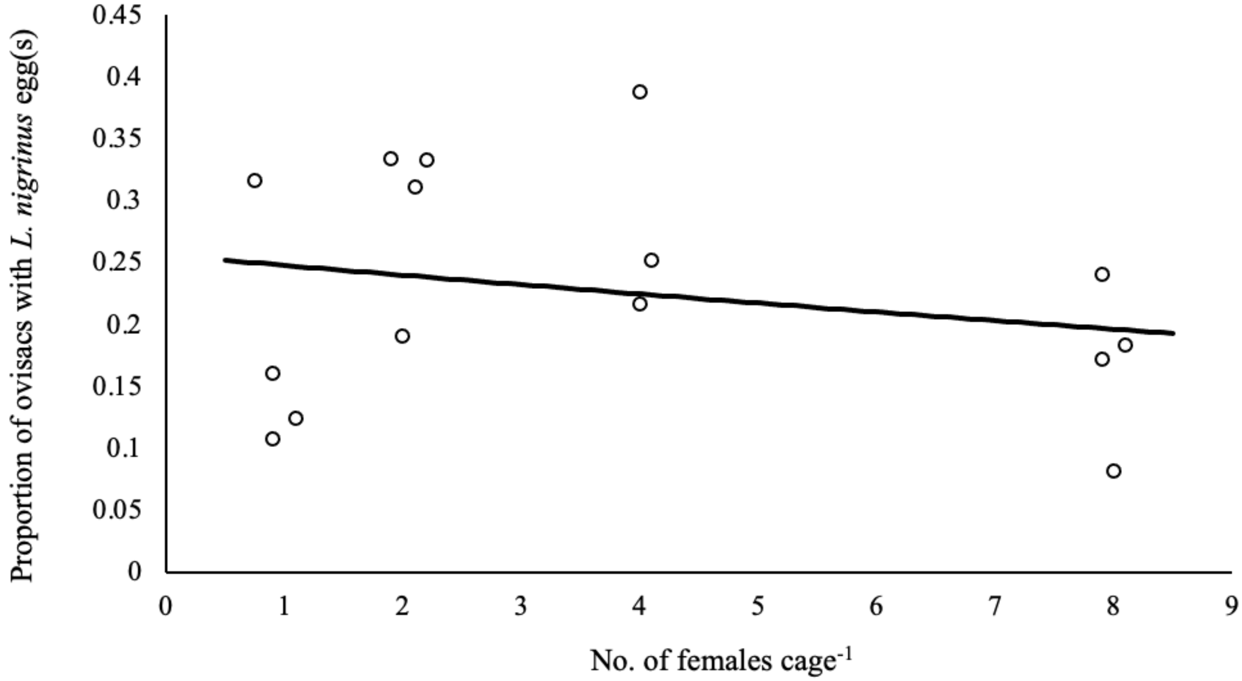

Figure 6. Relationship between the proportion of hemlock woolly adelgid ovisacs with *Laricobius nigrinus* egg(s) and the number of female *L. nigrinus* in that cage (p = 0.24, d.f. = 14).

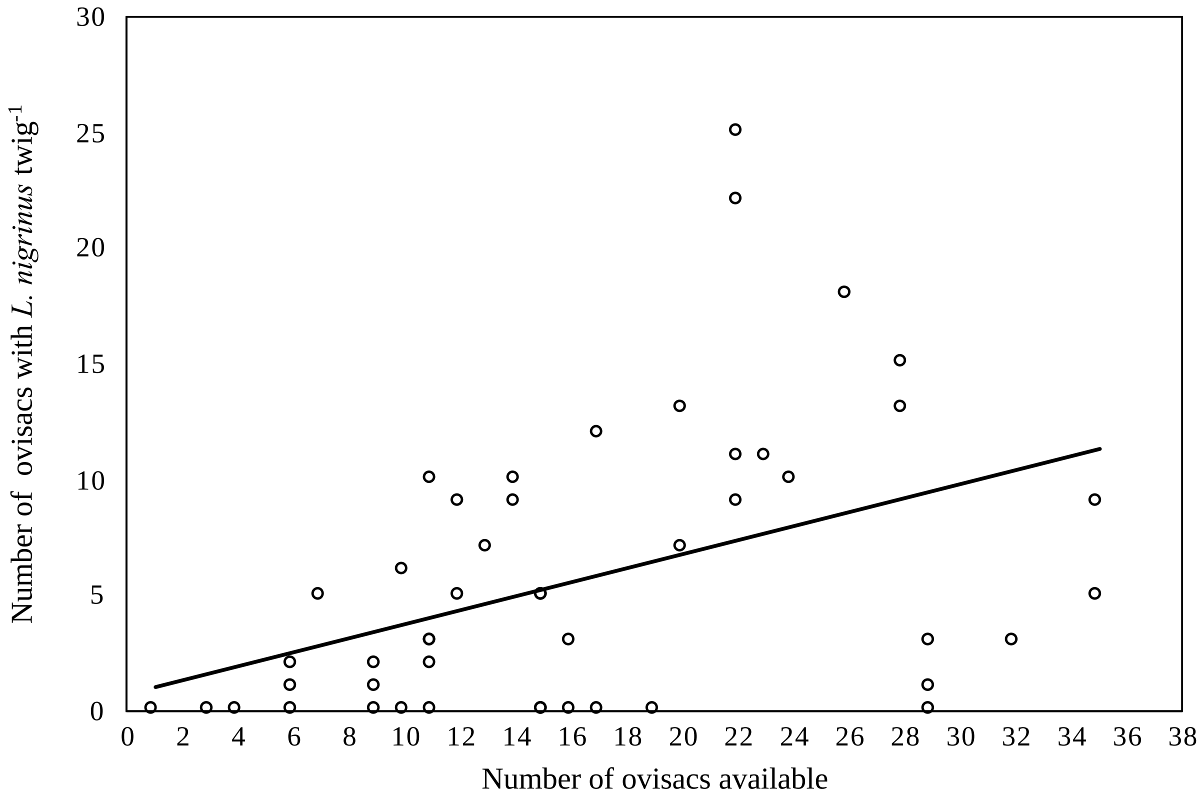

Figure 7. Positive relationship between the number of hemlock woolly adelgid ovisacs with *Laricobius nigrinus* egg(s) and the number of ovisacs available (p = 0.10, R^2 = 0.20).

Section 3.2: *Laricobius nigrinus* egg releases

A total of 277 *L. nigrinus* adults were recovered during the fall of 2020 from the 16 emergence tents located underneath release and control trees. Emergences began in early October, with the majority of adult *Laricobius* (74%) recovered during a single emergence event that occurred over a three-week period from mid-October through early November (Table 2; Fig. 8). The eight release tents caught an average of ~30 *L. nigrinus*, with three of the tents surrounding one of the release trees catching as many as 120 *L. nigrinus*. Some *L. nigrinus* adults emerged from tents under control trees confirming that there was a baseline population already in the stand prior to the experimental egg releases.

When looking at control trees and release trees, traditional measures such as tree height ($p = 0.68$) and volume ($p = 0.43$) were not drivers of emergence frequencies. However, the number of hemlock branches directly over each control tent was found to be significantly correlated with the number of *L. nigrinus* recovered per tent (Fig. 9, $p = 0.04$). In fact, the relationship between the number of branches over each tent and the number of *L. nigrinus* recovered per tent was similar regardless of treatment ($p = 0.009$), suggesting that this is a good predictor of beetle recovery (Fig. 9). It was also found that there was no significant difference when examining the effect of beetle catches as a function of branch density between release and control trees (i.e., similar slopes; $p = 0.96$; Fig. 9), indicating that differences in y-intercepts are the result of treatment (egg releases).

After standardizing trap catches to the per branch level, there was significantly more *L. nigrinus* per tent under trees which received egg releases compared to the control trees which did not (Fig. 10, $p = 0.002$). For every one *L. nigrinus* caught under the control trees, there were five caught under release trees (Fig. 10). However, due to a small sample size of release trees ($n = 3$), the recovery rate based on the estimated number of eggs released was highly variable by tree (29-93%).

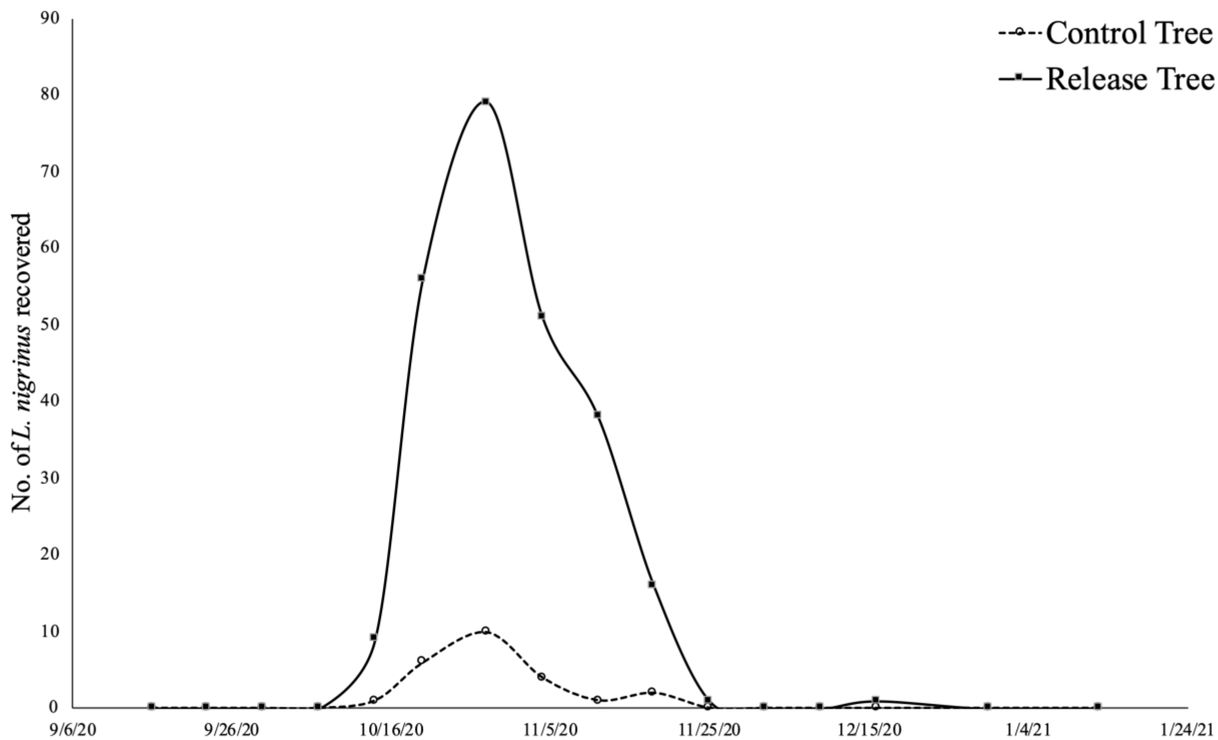

Figure 8. Emergence timeline of *Laricobius nigrinus* adults collected over a five-month period in Cashiers, NC between September 2020 and January 2021. Solid line represents beetle catches using soil emergence traps placed under three eastern hemlock trees that received *L. nigrinus* eggs in March 2020; dotted line represents catches from under three hemlock trees that did not receive egg releases.

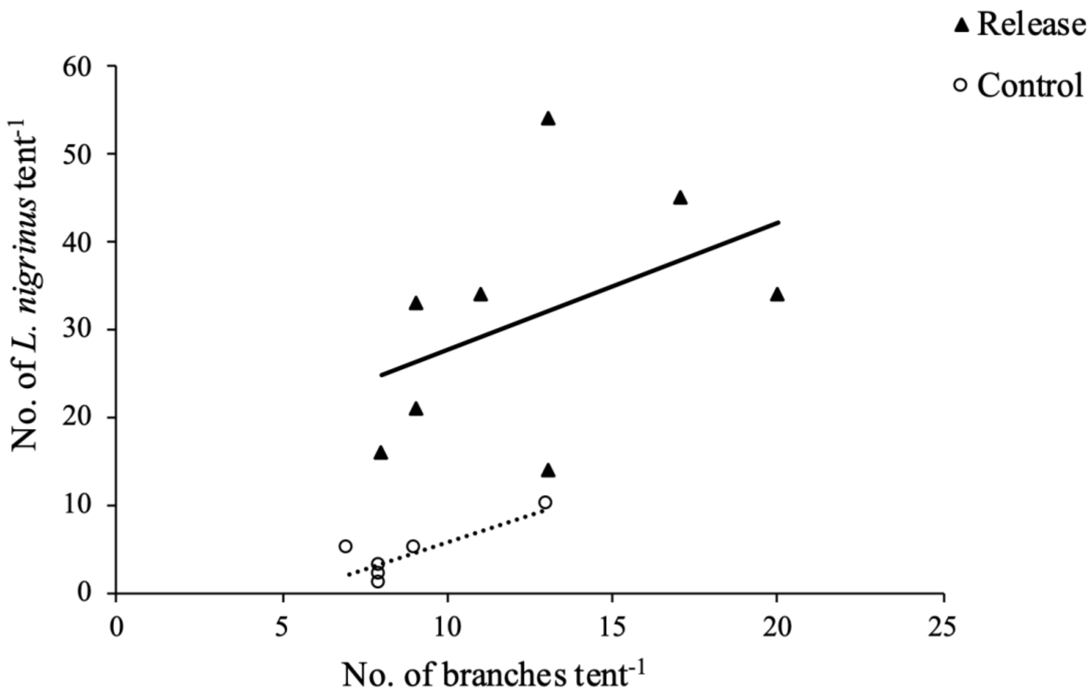

Figure 9. Relationship between the number of *Laricobius nigrinus* adults recovered per tent and the number of branches per tent at control and release trees. Black triangles represent beetles recovered in soil emergence tents underneath three eastern hemlock trees that received *L. nigrinus* eggs; open circles represent beetles recovered from under three hemlock trees that did not receive egg releases.

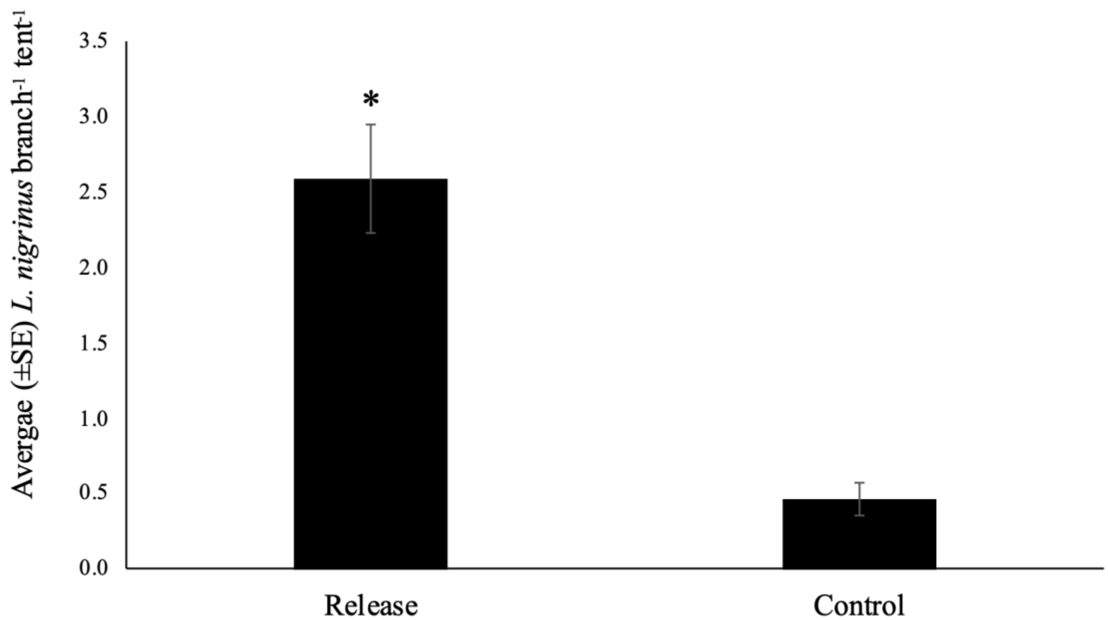

Figure 10. Average (± SE) number of *Laricobius nigrinus* adults per branch recovered per tent in both release trees that received *L. nigrinus* eggs and control trees that did not receive egg releases, * $p < 0.05$.

Table 2. Summary data regarding both release and control trees including the number of *Laricobius nigrinus* adults recovered.

Tree	Treatment	Tree height (m)	Volume (m^3)	No. of branches	No. of tents	No. of *L. nigrinus* recovered	Estimated eggs released
ET1	Release Tree	4.57	0.27	33	3	121	78
ET2	Release Tree	2.44	0.05	50	3	93	76
ET3	Release Tree	1.83	0.01	17	2	37	76
CT1	Control Tree	2.74	0.05	23	3	8	-
CT3	Control Tree	2.29	0.01	30	3	18	-

CHAPTER 4: DISCUSSION

The occurrence of multiple *L. nigrinus* eggs in a single HWA ovisac observed in this study could be evidence of intraspecific competition, with the total number of ovisacs available serving as the limited food resource for the larvae when they hatch. However, multiple oviposition was present in all cages containing only a single female and the number of *L. nigrinus* eggs per ovisac did not increase with the number of females per cage. Laying multiple eggs is costly for females, especially when considering that the first larvae to hatch will feed on the other *Laricobius* eggs as well as HWA eggs (Flowers et al. 2005). Alternatively, another explanation could be that this is a strategy to increase their fitness by ensuring enough food is available for at least one offspring to survive to adulthood. Increasing the number of eggs laid at one time in other insects has been proposed as a strategy to dilute predation or to increase lifetime reproductive success (Tallamy 2005). Some beetles (e.g. passalids) produce trophic eggs that function as a specialized food supply that delivers additional nutrients to larvae (Ento et al. 2008). Because there is no current evidence that *Laricobius* lay trophic eggs or of egg cannibalism by larvae, it would be interesting to look further at the multiple eggs laid by a single female in one HWA ovisac to see if they are all viable.

It was expected that as the number of females per cage increased that there would be an increase in the number of eggs laid, but this study found that there was actually a negative relationship between the average number of females and their average amount of eggs deposited into HWA ovisacs. The high-density of adult beetles could be leading to fewer total eggs laid as a result of intraspecific competition for the ovisacs, for each *Laricobius* egg (Than et al. 2020). This may suggest that rearing facilities could potentially reduce the number of mating pairs per cage and still receive similar numbers of beetle larvae, or increase the number of larvae by using the same number of mating pairs distributed across more cages. There is a potential that artificial environmental conditions created in a lab setting may affect *Laricobius* oviposition behavior and that this pattern may not occur in natural populations.

Although the overall proportion of HWA ovisacs with *Laricobius* eggs did not increase with the number of females per cage in my study, other research has shown that the number of eggs can be influenced by the number of females per branch (Lamb et al. 2006). Only between one and three pairs of *L. nigrinus* females were examined, however, so it is possible that three pairs may be below the threshold that would impact a density-dependent relationship (Lamb et al. 2006). The difference in eggs laid between two mating pairs and three mating pairs was only significant in March when oviposition was at its peak (Lamb et al. 2006). Considering this study only compared eggs laid by different numbers of mating pairs over one 18-day period, there could be slight variations in the relationships if observed for a longer time.

There was a significant relationship between the number of HWA ovisacs available and the number of *Laricobius* eggs laid; the more ovisacs available, the more ovisacs that had at least one *Laricobius* egg. As a biological control agent, *L. nigrinus* has high host specificity with HWA, relying on the population of their prey to continue to reproduce and survive. Experiments comparing oviposition of *L. nigrinus* to high and low densities of HWA found a rise in egg production with an increase in available prey (Lamb et al. 2005). This indicates that *Laricobius* are less likely to lay as many eggs if food is scarce. This information is helpful to scientists that are rearing generations of *Laricobius* in the lab, to prioritize food availability in their protocols for higher returns.

To ensure efficient inoculation of branches, it is important to understand the egg-laying behaviors of female *L. nigrinus.* Future experiments could include the species *L. osakensis*, which has also started to be used as a biocontrol agent against HWA. The lifecycle of *L. osakensis* is identical to that of *L. nigrinus*, so this experiment could be repeated using this protocol. Additionally, it may be beneficial to repeat this experiment with more than four groups of mating pairs, to try and get a more accurate estimate of when the increased density of *L. nigrinus* begins to negatively impact egg production. Overall, the lack of information on the oviposition behavior of *Laricobius* hampers the determination of the best inoculation density needed to ensure the highest number of viable beetle larvae possible.

This study also focused on the efficacy of *L. nigrinus* egg releases and is the first to quantify the method for establishing *L. nigrinus* populations for control of HWA. A new method of recovery, additional information regarding the biology of emergence, and an observed increase of *L. nigrinus* on release trees were documented. While an exact efficacy rate (ratio of adult beetles recovered to beetle eggs deployed) could not be determined, this research provides strong evidence that the egg stage is effective for *L. nigrinus* releases.

Based on temporal data gathered from emergence tents, there was a single mass emergence of beetles from mid-October to early-November during the fall of 2020, with 74% of adults recovered in a 3-week period (Fig. 8). This peak in adult emergence following aestivation is similar to emergence timelines observed in rearing facilities (Salom et al. 2012). The monitoring and detection of natural enemies is an important part of any biological control program. The use of soil emergence tents can reduce the amount of time and money spent looking for *L. nigrinus* establishment and have higher recovery rates compared to traditional collection methods, such as the beat sheet method where recovery of adult *Laricobius* is notoriously low (Jubb et al. 2021). This is partially due to the beetle's tendency to disperse vertically within trees, and beat sheeting is limited to the lower canopy of trees (Mausel et al. 2010). The branch clip method, another popular sampling technique, offers a higher recovery rate by quantifying larval densities on samples of HWA-infested branches but is costly because *Laricobius* larvae need to be genetically analyzed to determine species (Jubb et al. 2021).

Based on this experiment, we have developed an effective detection method for *L. nigrinus* by using soil emergence tents. It is recommended that multiple tents should be placed directly below HWA-infested branches about two weeks before estimated emergence. Although this research was conducted in North Carolina, these results can be extended to entire hemlock range in eastern North America (extending from northern Georgia to Canada). In western North Carolina, emergence occurs in mid-October, but at more northernly latitudes this could be even earlier as the temperature has a significant influence on how long *Laricobius* adults remain in the soil (Lamb et al. 2007). One of the control trees had no recovery of any *Laricobius*, likely a result of a low natural population of *Laricobius* in the stand in addition to the low sample size in this experiment. Therefore, it is recommended that several trees in a stand are sampled to account for variability among trees. When preparing the site for emergence traps, vegetation where traps will be placed should be trimmed to ensure that beetles fly up into the collection bottles instead of staying on plants and to allow the tent to lay flush with the ground. A blend of antifreeze and water can be used to fill the collection bottle, but if attracting wildlife is a concern, then plain water can replace the antifreeze mixture. It is also suggested that samples are collected weekly or bi-weekly to maintain the quality of the specimens. Based on emergence timelines, field teams looking for *Laricobius* establishment only need to sample during the few weeks where the majority of beetles are emerging, instead of sampling throughout the entire winter season. In future experiments, a degree-day model may provide more insight into a more precise range of peak emergence.

There are a few considerations regarding using tents for monitoring. Soil emergence tents are more expensive than beat sheets, with each individual tent costing approximately $300, but they are reusable and would take less time for technicians to sample. The tents used in this study were relatively sturdy against weather, but there were at least two instances where the tents were damaged by bears, so proximity to large wildlife should be considered. Lastly, all *Laricobius* beetles recovered can be broadly identified to species by morphology, but if you are interested in determining hybrid populations, DNA analyses would need to be completed. Regardless, soil emergence tents in this study have shown to be a reliable method in determining the presence and estimating the density of *Laricobius* in a hemlock stand.

Abundance of *L. nigrinus* adults per tent increased with the number of hemlock branches above each tent (Fig. 9), likely because more branches means that there is potentially more HWA ovisacs available for *Laricobius* larvae to feed upon. While branches are not an exact unit of measurement, only a single surveyor counted the total number of branches above each tent in order to reduce potential bias in the counts. This demonstrates that the number of branches on various areas of the tree should be considered when choosing a location for egg releases or when trying to detect *Laricobius* populations.

After standardizing per branch, there were significantly more *L. nigrinus* captured per tent on the trees that received egg releases compared to the trees that did not (Fig. 10). For every one *L. nigrinus* caught on the control trees, there were five caught on release trees. However, there was a lot of variability in recovery rates by tree, with the range of calculated recovery rates being 29-93% based on the estimated egg counts. Some of this variability could possibly be explained by the differences in the physical environment surrounding the trees. For instance, one release tree that had the lowest recovery rate had moss covering the soil below branches with *L. nigrinus* eggs as opposed to other release trees that had leaf litter completely covering the soil. It is assumed that *Laricobius* are able to inhabit the same environments that support hemlock growth as long as HWA is available, thus future studies should study the effect of soil cover and composition on the mortality rate of *Laricobius* in case that is another variable that should be considered when selecting trees to implement egg-releases on. There were also differences in the amount of sunlight each release tree received, which could have an impact on HWA populations on the trees (Miniat et al. 2020). Another potential explanation for the variation in recovery rates is that the number of eggs released was an estimate based on a subsample; there is a chance that release trees received more or less *Laricobius* eggs than estimated.

In order to achieve a better estimate of *L. nigrinus* recovery based on released eggs, a more controlled experiment would be needed involving a larger sample of similar-sized hemlock trees that allow for 360˚ access to branches and where oviposition from natural *Laricobius* populations could be prevented or better estimated. For example, if the number of branches are similar on each side of a tree, *L. nigrinus* could be released on half of the tree with soil emergence tents encircling the entire tree, allowing the other side of the tree where beetles were not released to act as the control. This would allow each tree to have both treatments and reduce the variability that could exist between trees.

In conclusion, this research provides a new cost-effective and less labor-intensive method to release *L. nigrinus* in the field in addition to an improved method to monitor and detect established *Laricobius* populations. While an exact efficacy rate cannot be determined at this time, there is strong evidence that egg releases work and should be considered in future HWA biological control management plans. The difference in *L. nigrinus* recovery rates between release and control trees in this study are most likely the result of egg releases, with five times as many beetles recovered from release trees relative to control trees. Calculated recovery rates were highly variable, but even with the most conservative estimate of recovery (29%), rates are similar to the success rates seen in rearing labs (25-35%), indicating that this new protocol could save current rearing labs both time and money. Simultaneously, higher estimates of recovery indicate that this method could increase the number of *L. nigrinus* established in the field compared to traditional release methods and improve the overall chances of combating an invasive forest pest.

This study provides strong evidence that *L. nigrinus* egg releases work, with eggs successfully hatching into larvae and developing into adults in the field. Reducing the number of mating pairs per cage does not affect beetle larval counts, thus rearing labs could use fewer *Laricobius* mating pairs per cage and still receive similar numbers of beetle larvae. This new information can be utilized by lab managers as well as field ecologists that work with biological control of HWA. Overall, the protocol described here reduces expenses, time, and potentially increases *L. nigrinus* in stands compared to current methods; this could have long-term benefits for the control of HWA. These results can be extended to entire hemlock range in eastern North America, providing new management tools for both domestic and international forest health professionals.

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