Laboratory rearing, establishment, subterranean survivorship, and thermal requirements of *Laricobius* spp. (Coleoptera: Derodontidae), biological control agents released for *Adelges tsugae* (Hemiptera: Adelgidae)

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Abstract: (Academic)

The hemlock woolly adelgid (HWA) (*Adelges tsugae* [Annand]) (Hemiptera: Adelgidae) is a non-native lethal pest to eastern hemlock (*Tsuga canadensis* [L.] (Pinales: Pinaceae), and Carolina hemlock (*Tsuga caroliniana* [Engelmann]) (Pinales: Pinaceae). In the early 2000's, a robust biological control initiative using *Laricobius* spp. (Insecta: Coleoptera) was launched with the goal of decreasing HWA's impact to hemlock and the associated ecosystems on landscape level. Since then, two *Laricobius* agents, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) have been evaluated, approved, and released. The production and subsequent release of these agents has largely occurred through mass rearing efforts by Virginia Tech. A descriptive analysis of these rearing and release data over the past 16 years at Virginia Tech revealed that the mass production of these agents has been consistently limited by: 1) excessively high rates of subterranean mortality and 2) early subterranean emergence. Very little is known about the subterranean life cycle of these species in the field in terms of survivorship and timing of emergence. Additionally, the thermal limits and rates of development are unknown for *L. osakensis* and were thus further investigated. In 2019, surveys within the urban environment in two counties in southwest Virginia were conducted to determine if these agents have dispersed from their original release location. *Laricobius nigrinus* was documented to have established outside of release sites in 100 and 75% of the surveyed grids containing HWA infested hemlocks. The establishments of these species in easily accessible locations allowed for the use of field-caught larvae, in addition to laboratory-produced larvae, for experiments.

Experiments were conducted in 2019-2020 to determine the overall subterranean survivorship in relation to site condition, differences in survivorship between field-caught vs. laboratory-reared, and the seasonal timing of emergence. We found that the average field subterranean survivorship (17.1 ± 0.4%) was significantly less than the historical production of these agents in the laboratory (37.5 ± 13.6%). We found that as soil pH and organic matter depth increased, survivorship decreased. While the majority of emergence occurred as expected in the fall, a surprising amount of emergence also occurred in spring and summer. The rate of development and the minimum threshold temperature for each life stage were determined through constant temperature experiments, and were used in the construction of a phenological forecast model. *Laricobius osakensis* minimum threshold temperature (3.6°) from egg to adult was similar to that of other *Laricobius* spp., but completed development at higher a constant temperature (22°C). Our results suggest *L. osakensis* can handle the same colder climates as *L. nigrinus*, but may be better adapted for warmer locations than *L. nigrinus*.

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Jeremiah Robert Foley, IV

Abstract: (General Audience)

Eastern and Carolina hemlocks are long lived, shade tolerant evergreen tree species that support a unique ecological assemblage of plants and animals. However, following the accidental introduction of a highly invasive aphid-like insect pest first found in the 1950's, known as the hemlock woolly adelgid (HWA), significant mortality of hemlocks in the eastern U.S. was eventually observed. In order to mitigate this pest's impact and to prevent the further collapse of this unique ecological system, a robust management program was initiated. Today, the primary management strategy to reduce HWA's deleterious effects is through the use of chemical and biological treatments. Biological treatments in the form of insect biological control agents, have focused on the use of two closely related beetle species in the genus *Laricobius* spp. These species were imported from their respective native ranges, biologically evaluated, and approved for release by federal and state agencies. Since then, the beetles have been mass-produced by multiple governmental and academic agencies and released on infested landscape on mostly public land. Key aspects of these species' biology in terms of their population patterns, development, distribution capabilities, and potential supplementary diet remains unknown. Therefore, investigations into these aforementioned aspects were conducted from 2018-2021 in both laboratory and field experiments. In order to supply beetle larvae for field experiments and to better understand how well these predatory beetles were establishing across the landscape, we investigated heavily urbanized areas for their presence. We found that *L. nigrinus* was established outside of release locations, in 100 and 75% of the surveyed grids containing HWA in southwest Virginia. *Laricobius* spp. spend a significant amount of their lives in a presumed dormant state beneath the soil during the summer months. Observations over the past 17 yrs. of rearing *Laricobius* spp. revealed relatively high rates of subterranean mortality and subterranean early emergence, both of which are substantial mortality factors. Through field experiments, the survivorship of these beneficial beetles was found to be 17.1 ± 0.4% and their seasonal emergence was not limited to fall.Comparing the average survivorship from our field study (17.1 ± 0.4%) to that of the past 17 yrs. of laboratory production (37.5 ± 13.6%), there was significantly less survivorship. The developmental and phenological biology of one of the two *Laricobius* spp., *L. osakensis* was determined and found to be similar to that of other beetles. However, *L. osakensis* was able to complete development at 22°C, the highest developmental temperature recorded for any *Laricobius* spp. The similar phenological biology of these two predators will allow for precise larval sampling as a metric for establishment. Results and conclusions from these data have provided further knowledge to scientists, land managers, and public stakeholders regarding the benefits of *Laricobius* spp.

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I would like to start off by acknowledging…myself. My journey from high school dropout to pizza maker to father to PhD candidate is unique and I am proud of all I have accomplished. Along the way, however, I was guided by countless people who've had a large impact on who I am today. A few individuals specifically, I can point to who truly inspired me, trusted me, and were there for me every step of the way.

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What good would an acknowledgements section be without a, "look mom, I did it" shout out. My mom showed me the importance of communicating through the science of art. She was an elementary school art teacher for, what seemed to be, all of Fort Lauderdale and routinely unofficially fostered every neighborhood kid. My mother always provided unconditional love and support but also had expectations for me which ensured I kept moving forward after dropping out of high school. She recently told my wife (Casey) that "Growing up, we couldn't keep him in school" to which Casey immediately replied "Yeah…now we can't get him out."

To Casey, this accomplishment is as much yours as it is mine. You are my best friend and over the past ten years, we have both grown in incalculable ways. You have motivated me to not only stay in school but pushed me to the highest echelons of academic achievements. Ten years and two children later, you are still the highlight of my life and I can't wait to make more pizzas with you and the boys.

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Finally, I dedicate this work to my first best friend and oldest sister Jessica Foley. Not a single day goes by that we are not thinking of you. You would be so proud of your daughter. We love you!

YOU NEVER SAID I'M LEAVING
By: Toni Kane
You never said I'm leaving
You never said goodbye
You were gone before we knew it
and only God knew why

A million times I needed you
A million times I cried
if love alone could have saved you
you never would have died

In life I loved you dearly
In death I love you still
In our hearts I hold a place
that only you can fill

It broke my heart to lose you
but you didn't go alone
as part of me went with you
the day God took you home.

Attributions:

Several colleagues aided in the data collection, analysis, and writing behind the following chapters presented as part of this dissertation. A brief description of their contributions is included here.

Chapter 2: Historical assessment and analysis of the mass production of *Laricobius* spp. (Coleoptera: Derodontidae), biological control agents for the hemlock woolly adelgid, at Virginia Tech

Carrie S. Jubb is the manager of the Virginia Tech mass rearing laboratory and a recent Masters student in the Dept. of Entomology at Virginia Tech. Carrie was responsible for all the data collection and conceptualization that allowed for this publication to take place. Carrie also assisted in writing of the original draft.

D. Austin Cole is a Ph.D. student in the Dept. of Statistic at Virginia Tech. Austin assisted in data curation, conceptualization, and analysis.

David Mausel, Ph.D. is a Regional Entomologist (Eastern Region) for the United Stated Forest Service and contributed to the data collection, conceptualization, and writing of the original draft.

Ashley L. Galloway, Ph.D. is an Entomologist and instructor at Roane State Community College. Dr. Galloway assisted in conceptualization, data collection, and writing of the original draft.

Rachel Brooks, Ph.D. is a Washington state research pathologist and assisted visualization, writing and reviewing of this manuscript.

Scott M. Salom, Ph.D. is a professor in the Department of Entomology, Virginia Tech. Dr. Salom provided funding, conceptualization, and writing of the original draft.

Chapter 3: The establishment and distribution of *Laricobius* spp. (Coleoptera: Derodontidae), a predator of hemlock woolly adelgid, within the urban environment in two localities in southwest Virginia

Thomas J. McAvoy is a senior laboratory specialist in the Department of Entomology at Virginia Tech. Mr. McAvoy assisted in conceptualization, experimental design, data collection, and provided editorial suggestions.

Seth Dorman, Ph.D. is a postdoctoral researcher at North Carolina State University and assisted in conceptualization, data analysis, and writing of the original draft.

Kyle Bekelja is a Ph.D. student in the Dept. of Entomology at Virginia Tech and assisted with experimental design, data collection, and writing of the original draft.

Timothy J. Kring, Ph.D is the Head of the Dept. of Entomology at Virginia Tech and assisted in experimental design and contributed editorial comments.

Scott M. Salom, Ph.D. is a professor in the Department of Entomology, Virginia Tech. Dr. Salom provided funding and writing of the original draft.

Chapter 4: Subterranean survivorship and seasonal emergence of *Laricobius* spp. (Coleoptera: Derodontidae), biological control agents for the hemlock woolly adelgid.

Thomas J. McAvoy is a senior laboratory specialist in the Department of Entomology at Virginia Tech. Mr. McAvoy was a co-author on this paper, and assisted in conceptualization, experimental design, and data collection.

Christopher Grubb is a Ph.D student at Virginia Tech and is a co-author on this publication, and assisted in data analysis.

Brian Strahm, Ph.D. is a professor in the Department of Forest Resources and Environmental Conservation at Virginia Tech and is a co-author on this publication, and assisted in experimental design and contributed editorial comments.

Albert E. Mayfield III, Ph.D. is a Research Entomologist for the USDA Forest Service and is a co-author on this publication, and assisted in experimental design and writing of the original draft.

Scott M. Salom, Ph.D. is a professor in the Department of Entomology, Virginia Tech. He provided funding as the principal investigator of the grant that supported this research. Dr. Salom is a co-author on this publication, and assisted in the experimental design and contributed to the writing of the original draft.

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Introduction: *Laricobius* spp. (Coleoptera): biological control agents of Adelgidae (Hemiptera) over the past century

The family Derodontidae LeConte (Insecta: Coleoptera), commonly known as the tooth-necked fungus beetles, are a primitive family of beetles that encompasses four genera: *Derodontus, Nothoderodontus, Peltastica,* and *Laricobius* (Lawrence and Hlavac 1979). All members within the family Derodontidae consume fungi, with the assumed exception of the genus *Laricobius* Rosenhauer. *Laricobius* are known to be specialist predators of Adelgidae (Insecta: Hemiptera) (Brown and Clark 1960, Lawrence and Hlavac 1979) and have been used as biological control agents across multiple systems for the past 100 years.

In 1847, in northern Europe, *Laricobius erichsonii* Rosenhauer (Coleoptera: Derodontidae) was first described and its predatory capabilities recognized. Between the 1930s to the 1950s, *L. erichsonii* was imported and released ("liberated") in North America against the highly invasive insect, balsam woolly adelgid (BWA), *Adelges piceae* Ratz (Clark and Brown 1958, Smith 1958). Since these initial releases, confusion on whether this species has fully established remains due to morphological similarities to its native North American congener, *Laricobius rubidus* LeConte (Clark and Brown 1958). Although *L. rubidus* is known to feed primarily on pine bark adelgid (*Pinus strobi*) Hartig, it has been documented to feed on other adelgid species, including BWA (Clark and Brown 1958) and HWA (Zilahi-Balogh et al. 2002, 2005).

The pursuit of other *Laricobius* agents began 50 years later, in the late 1990's, following the adventive arrival of one of North America's most destructive forest insects, the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Lovett et al., 2016). HWA is an exotic invasive pest of eastern hemlock (*Tsuga canadensis*) (L.) Carrière and Carolina hemlock (*Tsuga caroliniana*) Engelmann, in their respective distributions in the eastern United States (Fig. 1). HWA is thought to have been introduced from Japan via the ornamental hemlock nursery prior to its discovery in the early 1950's (Havill et al. 2006). It wasn't until 30 years later that HWA's impact and population density reached notable levels. Now, over 70 years since its discovery, HWA has spread throughout the entire Appalachian Mountains and up into coastal areas of Canada, and also to the Great Lakes Region in MI and Ontario (Kantola et al., 2019, Virginia Tech, 2021).

Within its introduced range of eastern North America, HWA has few natural enemies; all of which are more-or-less considered generalist and whose predatory impact is insufficient in maintaining HWA infestations at manageable levels (Montgomery and Lyon 1995, Wallace and Hain 2000). The pursuit of non-native, classical biological control agents began in the early 1990s and has continued to present day, with varying rates of establishment for each prospective species. In total, eight insect species, in four genera have been evaluated, approved, and released with Coleoptera accounting for the majority (75%). Establishment has been confirmed for three of the eight species (38%).

The first agent released was a ladybeetle, *Sasajiscymnus tsugae* Sasaji & McClure (Coleoptera: Coccinellidae) from Japan. Following release, *S. tsugae* establishment has been confirmed in 2 of the 16 (12%) release states (Hakeem et al. 2011, Jones et al. 2014). The release of three more ladybeetles; *Scymnus sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae), *Scymnus ningshanensis* Yu and Yao (Coleoptera: Coccinellidae), and *Scymnus coniferarum* Abbot & Smith (Coleoptera: Coccinellidae) occurred in 2004, 2007, and 2015, respectively. While the exact number of states that received these ladybeetles is unclear, establishment has not been confirmed in any. Most recently, two dipteran species in the family Chamaemyiidae from western North America have been evaluated (Grubin et al. 2011, Motley et al. 2017) and are currently being released in eastern North America. Establishment has yet to be confirmed for these two species.

Of the agents that have been evaluated and approved for release against HWA, *Laricobius* spp. have disproportionately received the most attention and has arguably been the most successful. *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) were evaluated and subsequently approved and released beginning in 2003 and 2012, respectively (Zilahi-Balogh et al. 2002, Mausel et al. 2010, Vieira et al. 2011, Mooneyham et al. 2016). Establishment of *L. nigrinus* has occurred in 13 of the 15 (86%) release states (Mausel et al. 2010, Virginia Polytechnic Institute and State University 2021). *Laricobius nigrinus* continues to disperse and establish outside of release locations and most recently has been documented within the urban environment (Foley et al. 2019). *Laricobius osakensis* has been released in six states with establishment occurring in two (30%) (Mooneyham et al. 2016, Toland et al. 2018). It is currently unknown if *L. osakensis* is dispersing and establishing outside of the release locations.

Laricobius nigrinus has a well-documented phenological synchrony with HWA in their introduced range of eastern North America (Zilahi-Balogh et al. 2003a, Mausel et al, 2008). Although data are not currently available on the field phenology of *L. osakensis* in its introduced range of eastern North America, it is understood to be similar to that of *L. nigrinus* and HWA (Vieira et al. 2013, Mooneyham et al. 2016, Toland et al. 2018). Both

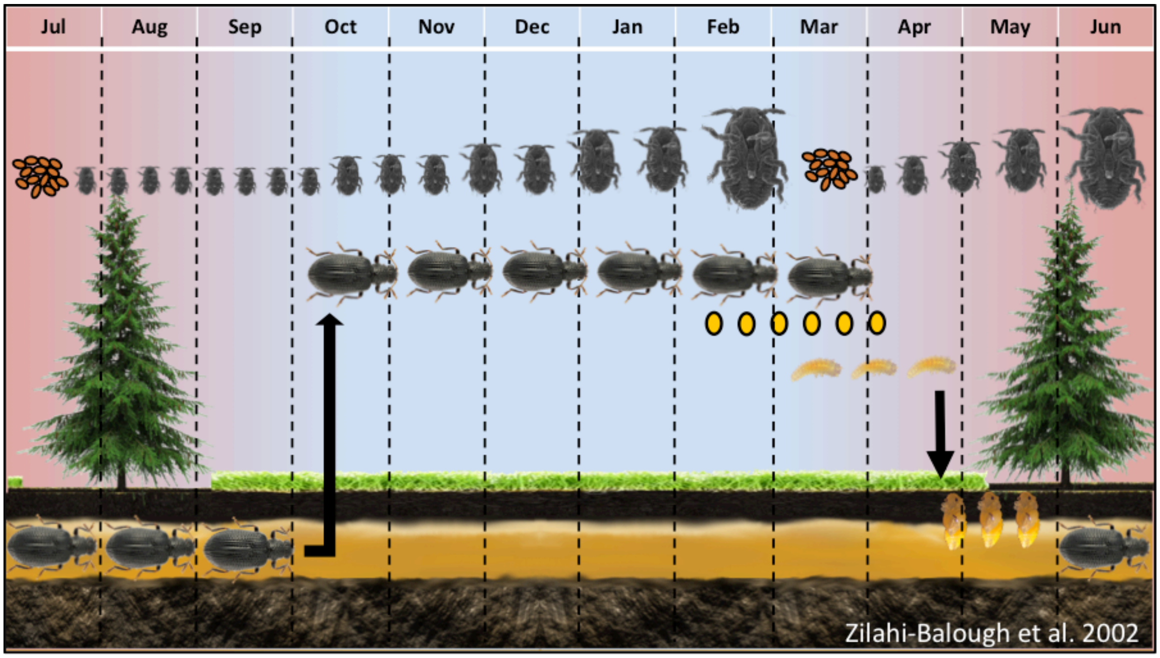
Laricobius spp. and HWA are active within their shared arboreal habitat during the cooler months from October to April (Figure 1). During the warmer months from May to September, *Laricobius* spp. are presumably dormant underneath hemlocks within the soil as adults (Salom et al. 2012, Wiggin et al. 2017), whereas, HWA is dormant as first instar nymphs at the base of hemlock needles (Salom et al. 2002). As HWA breaks its summer dormancy and starts developing, *Laricobius* spp. emerge from the soil, migrate to the canopy of hemlocks, and start feeding (Mausel et al. 2011, Arsenault et al. 2015).

Following the approval for the release of *Laricobius* spp., Virginia Tech and other state and federal agencies started mass-producing these agents with the goal of distributing them across the HWA infested landscape (Foley et al. 2021). As is the case for any rearing program, the ability to simulate environmental conditions (i.e., temperature, moisture, and habitat) and provide adequate nutrient requirements is crucial for the program's success (Leppla and Fisher 1989, Salom et al. 2002, Cohen and Cheah 2019). In order to rear *Laricobius* spp., we must mirror both the arboreal and subterranean life phases with respect to their seasonality, temperature, moisture, and nutrient requirements. Since their initial biological evaluation for the petition for release, many avenues of research have been pursued with the goal of further understanding how best to integrate these species into the larger HWA management strategy. However, despite the >285 peer-reviewed publications (Table 1.1) on *Laricobius* spp. over the past 50 years, many questions remain unanswered.

The purpose of this dissertation is to report on several studies aimed at further investigating how both *Laricobius* spp. released for HWA fit into an overall management strategy by understanding and analyzing:

1. The historical mass production of *Laricobius* spp. as biological control agents for the HWA at Virginia Tech.
2. The establishment and distribution of *Laricobius* spp. within the urban environment in two localities in southwest Virginia
3. The subterranean survivorship and seasonal emergence of *Laricobius* spp.
4. The temperature-dependent development and fecundity of *L. osakensis*

Figure 1. 1. Phenology of *Laricobius* spp. in relation to its host, *Adelges tsugae* (HWA). There are two generations of HWA per year. Sistens are present from July to early March. The progrediens are present from early March to the end of June. Adult *Laricobius* spp. are present in the subterranean habitat from April until October and the arboreal habitat from October until March. The color gradient represents the relative temperatures for the respective time of year (red = warm; blue = cold)



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Chapter 2: Historical assessment and analysis of the mass production of Laricobius spp. (Coleoptera: Derodontidae), biological control agents for the hemlock woolly adelgid, at Virginia Tech

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Abstract:

Laricobius nigrinus (Coleoptera: Derodontidae) Fender and *Laricobius osakensis* (Coleoptera: Derodontidae) Montgomery and Shiyake have been mass produced by Virginia Tech as biological control agents for the hemlock woolly adelgid (HWA), *Adelges tsugae* (Hemiptera: Adelgidae) Annand, for the past 15 and 9 yr, respectively. Herein, we describe modifications of our rearing procedures, trends and analyses in the overall production of these agents, and the redistribution of these agents for release to local and federal land managers. Based on these data, we have highlighted three major challenges to the rearing program: 1) high mortality during the subterranean portion of its life cycle (averaging 63% annually) reducing beetle production, 2) asynchrony in estivation emergence relative to the availability of their host HWA minimizing food availability, and 3) unintended field collections of *Laricobius* spp. larvae on HWA provided to lab-reared larvae complicating rearing procedures. We further highlight

corresponding avenues of research aimed at addressing each of these challenges to further improve *Laricobius* spp. production.

Introduction:

Adelges tsugae as a pest

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) is a non-native pest to eastern hemlocks, *Tsuga canadensis* L. (Pinales: Pinaceae), and Carolina hemlocks *Tsuga caroliniana* Engelmann (Pinales: Pinaceae). HWA was first observed in Richmond, Virginia in 1951 (Gouger 1971, Stoetzel 2002), and was presumably imported previously from Japan on ornamental hemlock nursery stock (Havill et al. 2006, Havill et al. 2016). HWA is native to Mainland China, Japan, Taiwan and western North America (Havill et al. 2006). *Adelges* spp. have a relatively complicated lifecycle that depends on the availability of a primary and secondary hosts to maintain sexual and asexual reproduction, respectively (Havill and Foottit 2007). Within the introduced range of eastern North America, HWA's primary host, tiger-tail spruce, *Picea torano* Voss (Siebold ex K. Koch) (Pinales: Pinaceae), is not present. The absence of tiger-tail spruce and the presence of HWA's secondary host, hemlock, has resulted in anholocyclic populations of HWA in its adventive range of eastern North America. HWA has two generations per year: 1) sistens and 2) progrediens. The sistens, or overwintering generation, is temporally the longest of the two. Sistens nymphs are present as aestivating first instars at the base of hemlock needles throughout summer and following the onset of cooler temperatures, start to develop through three more instars (McClure 1989, Salom et al. 2002, Zilahi-Balogh et al 2003a). Starting around February, HWA oviposition begins and the eggs of the next generation, the progredientes are laid. The shorter progrediens generation is present from March to late June.

Since its introduction, HWA has spread throughout much of the range of eastern and Carolina hemlocks and is currently established in 22 eastern states in the United States and in Nova Scotia, Canada (Kantola et al. 2019, Virginia Tech 2019). Hemlock mortality caused by HWA feeding can result in whole tree mortality, with larger trees succumbing to infestations more quickly (McClure 1991). Treatment options for managing HWA infestations vary in effectiveness, unwanted secondary environmental effects, and the temporal and spatial scales at which they can be applied (Steward et al. 1998, Silcox 2002, Havill et al. 2016, Mayfield et al. 2020). Of these, the principal tactics readily used are: 1) biological control agents, 2) chemical applications, 3) silvicultural applications, and 4) a combination of tactics through an integrated pest management (IPM) strategy (Mayfield et al. 2020). The emphasis of this manuscript will be on the use of biological control agents.

The mass production of Laricobius spp. as biological control agents for HWA

Laricobius spp. have received the most attention as biological control agents for HWA and are known to prey only on Adelgidae (Lawrence and Hlavac 1979, Zilahi-Balogh et al. 2002, Kok, et al. 2002, Havill and Foottit 2007). They have a univoltine life cycle, in which both the adults and larvae feed on adelgids (Zilahi-Balogh et al. 2002, Kok, et al. 2002, Vieira et al. 2011, Salom et al. 2012), exhibit significant rates of predation (Jubb et al. 2020a), and are associated with their host in both forested and urban environments (Mausel et al. 2010, Toland et al. 2018, Foley et al. 2019, Jubb et al. 2021.

The only *Laricobius* species endemic to eastern North America is *Laricobius rubidus* LeConte (Coleoptera: Derodontidae). The primary and preferred host of *L.*

rubidus is the also endemic pine bark adelgid (PBA), *Pineus strobi* Hartig (Hemiptera: Adelgidae). The host of PBA is eastern white pine, *Pinus strobus* L. (Pinales: Pinaceae), which often occurs sympatrically with hemlock in both natural and urban landscapes.

Laricobius nigrinus was the first *Laricobius* species recognized for its potential as a biological control agent following field observations in the coastal rainforests of western North America (Humble 1994, Montgomery and Lyon 1996). They were first collected and imported to a United States Department of Agriculture (USDA) approved Beneficial Insects Containment Facility (BICF) at Virginia Tech in 1997. Following biological evaluation studies (Zilahi-Balogh et al. 2002), *L. nigrinus* was approved for release in 2000. Over the years, multiple universities and governmental agencies have initiated *Laricobius* spp. mass rearing programs, with varying degrees of success and production. Currently, Virginia Tech, University of Tennessee, and University of Georgia are the only entities with colonies of *Laricobius* spp. agents produced for field release. Following the release and establishment of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) as a biological control agent in eastern North America, hybridization between the native congener *L. rubidus* and imported *L. nigrinus* was observed at a proportion of 11-15% (Havill et al. 2012, Fisher et al. 2015, Mayfield et al. 2015, Wiggins et al. 2016).

In 2006, an additional *Laricobius* spp., *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae), was collected in Japan and was also brought to the BICF at Virginia Tech for biological evaluations (Montgomery et al. 2011, Vieira et al. 2011, Story et al. 2012). The goal was to have a complementary agent to *L. nigrinus* that co-evolved with the pest, HWA, in its native range of Japan (Havill et al. 2006).

Following host-range testing and potential impact assessments, *L. osakensis* was approved for release in 2010 (Fischer et al. 2014, Mooneyham et al. 2016, Toland et al. 2018). However, due to the presence of a cryptic second species within the colony, *Laricobius naganoensis* Leschen (Coleoptera: Derodontidae)*,* releases were deferred until strict colony purification procedures were implemented (Fischer et al. 2014). Although *L. naganoensis* was approved for release from quarantine in 2017 (USDA 2017), no releases have occurred and colony purification protocols continue to be used when rearing wild-caught collections of *L. osakensis*. Rearing requirements for *L. osakensis* followed the protocol developed for *L. nigrinus*. It was assumed that the two congeners shared similar thermal and moisture requirements based on climate matching data (Vieira et al. 2013).

With the approval for release of two *Laricobius* spp. granted, the Insectary at Virginia Tech was the first lab to develop and implement mass rearing protocols (Salom et al. 2012), with the goal of supplying biological control agents to federal and state land managers. In order to produce consistent and reliable specimens for release, specific biological and environmental requirements must be met. This includes mirroring the two distinct life phases (arboreal and subterranean) of *Laricobius* spp. and adequate provisioning of temperature, light, humidity, and primary and secondary nutrients. Development of the rearing procedures was initially based on the best available knowledge of the biology and environmental conditions of the natural systems. These procedures have evolved over time through scientific testing to optimize production. The long-term nature of this rearing program and the lessons learned have produced a considerable amount data that are analyzed here to better understand our successes and failures. In addition, we aim to highlight potential avenues of research to further increase laboratory production, quality, efficiency, and consistency.

Materials and methods:

Overview of the past and present standard operating procedures (SOP) for the mass production of Laricobius spp. agents

Laricobius spp. adult collections and importations to Virginia Tech

Inherent in the success of many biological control programs is the ability to mass-produce natural enemies of target insect pests or plant herbivores of weeds within a laboratory insectary. This requires efficient rearing procedures with precise knowledge of a natural enemy's lifecycle, dietary and thermal requirements, reliable personnel, and quality control (Leppla and Fisher 1989, Cohen and Cheah 2019). Beginning in 1997, the first shipments of *L. nigrinus* were sent to the Virginia Tech's BICF in Blacksburg, VA. Here, incipient colonies were established and host-range and developmental biological studies were conducted (Zilahi-Balogh et al. 2002, Salom, et al. 2002, Zilahi-Balogh et al. 2003a, 2005). Over the next four years, a colony was maintained in the BICF, however, due to high rates of mortality, further scientific studies were conducted with the goal of increasing colony survivorship in order to have a sufficient number of specimens for research use (Lamb et al. 2005, Salom et al. 2012).

With the approval for release granted and rearing protocols further streamlined, *L. nigrinus* was removed from quarantine and brought to the Virginia Tech Mass Rearing Insectary in Blacksburg, VA. Mass rearing protocols were put in place in 2004 (Salom et al. 2012). At this time, a field insectary was also established at Kentland Farm, Blacksburg, VA (Mausel et al. 2008, Salom et al. 2011). The long-term goal of our field insectary was to passively produce sufficient field reared specimens without artificially introducing laboratory domestication effects and reducing rearing costs. From 2005 to

2015, in attempts to avoid inbreeding depression through genetic bottlenecking and laboratory domestication, *L. nigrinus* rearing colonies were restocked annually with wild-caught specimens from either the Puget Sound region in Washington, or from Idaho, USA. It's been documented that ecotype of biological agents can vary in weight (Foley et al. 2016), thermal tolerance (Mausel et al. 2011), and morphology (Tipping et al. 2010). Therefore, two ecotypes of *L. nigrinus* (coastal vs. interior) were collected from climatically distinct areas in the Pacific Northwest with the goal of establishing each ecotype in the eastern United States with respect to their cold tolerant thresholds (Mausel et al. 2011).

Following the original collections in 2006 and the subsequent approvals for release of *L. osakensis* in 2010 and 2012, there have been four additional overseas collections (2010, 2012, 2015, 2019). Those specimens were sent to Virginia Tech BICF for colony purification, mass rearing, and experimental testing (Fisher et al. 2014).

While, for the most part, the rearing SOP for *L. nigrinus* outlined by Salom et al. (2012) is still in effect at Virginia Tech, we are now rearing *L. osakensis*, and there have been incremental changes to the equipment used, changes in the order of operations, the addition of artificial diets for early emerging adults, shifts in temperature requirements, and timing of temperature treatments throughout the rearing season (Salom et al. 2012). For a general diagram on the rearing procedures for *Laricobius* spp. for each respective life stage see Figure 2.1, and for more detailed descriptions of the SOP see Salom et al. (2012).

HWA field collections as host material for Laricobius spp.

In order to supply developing *Laricobius* spp. colonies with sufficient prey, week-to-bi-weekly collections of HWA infested eastern hemlocks are made from field sites in Virginia and surrounding states between the months of October and June. Hemlock branches infested with HWA are cut, brought back to the mass rearing lab, and are stored in 18.9 L buckets of H_2O. From these branches, individual bouquets of hemlock twigs (20-25 cm long) with high densities of HWA (2-3 per cm) are bundled by securing hemlock twigs in 29.6 ml Waddington North America (WNA) P10 plastic cups filled with Instant Deluxe Floral Foam (Smithers-Oasis North America, Kent, OH) saturated with H_2O and wrapped in Parafilm M (Beemis N.A., Neemah, WI). Field collecting HWA as food for the developing colony, without the presence of *L. nigrinus* and *L. rubidus* larvae and/or adults on hemlock branches, has been a continuous challenge. This is due to the dispersal of *L. nigrinus* from original release sites and the presence of *L. rubidus* on HWA in areas where white pine and hemlock co-occur. Steps are taken to minimize the occurrence of field collected *Laricobius* larvae and adults as HWA is brought in from the field, details of which are discussed later on.

Oviposition and egg transfer

Laricobius nigrinus start oviposition shortly after HWA sistens adults begin oviposition (Zilahi-Balogh et al. 2003a). *Laricobius osakensis* start oviposition shortly before HWA sistens adults begins oviposition (Vieira et al. 2013). *Laricobius* spp. densities in feeding containers are then reduced from 50 to approximately 20-25 adults per container to maximize feeding and oviposition opportunities within the container (Figure 2.1). Hemlock/HWA bouquets now containing *Laricobius* spp. eggs are removed every week from the feeding/oviposition containers and transferred into Berlese larval funnels with additional fresh foliage (Figure 2.1). Adult oviposition temperatures during this period are incrementally increased from 4°C in January to a maximum of 10°C in March which coincides with the period of peak *Laricobius* oviposition.

Larval development and drop

The transferred hemlock bouquets containing *Laricobius* spp. eggs are held in rearing funnels at 13° ± 2°C (12:12) for the duration of egg and subsequent larval development. When the larvae reach the fourth instar pre-pupal stage, they drop from the branch into four-ounce Mason jars (Jarden Corporation, Rye, NY) attached to the bottom of the larval funnels. In the early years of rearing *Laricobius* spp., pupation medium (soil mix) was directly placed at the bottom of the Mason jars three weeks after larval funnel initiation. However, this approach is no longer used (due to the difficulty of separating out the fallen larvae from the soil) and Mason jars are left empty and checked once daily for the presence of pre-pupae. If premature larvae (not yet pre-pupae) have fallen into the Mason jars, they are placed back on HWA infested hemlock to continue feeding and developing and are recollected when they drop as mature larvae. Any pre-pupae located are removed, counted, and placed onto the soil in an aestivation container with 5-7 cm of soil media composed of 2:1 peat moss:sand. Prior to adding pre-pupae, the soil media is saturated with distilled water at ~35% by weight. The weight of each soil container is then maintained throughout the season. Once in the aestivation container, pre-pupae burrow into the soil and begin pupation. They are kept in aestivation containers at a density of approximately 200 individuals per 820 cm^3 of soil (Figure 2.1).

Pupation and aestivation

Pupating *Laricobius* spp. are held in soil aestivation containers at 13 ± 2°C (12:12) for approximately 6-7 weeks until pupation is complete. Temperatures are then adjusted to 19°C for adult summer aestivation.

Adult emergence and feeding

As HWA breaks its summer dormancy and develops through its four nymphal stages, *Laricobius* adults emerge from the soil and begin predation. It is precisely at this time, when HWA is breaking dormancy, when the temperature is deceased from 19° to 13°C in the insectary to simulate seasonal changes in temperature. This temperature decrease prompts *Laricobius* spp. to emerge from the soil (Lamb et al. 2007, Salom et al. 2012). From 2004 to 2007, following *Laricobius* spp. adult emergence prior to aestivation break of HWA, beetles were given bouquets of hemlocks infested with first instar aestivating HWA nymphs as a nutrient source (Figure 2.1). From 2008 until present, the early emerging adults have been offered an artificial diet; Lacewing and Ladybug Food (Wheast, Planet Naturals, Bozeman, MT) or the CC diet (egg-based), in addition to bouquets of hemlocks containing aestivating first instar HWA nymphs (Cohen and Cheah 2015). A quarter-sized spread of artificial diet is offered on filter paper, which is taped to the side of each feeding/oviposition container. The diet is replaced every 2 weeks until HWA reaches the second instar stage. The early emerging adults are held at temperatures of 4 °C, 12:12 L:D, and at densities of approximately 50 adults per container (Figure 2.1). Host material and artificial diets are replaced every 2 weeks. Following emergence, adults are identified to species based on their morphology (coloration, size, and presence, absence, and shape of their pronotal tooth) using a dissection microscope (Zilahi-Balogh et al. 2006, Leschen 2011).

Data analysis:

Rearing data were collected over 15 years; from 2004 – 2019. Statistical analysis of the data was conducted using R version 3.6.1 and JMP version 15 and a $p \leq 0.05$ was considered significant for all of the following analyses. Data such as larval drop Julian date (JD), adult emergence JD, and subterranean survivorship are reported for each container (Tables 2.1, 2.2, and 2.3). Calendar dates reported for corresponding JD are for non-leap years. The larval drop date for each container is the last JD that larvae were placed in the container (before reaching capacity), and the container's adult emergence date is the first day that adults emerged from that container (Tables 2.1 and 2.2).

The first day larvae went underground and the number of days larvae spent underground were correlated against percent subterranean survivorship using the Pearson's correlation test for *L. nigrinus*, *L. osakensis*, and both *L. nigrinus* and *L. osakensis* combined from 2005 to 2019 (Tables 2.1 and 2.3). Averages of each Pearson's correlations were calculated using a Fisher's r-to-Z transformation (Tables 2.1 and 2.3).

The larval drop JD, adult emergence JD, and the total days underground data did not follow normal distributions, resulting in the use of the non-parametric Kruskal-Wallis and Friedman tests. Kruskal-Wallis tests were conducted to determine if there was a difference in the median larval drop JD, adult emergence JD, and total days underground across years. A Friedman test, using year as the blocking variable, was used to determine if there was a difference in the median larval drop JD, adult emergence JD, and total days underground, between the two species. Due to the unequal number of observations across the different species/years, an approximate Friedman test with repeated measures was performed in JMP by conducting a Wilcoxon rank sum test on the ranks of the response blocked by year.

Results:

Laricobius nigrinus and *L. osakensis* have been mass-produced by Virginia Tech since 2004 and 2010, respectively. To date, Virginia Tech has produced 264,552 larvae and 108,992 adults of *L. nigrinus* and 210,143 larvae and 70,850 adults of *L. osakensis* (Tables 2.1 and 2.2). Following emergence and prior to release or experimentation, 70,307 (39%) additional *L. nigrinus* and *L. osakensis* adult deaths occurred across all years. The total number of *L. nigrinus* reproductive adults (P_1) used for colony foundation from 2005 to 2019 was 8,594 and ranged from 26 in 2019 to 713 in 2005 (Table 2.1). The total number of *L. osakensis* reproductive adults (P_1) used for colony foundation from 2011 to 2019 was 5,560 and ranged from 342 in 2015 to 1,200 in 2011 (Table 2.1).

Larval Drop

The average total number of days *L. nigrinus* larvae dropped from 2004 to 2019 was 114, and ranged from JD 76 (March 17th) to 190 (July 9th) with a median of 128 (May 8th) (Table 2.1 and Figure 2.2). The average total number of days *L. osakensis* larvae dropped from 2010 to 2019 was 120 and ranged from JD 66 (March 7th) to 186 (July 8th) with a median of 116 (May 26th).

Due to year-to-year variability in the timing of temperature treatments, the unitization of wild-caught and laboratory reared P_1 reproductive adults, and host availability and quality, we expected to find differences in the responses of interest (median larval drop JD) across the years and between the two species. Kruskal-Wallis analysis on JD larvae drop, from 2005 to 2019, showed a significant difference across the years for *L. nigrinus* (X^2 = 131.76, d.f. = 13, P <0.001) and for *L. osakensis* (X^2 = 236.34, d.f. = 8, P <0.001) from 2011 to 2019.

When it came to comparing the two species across years, only data from 2011 to 2017 and 2019 were included due to not having data for both species in other years. Friedman's pairwise comparison test showed a significant difference between both species (X^2 = 11.56, d.f. = 1, P =0.007). The result of this test supports the observation that the median larval drop date is later for *L. nigrinus* (JD 128) than for *L. osakensis* (JD 116) across the years.

The first day underground (i.e. last larvae drop date for each container) for *L. nigrinus* and *L. osakensis* was significantly negatively correlated with the percent survivorship from 2007-2011, 2013 and 2015 and from 2011, 2014-2017, respectively (Table 2.1). From 2004 to 2019, the average Pearson's correlation coefficient and corresponding p-value for *L. nigrinus* was -0.50 and <0.001, respectively (Table 2.1). From 2010 to 2019, the average Pearson's correlation coefficient and corresponding p-value for *L. osakensis* was -0.18 and <0.001, respectively (Table 2.1). The average correlation coefficient of survivorship vs. first day underground for *L. nigrinus* is 64%

larger than for *L. osakensis* (Table 2.3). These results suggest, especially for *L. nigrinus*, that the earlier each larvae cohort drop to the soil the higher their survivorship.

Subterranean duration and survivorship

The average median number of days spent underground for *L. nigrinus* was 198 and for *L. osakensis* was 214, and ranged from 165 to 237 and 178 to 250, respectively (Table 2.3). The average subterranean survivorship, which includes both pupation and adult aestivation, for *L. nigrinus* and *L. osakensis* was 39.7 and 33.9%, respectively.

Kruskal-Wallis analysis on number of days underground, from 2005 to 2019, showed a significant difference between each year for *L. nigrinus* (X^2 = 229.38, d.f. = 13, P <0.001) and from 2011 to 2019 for *L. osakensis* (X^2 = 403.14, d.f. = 8, P <0.001). Friedman's pairwise comparison test showed a significant difference between *L. nigrinus* and *L. osakensis* (X^2 = 27.54, d.f. = 1, P <0.001). This test result supports the observation that the median number of days spent underground across the years is higher for *L. osakensis* compared to *L. nigrinus.*

The median days spent underground was significantly positively correlated with subterranean survivorship for a majority (71%) of the rearing years for *L. nigrinus* (from 2005 to 2011, 2013, 2015, and 2017 (Table 2.3). From 2005 to 2019, the average Pearson's correlation coefficient and corresponding p-value for *L. nigrinus* was 0.58 and <0.001, respectively (Table 2.3). The median days spent underground was significantly positively correlated with subterranean survivorship for a majority (70%) of the rearing years for *L. osakensis* (from 2011, 2013 to 2018 (Table 2.3). From 2011 to 2019, the average Pearson's correlation coefficient and corresponding p-value for *L. nigrinus* was

0.37 and <0.001, respectively (Table 2.3). The average correlation coefficient of survivorship vs. median days underground for *L. nigrinus* is 36% larger than for *L. osakensis* (Table 2.3). These results suggest that the longer *Laricobius* spp. are underground the higher their survivorship.

Adult Emergence

The JD window of emergence for *L. nigrinus* ranged from 153 (June 2nd) to 345 (December 11th) and for *L. osakensis* was 158 (June 7th) to 351 (December 17th). The average median adult emergence JD for *L. nigrinus* ranged from 256 (September 13th) to 318 (November 14th) (Δ = 62 days) with mean and standard deviation of 296 (October 23rd) ± 18.4 from 2004 to 2019 (Table 2). The average median adult emergence JD for *L. osakensis* ranged 281 (October 8th) to 313 (November 9th) (Δ = 32 days) with mean and standard deviation of 297 (October 24th) ± 20.8 from 2011 to 2019 (Table 2.2).

Kruskal-Wallis analysis on median emergence JD, from 2005 to 2019, showed a significant difference across each year for *L. nigrinus* (X^2 = 394.33, d.f. = 13, P <0.001) and from 2011 to 2019 for *L. osakensis* (X^2 = 379.12, d.f. = 8, P <0.001). Friedman's pairwise comparison test showed a significant difference between both species' median adult emergence JD (X^2 = 6.85, d.f. = 1, P =0.009).

Discussion:

When the mass production of *Laricobius* agents began, the goals were to supply local, state, and federal land managers with biological control agents for release, and to have enough live insects to conduct related experiments regarding the biological control of

HWA. The tandem pursuit of these two goals has allowed us to continue the mass production of *Laricobius* spp. agents over the past decade and a half at Virginia Tech. From inception of our rearing program in 2004 until present, we have sent an average, 693 *Laricobius* spp. per shipment to 43 collaborators across 15 states (Table 2.4). The states that received *Laricobius* spp. are GA, KY, MA, MD, ME, NC, NH, NJ, NY, OH, PA, RI, VA, VT, and WV. These collaborators have played a pivotal role in dispersing these agents across the eastern and Carolina hemlock landscape in eastern North America with 13 of the 15 states who've received beetles having confirmed establishment (Virginia Tech 2019).

Over the past decade and half of rearing *Laricobius* spp. at Virginia Tech, the observation of "premature" larvae found in the Mason jars attached to the bottom of the funnels has consistently been noted. Salom et al. (2012), described these premature larvae as smaller, darker in color, and less mobile than mature larvae. It is unclear why these larvae premature drop from the infested plant material. The premature larvae are typically found throughout the larval rearing season and are placed back onto the hemlock foliage containing HWA, whereby they presumably resume predation and larval development before dropping back down into the funnels. Following the observation of larvae in the funnels, a technician visually determines based on size, color, and mobility if the specimens are premature or not. However, the weight difference between 2nd and 3rd instars to the 4th pre-pupal instar is on the scale of milligrams and not always easily discernable by the naked eye. It is possible these larvae are being placed onto the soil and do not have enough resource, measured by biomass, to make it thorough pupation and/or remain in aestivation.

While there is variability in the starting number of adults for each species and from year-to-year, based on our experience, the ideal starting number of reproductive adults range is between 800-1000 P_1 at a sex ratio of roughly 1:1. This rearing capacity is limited by the physical space available in the rearing facility as well as available personnel. The lower end of the range is more suitable for colony purification of *L. osakensis*, as physical space requirements increase with the need to keep individual groups separated (Fisher et al. 2014). The higher end of the range is more appropriate for *L. nigrinus*, which are reared using standard protocols.

The average median JD on which 50% of the larval population dropped and were placed onto soil is later for *L. nigrinus* compared to *L. osakensis*. A possible explanation for these results is the timing of when oviposition starts for the respective species. *Laricobius osakensis* have been observed starting oviposition as early as mid-December (Vieira et al. 2013, Personal obs.) in its native range of Japan, in the laboratory, and at release sites, whereas oviposition for *L. nigrinus* has been observed as early as late January (Zilahi-Balogh et al. 2003b). The phenological rate of development for *L. osakensis* is not fully understood within its introduced range of eastern North America and warrants further investigation.

Based on our analysis of these data, we have highlighted three major challenges in *Laricobius* spp. mass production and corresponding potential avenues of research. These need to be adequately addressed and understood in order to further increase laboratory quality, consistency, and production of *Laricobius* agents. The first major challenge is higher than desired colony mortality during pupation and aestivation, when the insects are in their subterranean environment (Table 2.3). A second major challenge has been the early emergence of *Laricobius* spp. relative to their host, HWA (Table 2.2 and Fig. 2.2). The final challenge involves the presence of field-collected larvae and adults on HWA infested branches used to feed lab-reared colonies. Because *L. nigrinus* has established at and dispersed from many original release sites, finding locations where *L. nigrinus* is not present is a continuous issue that complicates our rearing efforts.

Challenge 1: Subterranean duration and survivorship

During the subterranean portion of the *Laricobius* spp. lifecycle (~ 6 months), the insect pupates and enters into a period of presumed aestivation. From 2004 until 2019, the average subterranean colony mortality for *L. nigrinus* and *L. osakensis* was 40 and 34%, respectively (Table 2.3). The reason for such severe mortality is unclear. Some variables we might consider are soil moisture and larval density per soil container. Lamb et al. (2007) reported a decrease in adult emergence at soil moisture levels outside of the 40-50% range. However, our moisture levels are consistently monitored and maintained at or close to recommended levels and does not explain our results. Salom et al. (2012) did not see a density effect on survivorship when evaluating 120, 240, and 360 larvae per container. As we have maintained our larval densities at ~200 per soil container, it is unlikely larval densities explain our mortality rates.

Laricobius spp. subterranean mortality in a field setting is not currently well understood. Jones et al. (2014) experimentally tested the subterranean survivorship of *L. nigrinus* in northern Georgia, US, which corresponds to the southern limit of eastern hemlock, and recovered four adults from the estimated 1,440 larvae released. Jones et al. (2014) contributed their findings and lack of recoveries to the thermal developmental limit of 21°C for *L. nigrinus* (Zilahi-Balogh et al. 2003b). Additional studies need to be conducted to accurately document the subterranean survivorship of *L. nigrinus* and *L. osakensis* across their established range in relation to site factors and thermal requirements.

Avenues of research that could serve to increase colony production and subterranean survivorship is to determine the life stage (pupation or aestivation) most susceptible to mortality, the effect of 4th instar larvae biomass on *Laricobius* spp. subterranean duration and survivorship, the effect of pre-pupa handling time on subterranean survivorship, and the nutrient quality of HWA in relation to tree age, health, and stage of infestation.

Challenge 2: Timing of emergence

The median emergence time for *L. nigrinus* was significantly different from that of *L. osakensis*. Moreover, the average median range (the number of days during which 50% of the population emerges) for *L. osakensis* (32 days) is almost half compared to that of *L. nigrinus* (64 days). Based on these data, *L. osakensis* also remains in the soil longer than *L. nigrinus*.

When *Laricobius* spp. adults emerge before HWA breaks aestivation, additional colony mortality occurs. Early emergence has been and continues to be an issue and suggests there are underlying biological variations that are not fully understood (Figure 2.1). The total number of adults for both species that emerged early from aestivation from 2004-2019 was 179,842. Of those, 39% (70,307) died prior to field release or experimental research. To decrease further colony mortality following early emergence, the use of interim diets and slight changes to temperature treatment and timing of temperature treatments have been implemented or recently suggested.

Interim diets used by Virginia Tech have two main forms: 1) various artificial yeast, egg, and protein mixtures, and 2) adelgid eggs of either HWA from the previous generation kept at their minimal developmental threshold of 5˚C (Zilahi-Balogh et al. 2003b), to slow development, or from secondary adelgid host such as PBA. The artificial diet currently used is "Lacewing and Ladybug Food". Cohen and Cheah (2015) concluded the diet as "highly effective in extending the survival of adults". In attempts to decrease any further mortality of *Laricobius* spp. adults, HWA sistens and PBA eggs are supplied. Although, *Laricobius* spp. cannot complete development on PBA eggs solely (Zilahi-Balogh et al. 2002, Vieira et al. 2011), feeding still occurs. There are no data available on the effects that consumption of older stored HWA progrediens or freshly collected PBA eggs have on increasing *Laricobius* spp. survivorship. However, based on anecdotal observation, we believe there is a net positive effect.

From 2004 until 2019, the average median number of days spent underground for *L. nigrinus* and *L. osakensis* was 198 and 214, respectively (Table 2.3). Salom et al (2012) determined that both moisture and temperature influence the number of days spent underground. The decision for when to shift from simulated summer temperatures (19˚C) to simulated fall temperatures (13˚C) is based on field observations of HWA breaking aestivation in southwest Virginia. This reduction in laboratory temperature is usually initiated in early to mid-October around JD 274 (Figure 2.2). Following this shift in temperature at the insectary, the median JD at which 50% of the adults emerged for *L. nigrinus* and *L. osakensis* was 298 and 300, respectively (Table 2.2). Therefore, while there is substantial variability in the timing of initial emergence, half of the colony consistently emerges from the subterranean environment following the shift to cooler temperatures (Table 2.2 and Figure 2.2).

Based on these data, a potential technique that could be used to subvert the phenomenon of early emergence and to increase subterranean survivorship of *Laricobius* spp. is to allow the beetles to have a longer subterranean period with respect to how long each larval cohort is in the soil. For example, the Pearson's correlation between days spent underground and survivorship shows a significant positive relationship, for most years (Table 2.3). These results suggest that the longer the beetles are in the soil the greater their survivorship, on a per soil container basis. In addition, the Pearson's correlation between first day underground and survivorship shows a significant negative relationship, for most years (Table 2.1). These results suggest that the first cohort of larvae to make it to the soil, and thereby having a longer subterranean period, also has a higher survivorship.

Together, these results indicate the potential to increase subterranean survivorship. Instead of making the temperature shift on the same date for all soil containers (based on field-observed aestivation break), the temperature-shift date could be varied by container, based on the number of days each cohort have been in the soil. This ensures a more uniform subterranean period (of adequate length) for each aestivation container.

Challenge 3: Unintended field collections of Laricobius spp. larvae

Laricobius nigrinus was first released from 2003-2005, in 22 localities from Georgia to Massachusetts (Mausel et al. 2010). Following a three-year sampling period, establishment was confirmed at 13 locations in plant hardiness zones 6a, 6b, and 5b (Mausel et al. 2010). Since 2005, *L. nigrinus* releases have continued and this species is now established >13 states in both forest and urban environments (Foley et al. 2019, Virginia Tech 2019, Jubb et al. 2021). The dispersal and subsequent establishment of *L. nigrinus* from field release sites is likely larger than previously reported (Davis et al. 2012, Foley et al. 2019). During weekly to bi-weekly food collections, it is challenging to find locations in southwest Virginia and the surrounding area where *Laricobius* spp. are not present. Unintended field collections of *Laricobius* spp., whether *L. nigrinus, L. rubidus*, or hybrids thereof, further complicate rearing procedures by reducing the food availability for the lab reared colony, by disrupting the synchrony of larval developmental progression, and increasing the handling time for technicians when identifying species morphologically following emergence.

Laricobius nigrinus has overwhelmingly been considered the focal predator of HWA sistens by numerous governmental agencies, universities, and private stakeholders, each with the goal of releasing as many agents as possible. This was either done through the mass production of these agents (Salom et al. 2012), or through the relocation of these agents directly from the PNW (McDonald et al. 2011). As a result, *L. nigrinus* is now widespread across most of the HWA infested eastern hemlock and Carolina hemlock range (Foley et al. 2019, Virginia Tech 2019, Jubb et al. 2021). This same initiative has not been as exhaustive for *L. osakensis* as it has been for *L. nigrinus.* Therefore, we foresee the rate of establishment of *L. osakensis* across the eastern and Carolina hemlock range occurring at a slower pace.

Following HWA field collections, where the presence of *Laricobius* spp. is noted, efforts are made to avoid those collection areas in the future. In attempts to capitalize on regular cycles of quick population growth and decline of HWA, we also try to find new source populations of HWA, hoping that *Laricobius* spp. are not yet established there. From a mass rearing perspective, we foresee the unwanted field collection of unidentified species of *Laricobius* larvae as a continuing disruption within the rearing process, and therefore, we must adapt accordingly. As food is brought in from the field to the Insectary and stored in 18.9 L buckets, prior to bouquet construction, sub-samples are scouted for the presence of *Laricobius* spp. eggs and larvae. In addition, when scouting is neither effective nor sufficiently comprehensive, phenological anomalies can aid detection of field-collected insects. The date at which larvae start appearing at the bottom of the funnels, in relation to the entire colony's phenology, is particularly informative. For example, if 4th instar pre-pupal larvae are found in mason jars when the majority of the lab colony larvae are still developmentally younger (i.e., 2nd instar), they are considered field-caught larvae.

Laricobius nigrinus is an important species in the biological control effort against HWA. However, limited laboratory resources and the widespread release and subsequent establishment of *L. nigrinus* across the landscape raises an earnest question: is there a need for continued mass production of this species? It is precisely with this question in mind that Virginia Tech discontinued the mass production of *L. nigrinus* in 2018 and focused on a species that is not already widely established, *L. osakensis*. However, in subsequent years, field-caught 4th instar pre-pupal *Laricobius* spp. larvae continued to be found in Mason jars, and therefore soil aestivation arenas were prepared so that those individuals could be reared to the adult stage and later released. Moving forward, mass production at Virginia Tech will continue to be focused on *L. osakensis*, however, retaining, rearing, and releasing field caught *L. nigrinus* is a worthwhile side effort.

From an applied perspective, the ubiquity of *L. nigrinus* throughout its introduced landscape of eastern North America is promising. An operational metric often used to help define a successful biological control agent is its establishment and subsequent dispersal success and capabilities (Messenger et al. 1976, Goode et al. 2019). Evidence suggests that *L. nigrinus* is established at most release sites, is dispersing from those sites into new environments that contain hemlocks infested with HWA, and is exhibiting significant predation of HWA (Mausel et al. 2008, Mayfield et al. 2015, Jubb et al. 2020a). However, *Laricobius* spp. by themselves are not sufficient in reducing HWA populations to acceptable levels and are but one tool in the overarching IPM strategy for HWA (Mayfield et al. 2020).

Lastly, it is with the undesirable field-caught *L. nigrinus* and/or *L. rubidus* in mind, that these data must be examined carefully. During larval production, species identity and the number of undesirable field-caught specimens brought into the lab are difficult to discern. *Laricobius* larval species determination, based on morphology, is not possible. However, when *L. nigrinus* and *L. osakensis* become adults, morphology can be used to separate these species. The aforementioned steps of scouting host material as it's brought into the lab and observing phenologically asynchronous pre-pupal larval drops are taken to help identify the presence of undesirable field-caught *Laricobius* spp., but does not completely stop the input of field-caught insects into the insectary.

Overall, the production of *Laricobius* spp. at Virginia Tech has been a successful endeavor that has not only served our local forest and urban ecosystems, but also those numerous collaborators in multiple states who have received shipments of predatory beetles. Based on these analyses and results of rearing *Laricobius* spp. over the past 15 years, we recommend several areas of research in order to understand their biological requirements and to increase laboratory production. These include: 1) constant temperature experiments of *L. osakensis* to determine the developmental rate and ideal temperatures for rearing with respect to each life stage and phase, 2) evaluate the effect of *Laricobius* spp. larval biomass on their subterranean survivorship and timing of emergence, 3) study *Laricobius* spp. subterranean survivorship in a field setting as it relates to site factors, 4) assess the effect of handling time for pre-pupae, 5) determine the nutrient quality of HWA in relation to tree health, age, and stage of infestation, and 6) stagger the changes in temperature with respect to how long each larval cohort is in the soil.

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Table 2. 1. Summary of the total number of *Laricobius* spp. reproductive adults and larvae produced by Julian date at the Virginia Tech insectary and Pearson's correlation for each year and species relating survivorship of emerging adults to date they first went into the soil.

				Julian Date of Larvae Drop						Survivorship vs. First Day Underground (Pearson's correlation)		
Year	Spp.	Fecund Adults	Total larvae	Min	25%	50%	75%	Max	Mean no. ± SD	Soil containers (n)	Coeffi -cient (r)	p-value
2004	LN	NA	24,803	20	62	93	112	159	88 ± 29.3	NA	NA	NA
2005	LN	713	19,285	95	123	133	152	192	137 ± 17.7	16	-0.18	0.483
2006	LN	1,067	13,205	102	126	144	156	214	143 ± 22.0	34	-0.13	0.47
2007	LN	1,231	40,912	72	121	133	145	190	133 ± 17.2	199	-0.41	<0.001*
2008	LN	1,200	45,985	72	128	139	158	198	140 ± 23.1	237	-0.47	<0.001*
2009	LN	1,230	32,009	86	123	135	145	202	135 ± 18.7	160	-0.76	<0.001*
2010	LN	1,070	38,352	76	114	127	138	196	126 ± 19.7	191	-0.55	<0.001*
2011	LN	300	8,039	93	117	132	149	199	134 ± 21.9	40	-0.81	<0.001*
	LO	1200	27,987	79	114	124	137	183	127 ± 18.4	40	-0.47	0.002*
2012	LN	245	2,823	72	109	125	144	191	129 ± 28.2	16	0.01	0.968
	LO	800	10,691	60	89	110	125	180	109 ± 26.6	86	0.03	0.752
2013	LN	470	11,561	84	109	123	139	171	124 ± 18.5	72	-0.31	0.008*
	LO	440	32,389	54	92	108	128	184	110 ± 24.4	176	-0.02	0.749
2014	LN	336	5,803	65	114	130	146	182	130 ± 21.2	31	-0.21	0.268

	LO	735	29,812	61	119	135	152	184	135 ± 23.0	162	-0.47	<0.001*
2015	LN	387	7,622	61	103	118	132	176	117 ± 21.3	43	-0.50	<0.001*
	LO	342	11,944	61	92	105	120	176	107 ± 21.8	76	-0.53	<0.001*
2016	LN	42	1,556	66	103	118	134	182	119 ± 23.4	14	-0.37	0.197
	LO	500	21,420	54	78	94	114	180	99 ± 22.1	204	-0.27	<0.001*
2017	LN	277	11,805	57	97	115	129	195	114 ± 26.8	63	-0.17	0.178
	LO	600	18,612	58	91	113	127	188	111 ± 25.8	145	-0.41	<0.001*
2018	LO	365	42,753	65	105	114	126	189	117 ± 18.6	201	0.05	0.453
2019	LN	NA	792	115	135	150	165	198	152 ± 20.8	4	0.83	0.17
	LO	578	14,535	99	123	138	159	213	142 ± 21.8	71	0.64	<0.001*
2004-2019	LN	8,594	264,552	76	112	128	143	190	128 ± 22.0	1120	-0.50[a]	<0.001*[a]
2011-2019	LO	5,560	210,143	66	100	116	132	186	117 ± 23.1	1161	-0.18[a]	<0.001*[a]
2004-2019	LN+LO	14,154	474,695	72	108	123	139	188	124 ± 22.4	-	-	-

LN = *Laricobius nigrinus*
LO = *Laricobius osakensis*
Min = Minimum
Max=Maximum
* Statistically significant p-value (< 0.05)
n = total number of soil containers
[a] calculated using Fisher's z' transformation
\- analyses were not conducted

Table 2. 2. Summary of the total number of *Laricobius* spp. adults produced at the Virginia Tech insectary for each year and Julian data quantiles of emergence with the mean ± SD

Year	Species	Total adults	Soil containers (n)	Julian Date of Adult emergence					
				First	25%	50%	75%	Last	Mean ± SD
2004	LN	7,828	NA	233	293	300	307	326	300 ± 13.2
2005	LN	3,416	16	262	293	301	308	341	301 ± 10.8
2006	LN	1,995	34	264	293	300	308	365	300 ± 21.0
2007	LN	15,136	199	199	291	305	314	355	301 ± 18.1
2008	LN	20,526	237	211	261	276	302	339	280 ± 25.6
2009	LN	13,060	160	238	301	308	315	348	307 ± 12.5
2010	LN	26,774	191	204	299	308	315	363	304 ± 18.9
2011	LN	3,757	40	231	305	311	318	340	311 ± 11.3
	LO	5,896	40	265	302	309	317	341	309 ± 12.1
	LN+LO	9,653	80	231	303	310	317	341	310 ± 11.8
2012	LN	1,248	16	256	295	308	318	351	306 ± 16.5
	LN+LO	3,639	102	163	294	304	314	351	302 ± 21.2
2013	LN	5,918	72	163	287	304	314	356	300 ± 18.9
	LO	13,896	176	158	283	300	313	364	298 ± 23.1
	LN+LO	19,814	248	158	284	302	313	364	298 ± 21.9
2014	LN	2,862	31	166	301	311	317	349	307 ± 16.0
	LO	8,680	162	164	272	292	304	348	288 ± 21.0

	LN+LO	11,542	193	164	277	297	310	349	293 ± 21.5
2015	LN	1,750	43	182	246	256	274	331	260 ± 25.3
	LO	6,876	76	163	302	313	321	364	311 ± 16.7
	LN+LO	8,626	119	163	289	308	319	364	301 ± 27.6
2016	LN	870	14	258	307	318	327	364	316 ± 22.1
	LO	8,385	204	182	263	281	302	364	281 ± 27.4
	LN+LO	9,255	218	182	265	286	306	364	284 ± 28.8
2017	LN	3,543	63	205	263	283	296	315	278 ± 23.5
	LO	5,029	145	205	275	291	302	316	288 ± 18.0
	LN+LO	8,572	208	205	271	287	300	316	284 ± 21.0
2018	LO	15,322	201	222	274	304	317	361	296 ± 27.9
2019	LN	309	4	226	254	277	290	328	273 ± 22.1
	LO	4,375	71	226	294	304	311	349	300 ± 17.8
	LN+LO	4,684	75	226	291	303	311	349	298 ± 19.3
2004-2019	LN	108,992	1120	163	286	298	308	345	296 ± 18.4
2011-2019	LO	70,850	1161	158	284	300	311	351	297 ± 20.8
2004-2019	LN+LO	179,842	2281	158	285	298	309	347	297 ± 19.3

LN = *Laricobius nigrinus*

LO = *Laricobius osakensis*

n = total number of soil container

Table 2. 3. Summary of *Laricobius* spp. adult subterranean survivorship at the Virginia Tech insectary for each year and species and Pearson’s correlation coefficient (Survivorship vs. Median Days Underground). Correlation coefficients averages from 2005 to 2019 are weighted by the number of data points used to calculate each year’s correlation.

Year	Species	Subterranean survivorship (%)	Median days Underground[1]	Survivorship vs. Median Days Underground (Pearson's correlation)		
				Soil containers (n)	Coefficient (r)	p-value
2004	LN	31.6	NA	NA	NA	NA
2005	LN	17.7	197	16	0.51	0.043*
2006	LN	15.1	184	34	0.44	0.009*
2007	LN	37.0	193	199	0.54	<0.001*
2008	LN	44.6	178	237	0.46	<0.001*
2009	LN	40.8	191	160	0.84	<0.001*
2010	LN	69.8	199	191	0.63	<0.001*
2011	LN	46.7	196	40	0.81	<0.001*
	LO	21.1	219	40	0.63	<0.001*
2012	LN	44.2	211	16	0.02	0.951
	LO	22.4	227	86	-0.04	0.720
2013	LN	51.2	207	72	0.36	<0.001*
	LO	42.9	221	176	0.31	<0.001*
2014	LN	49.3	213	31	0.25	0.179
	LO	29.1	187	162	0.59	<0.001*
2015	LN	23.0	202	43	0.51	<0.001*
	LO	57.6	250	76	0.50	<0.001*
2016	LN	55.9	237	14	0.28	0.341
	LO	39.1	227	204	0.45	<0.001*
2017	LN	30.0	195	63	0.36	0.004*
	LO	27.0	205	145	0.50	<0.001*
2018	LO	35.8	216	201	0.37	<0.001*
2019	LN	39.0	165	4	-0.86	0.136
	LO	30.1	178	71	-0.53	<0.001*
2004-2019	LN	39.7	198	1120	0.58[a]	<0.001*[a]
2011-2019	LO	33.9	214	1161	0.37[a]	<0.001*[a]

LN = *Laricobius nigrinus*; LO = *Laricobius osakensis*; n = Total number of soil containers

[1] = Julian date; * Statistically significant p-value (< 0.05); [a] calculated using Fisher’s z’ transformation

Table 2. 4. Number of *Laricobius* spp. shipments from the Virginia Tech insectary and the number beetles released per State and overall, from 2004 to 2019.

Release State	Total no. of shipments	Total no. released
GA	1	600
KY	4	6,500
MA	7	5,200
MD	18	13,850
ME	12	6,510
NC	1	200
NH	14	6,650
NJ	7	5,800
NY	7	4,010
OH	10	4,950
PA	30	17,810
RI	1	300
VA	20	16,755
VT	2	1,000
WV	24	19,400
Total	158	109,535

Figure 2. 1. Diagram of the specific rearing temperatures, shift in temperature treatments, and arenas used with respect to each distinct life stage (Center: egg, larva, pupa, and adult) for the production *Laricobius* spp. agents. There are three arenas used (bottom): 1) Larval funnels, 2) Oviposition containers, and 3) Soil estivation containers. The process begins with adult emergence and feeding: A) adults are either field collected or laboratory reared and used as reproductive adults, B) adults are given bouquets of first early instar HWA and an artificial diet. Once oviposition begins: C) Hemlock plant material containing *Laricobius* spp. eggs embedded in the HWA woolly flocculent are transferred to D) larval funnels. Here larvae develop to the fourth instar prepupa stage and E) drop to the bottom the funnel where they are collected and placed onto the soil in the F) soil estivation arenas. Following pupation and estivation, G) *Laricobius* spp. adults emerge. A selective cohort is used as P1 reproductive adults for subsequent colony production and the rest are shipped to land mangers throughout the range of HWA infestations.

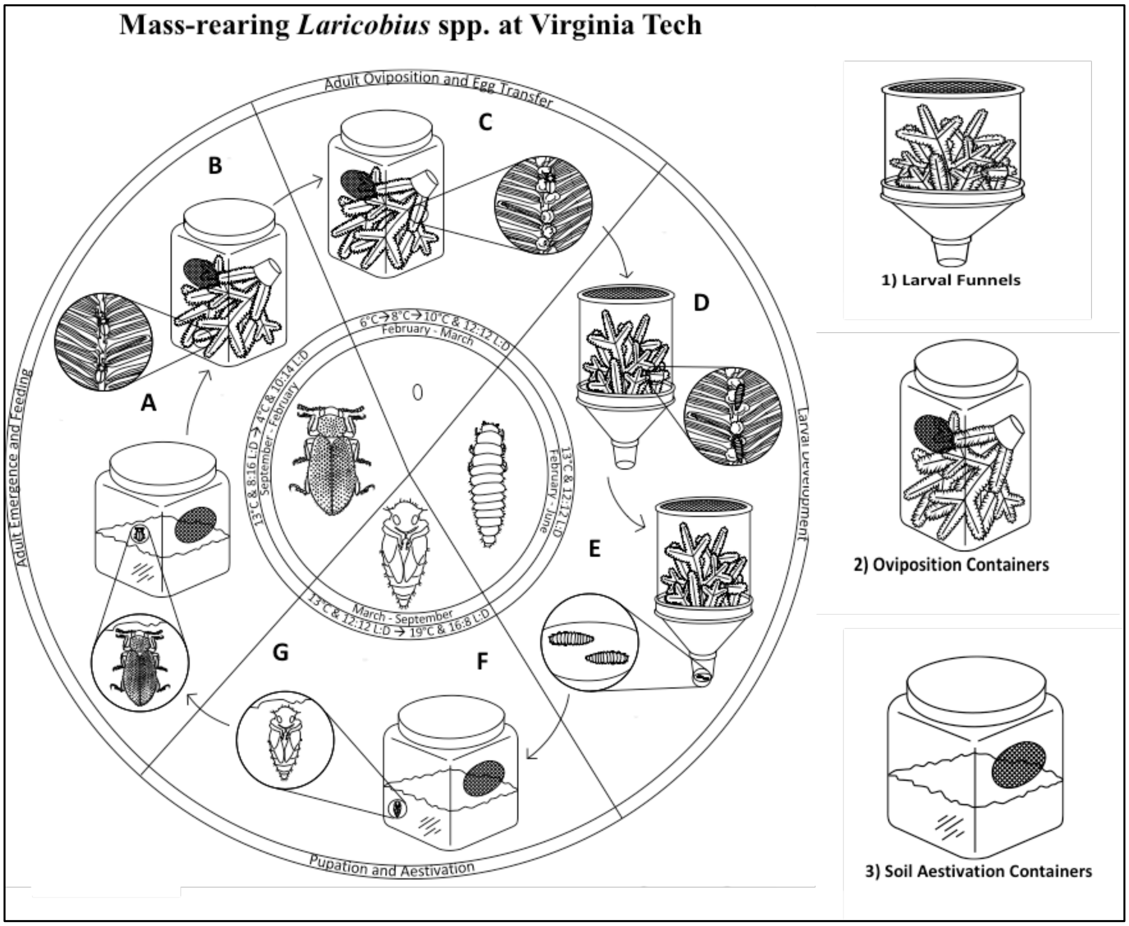
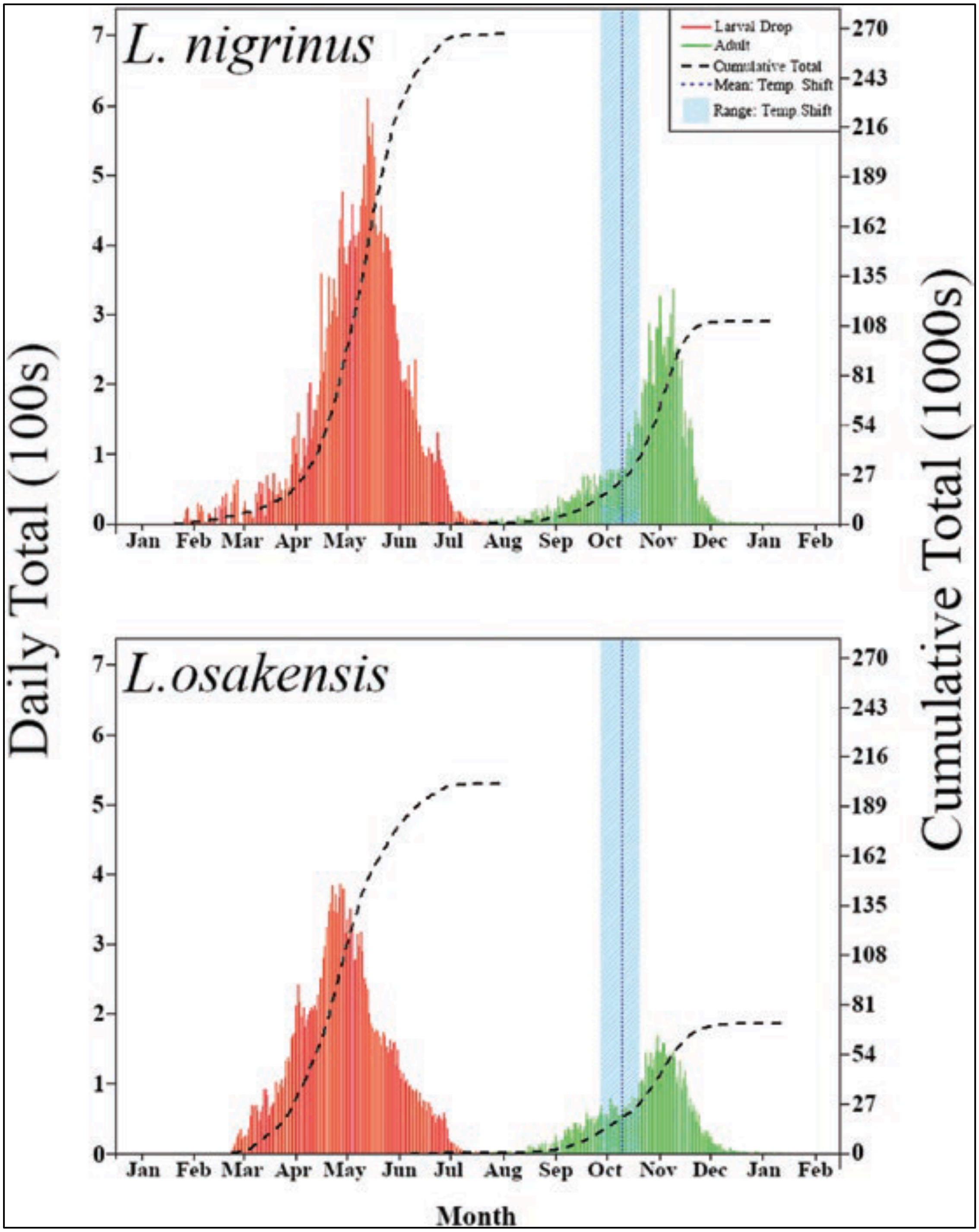


Figure 2. 2. Daily and cumulative larval drop (red) and subsequent adults emerged (green) from 2014 to 2019 for *L. nigrinus* (top) and *L. osakensis* (bottom). The blue dotted line (mean) and the surrounding light blue band shows the range in which the temperatures were changed from 19 to 13°C to stimulate emergences based on the field observation of estivation break of HWA.



Chapter 3: The establishment and distribution of *Laricobius* spp. (Coleoptera: Derodontidae), a predator of hemlock woolly adelgid, within the urban environment in two localities in southwest Virginia

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Abstract:

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is a highly destructive non-native pest lethal to eastern hemlock, *Tsuga canadensis* (L.) Carrière, and Carolina hemlock, *T. caroliniana* Engelmann. Fifty years following the first observation of HWA in eastern North

America, a predatory beetle, *Laricobius nigrinus* Fender, was evaluated, approved, and released as a biological control agent. Efforts were made to mass rear *L. nigrinus* with the goal of redistributing the beetles on public lands, typically in forest ecosystems, as a secondary option to silvicultural and chemical controls. The majority of *L. nigrinus* releases has occurred on public lands. Herein, we report the observation of *L. nigrinus* within the urban environment outside of known release locations. Two towns, Blacksburg and Radford, were divided into 0.40 km^2 grids. A total of 27 and 19 grids were randomly selected from each town, respectively. Hemlocks were present in 44 and 42% of the grids

surveyed in Blacksburg and Radford, respectively. In Blacksburg and Radford, 86 and 100% of the grids with hemlocks were infested with HWA, and of those infested hemlocks, *Laricobius* spp. was present in 100 and 75% of grids, respectively. A total of 154 *Laricobius* spp. (98% *L. nigrinus* and 2% *Laricobius rubidus*) adults were collected between each town. While it is unclear the level of control *L. nigrinus* has on reducing HWA's impact, the establishment of this biological control agent in the urban environment is an additional level of predation, that would otherwise not be present, for homeowners with HWA infested trees.

Introduction:

*Adelges tsu*gae Annand (Hemiptera: Adelgidae), commonly known as the hemlock woolly adelgid (HWA), is a destructive invasive, non-native pest of eastern hemlock, *Tsuga canadensis* (L.) Carrière, and Carolina hemlock, *T. caroliniana* Engelmann (Jenkins et al. 1999, Havill at al. 2006). *Adelges tsugae* was first reported in the urban environment in the early 1950s in Richmond, Virginia on ornamental hemlock (Gouger 1971, Souto 1996), presumably imported from Japan (Havill et al. 2006). Since then, HWA has spread into the indigenous hemlock distribution across 20 states and portions of Canada, covering over half of the extant range of eastern hemlocks (USDA Forest Service, 2019). The continued spread of HWA into urban and forest landscapes jeopardizes the health and integrity of ecosystems that depend on these keystone species (Ellison et al. 2014), as well as threatens the aesthetic nature of these ecosystems (Stadler et al. 2005).

Laricobius nigrinus Fender (Coleoptera: Derodontidae) and *Laricobius rubidus* LeConte (Coleoptera: Derodontidae) are native predacious species of Adelgidae, occupying the western and eastern portions of the United States, respectively (Zilahi-Balogh et al. 2002, Onken and Reardon 2011). *Adelges tsugae* is the preffered prey of *L. nigrinus* and following its introduction in the eastern US, has become secondary prey of *L. rubidus*. The primary prey of *L. rubidus* is the pine bark adelgid (PBA), *Pinus strobi* (Hartig) (Wantuch et al. 2017). *Laricobius osakensis* Shiyake and Montgomery (Coleoptera: Derodontidae) is native to Japan and was imported into the United States under quarantine in 2006, approved for release in 2010, and subsequently released in the eastern U.S. beginning in 2012 (Vieira et al. 2011, Fischer et al. 2014, Mooneyham et al.

2016). Both *L. nigrinus* and *L. osakensis* are now established in Virginia (Mausel et al. 2010, Toland et al. 2018).

Following the approval for release of *L. nigrinus*, the University of Tennessee and Virginia Tech initiated a robust mass rearing effort (Lamb 2002, Jubb 2011). The goal was to supply public land managers with biological control agents as an additional control option, where chemical insecticide treatments are not feasible due to ecological and/or economical issues. Since the approval for release of *L. nigrinus* in 2003, releases have been made from Georgia to Maine, totaling over 400,000 beetles (Jubb 2019). Establishment and subsequent recoveries have occurred within every release state. The majority of releases have occurred within forest settings. For homeowners, there is only a handful of effective registered insecticides that can safeguard individual infested trees from succumbing to HWA infestations (Vose et al. 2013). Chemical control options are more plausible for homeowners and municipalities targeting individual trees than for land managers targeting trees at the ecosystem level. Insecticide application also requires a base knowledge of how to apply the insecticides (e.g., foliar sprays, soil drench, or trunk injections), requires re-application every four to seven years, and costs may limit the number of trees that can be treated (Cowles et al. 2006, Benton et al. 2016). In spite of these limitations, chemical control is the principal tactic employed in urban environments. Beetles produced by laboratories or collected by government employees are destined for release on public lands. The only case of a focused effort to release beetles in communities or on private lands was done through consultants. In that situation over 14,000 *L. nigrinus* collected from the Seattle, WA area were released in the area surrounding Boone, NC (McDonald et al. 2011). Other than this example, no other known intentional releases of *Laricobius* spp. in urban environments has been reported. The goal of this study was to determine if *Laricobius* spp. have become established at non-release sites within the urban environment in two localities in southwest Virginia: Blacksburg, and Radford.

Materials and Methods:

Blacksburg and Radford, VA were divided into 0.40 km^2 grids (Fig. 3.1) Individual grids that did not completely fall within each respective town limits were excluded. A total of 27 and 19 grids were randomly selected from the two towns, respectively. Each town was surveyed between 21 February and 12 March 2019. Each individual grid was surveyed for the number of hemlocks, number of hemlocks with HWA, and the number of hemlocks with HWA and *Laricobius* spp. *Laricobius* adults were sampled using the beat sheet method for a collection time of five minutes, and if beetles were present, they were manually aspirated and collected. While this method historically underestimates the population of *Laricobius* adults (Mausel et al. 2010), it is quick and easy to employ. Species determination was based on morphology using a dissection microscope (Leschen 2011). *Laricobius* adults sampled within surveyed grids were mapped using GIS (Esri ArcGIS Version 10.1.6). The National Land Cover Database (NLCD) 2016 land cover raster layer, was used to visualize forest composition and development intensity within surveyed grids (Fig. 3.1) (Yang et al. 2018).

Results:

Hemlocks were present in 44 and 42% of the grids surveyed in Blacksburg and Radford, respectively. In Blacksburg and Radford, 86 and 100% of the grids with hemlocks were infested with HWA, and of those infested hemlocks, *Laricobius* spp. was present in 100 and 75% of grids, respectively. A total 154 *Laricobius* spp. (98% *L. nigrinus* and 2% *L. rubidus*) adults were collected from Blacksburg and Radford.

Discussion:

This is the first documentation of *L. nigrinus* in the urban environment in the eastern United States where beetles have not been intentionally released. During the initial scouting for biological control agents of HWA in the Pacific Northwest, a common, reliable, and convenient location to collect *L. nigrinus* is in the urban environment which includes neighborhoods, parks, and cemeteries (McDonald et al. 2011). The relatively high rates of recovery suggest that *L. nigrinus* can successfully establish in the urban environment within its newly introduced range. This supports our hypothesis that once released, *L. nigrinus* can disperse, locate its prey, and establish in environments outside of the forest setting. Following emergence, *L. nigrinus* uses a combination of olfactory cues, visual cues, and semiochemicals from infested trees to locate its prey (Mausel et al. 2011, Wallin et al. 2011)

The source of *L. nigrinus* found in each respective town is unknown. The closest release sites of *L. nigrinus* were at two locations in 2003. One site was located at Price's Fork (37.2121 N, -80.4894 W), 6.9 km from Blacksburg and 11.8 km from Radford. The second release site was Kentland Farm in Blacksburg, VA (37.2075 N, -80.5895 W) located 15 km from Blacksburg and 9.3 km from Radford. *Laricobius nigrinus* adults may have dispersed to these adjacent towns 16 years after their initial release. Another source of *L. nigrinus* collected in Blacksburg may have been the Virginia Tech's mass rearing facility located in the town limits of Blacksburg (37.2206 N, -80.4234 W). Hemlock branches infested with HWA are collected from other localities in Virginia and further south, and bought to Virginia Tech's rearing facility for mass rearing of *Laricobius* spp. These HWA infested branches may have been collected from sites that supported *L. nigrinus* and *L. rubidus* populations, and adults may have been present on the branches. These branches were kept outside the rearing facility and adults present on the branches may have flown from them and dispersed into Blacksburg. The ubiquity of *L. nigrinus* throughout both towns, their relative location to the closest known releases, and their univoltine life cycle suggest the beetles had been established for many years.

The establishment and distribution of *L. nigrinus* in the urban environment is an added level of protection for homeowners who have HWA infested hemlocks. After learning about biological control, homeowners often ask, "How do I get beetles for my trees?" Due to the apparent ability of *L. nigrinus* to successfully disperse over reasonably long distances, other urban areas close to *L. nigrinus* release sites may already have this species established, or will, soon. Monitoring for this predator in urban habitats can help homeowners and municipalities determine how best to address the presence and potential impact of HWA infested hemlocks.

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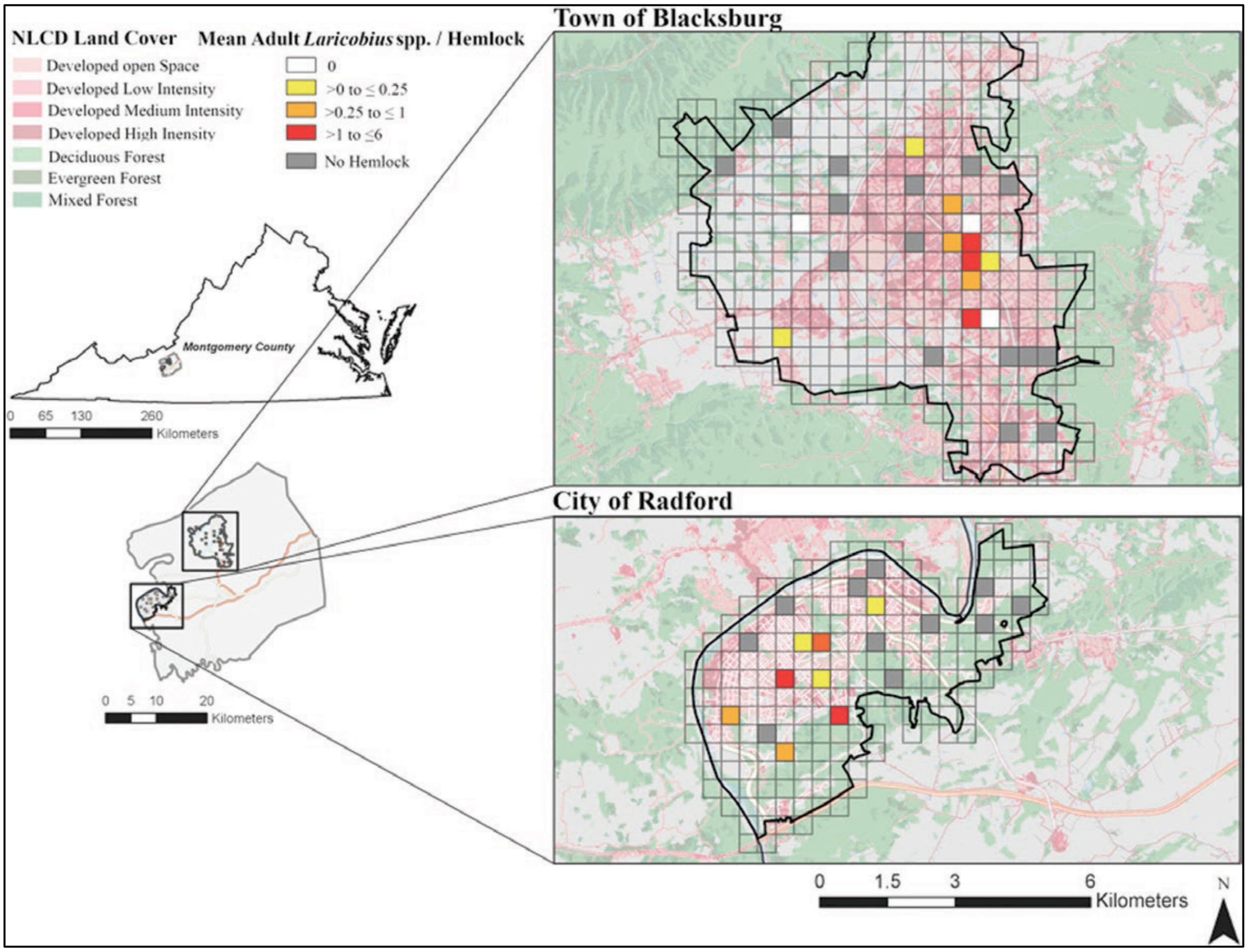
Figure 3. 1. Randomly selected survey grids (0.40 km^2) sampled in the town of Blacksburg (n =27) and Radford City (n =19), VA. Selected grids were surveyed for the presences of hemlocks, HWA, and *Laricobius* spp. in 2019. Color of each grid represents mean adult *Laricobius* sampled per hemlock tree. The National Land Cover Database (NLCD) 2016 land cover raster data layer illustrates forest composition and development intensity at each survey site.



Chapter 4: 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 ± 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.

Introduction:

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* Engelmann (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 first identified in the eastern U.S., HWA has 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 2021). 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), and a secondary host hemlock (*Tsuga* spp.), to maintain sexual generations (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 (Fig. 4.1). (Fernald 1898, Gillette 1909).

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 stands 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, and 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. *Laricobius 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; Fisher 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 (Fisher 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 2021). 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; Virginia Tech 2021; Jubb et. al., 2021). 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 (Vieira et al. 2011; Zilahi-Balogh et al. 2002; Zilahi-Balogh et al. 2003). *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, -80.4090° W) in 2019 and 2020. Branch clippings containing 3rd and 4th 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, post-hoc 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 (Figure 4.2, Table 4.1) 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 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 diam, with a wall thickness of 2 cm (schedule 40). The upper half of each trap was internally lined with a 7.6 cm yellow 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 (Figure 4.3). The yellow 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 diam.) 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 where 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 *Laricobius nigrinus*, *L. osakensis*, and *Laricobius 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, replicate, 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 (C^{o}). 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 used organic matter depth because 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 analogue 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 ± 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 4.2). There was no detectable difference in subterranean survivorship between *Laricobius* spp. that were field-collected to those that were laboratory-reared (Table 4.2). 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 mean (± SD) organic layer depth was 3.54 ± 3.06 cm. 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 4.2). 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 4.2).

The timing of seasonal emergence was not limited to fall, regardless of collection/rearing technique or the adjustment factor (Table 4.3). Emergence within the control treatment occurred at two of the five sites (McCoy and Price's Fork Research Station) across both years. No emergence occurred in control traps in summer and therefore no adjustment factor for this time period was necessary (Table 4.3). Regardless of the adjustment factor, most of the emergence occurred in fall (Table 4.3). In spring, the total percent emergence was reduced by more than half when adjusted for emergence within the controls (Table 4.3).

The control-adjusted combined mean (± SD) field subterranean survivorship for laboratory-reared *Laricobius* spp. was 18.0 ± 1.1% and for field-collected *Laricobius* spp. was 16.2 ± 1.1%, with an overall mean of 17.1 ± 0.8%. This was significantly less than the historical laboratory subterranean survivorship of 37.5% ± 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. *Laricobius 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 ± 0.8%) in this field study was higher then 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 4.3).

In this study, as pH increased subterranean survivorship decreased (Table 4.2). 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* with in 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 ≥ 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, handling the insects 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 ± 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 though 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 details of 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 years 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 ± 13.6%) was more than double the average of this field study (17.1 ± 0.8%). Although conclusions from this study are based on rearing/collection technique rather then species, *L. nigrinus* had the highest proportional presence within the field-collected colonies. Therefore, it is likely that the results form the field-collected cohorts are suggestive of the natural biology of this species. However, the same overarching conclusions cannot be made for what was the 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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Table 4. 1. *Laricobius* spp. subterranean survivorship monitoring sites in western Virginia with five sites tested over two years.

			GPS	
Site	Plot	Year	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

Table 4. 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[1]	Estimate (SE)[2]	Pr(>|z|)[3]
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[4]	1	0.0207 (0.012)	0.074

[1] = df: degrees of freedom. [2] = SE: Standard error. [3] = Pr(>|z|) represents p-value significance. [4]= Moisture: April-to-December average. * = Significant (P<0.05).

Table 4. 3. *Laricobius* spp. emergence partitioned based on season and treatment.

Treatment	Spring (4/1-6/30)				Summer (7/1-9/30)		Fall (10/1-12/31)				Total	Total*
	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.

Figure 4. 1. Scanning electron microscope (SEM) image of HWA progrediens chronic tether.

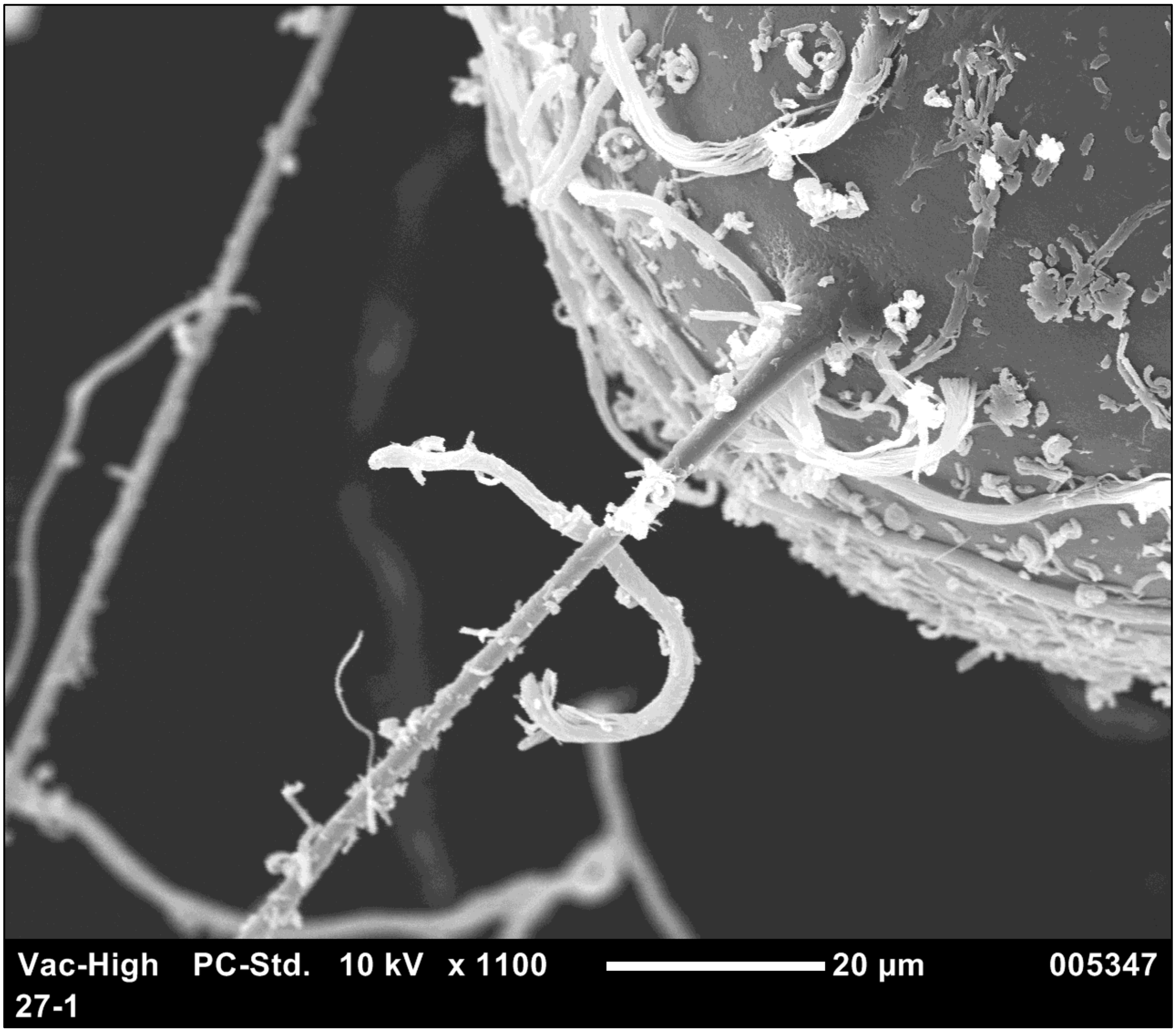


Figure 4. 2. Locations of five study sites (blacks dots) used to assess the subterranean survivorship of *Laricobius* spp. predators of HWA from 2019-2020 comparing to collection sources (laboratory-reared vs. field-collected). KL = Kentland Farms, MC = McCoy, ML = Mountain. Lake, PC = Poverty Creek, and PFRS = Price’s Fork Research Station.

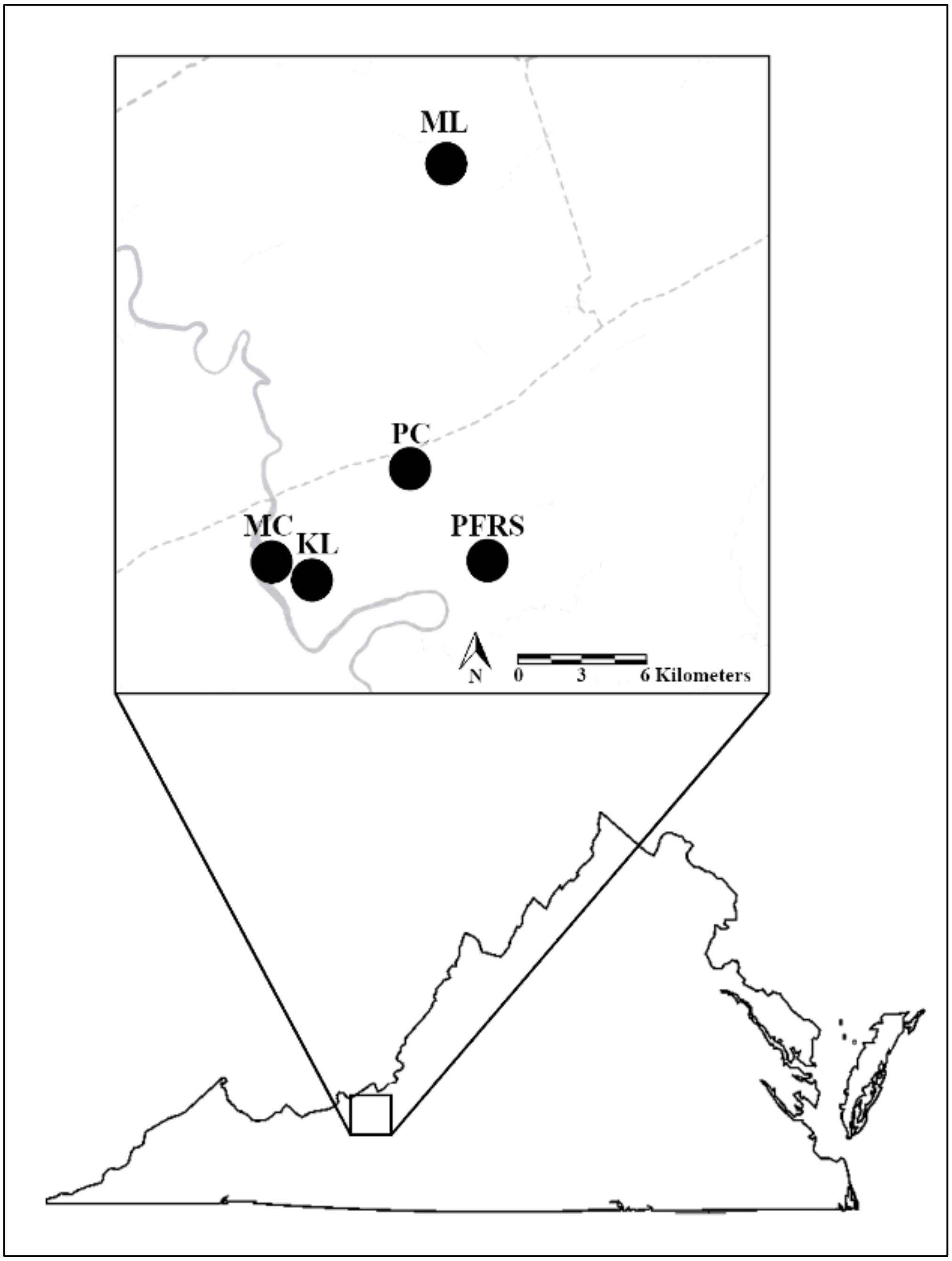
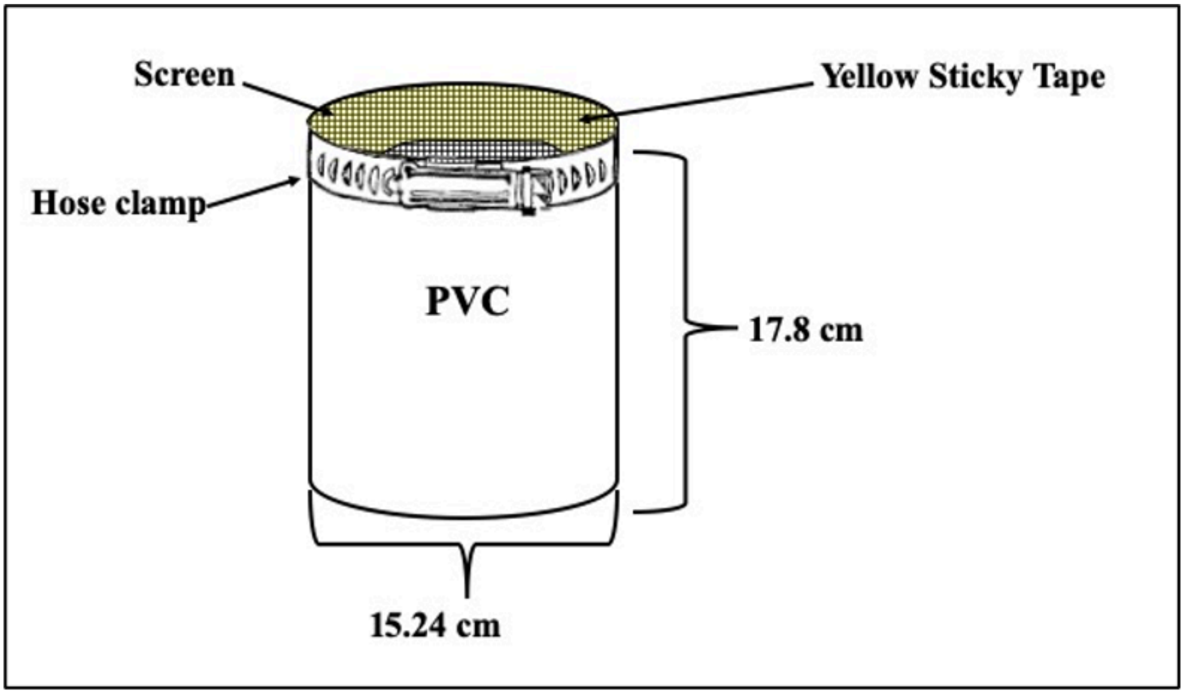


Figure 4. 3. *Laricobius* spp. subterranean emergence trap. Individual traps were schedule 40 PVC, internally lined with a 7.62 cm wide yellow sticky card, topped with a fine mesh screen, and secured witha hose clamp.



Chapter 5. Temperature-dependent development, survival and oviposition of *Laricobius osakensis* (Coleoptera: Derodontidae); a specialist predator of *Adelges tsugae* (Hemiptera: Adelgidae)

Abstract:

In North America, the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), continues to spread and impact populations of *Tsuga canadensis* (L.) Carrière and Carolina hemlock (*Tsuga caroliniana*) Engelmann. A predatory, *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae), is being mass-produced and released for the biological control of HWA. Developmental rate of *L. osakensis* on HWA was determined at six constant temperatures ranging from 5 to 22°C. Temperature response predictions were confirmed through routine field collections. Oviposition was also assessed at five constant temperatures ranging from 5 to 25°C. Head capsule width for each respective larval instar was also measured. *Laricobius osakensis* did not complete development from egg to adult at the two lowest temperatures tested, 5 and 8°C, but did so at each of the other temperatures tested (11, 15, 19, and 22°C). The minimum developmental thresholds were estimated for eggs (4.2°C), 1st (1.8°C), 2nd (5.5°C), 3rd (4.6°C) and 4th instar (4.1°C), prepupa (3.6°C) and pupa (7.5°C). The lower developmental threshold from egg to adult for *L. osakensis* (3.6 °C) was similar to that of *Laricobius nigrinus* (Fender) (3.7°C) and *Laricobius kangdingensis* (Zilahi- Balogh and Jelínek) (2.8°C), and to that of their HWA host (3.9°C). The upper developmental and survival threshold temperature is higher for *L. osakensis* than for *L. nigrinus* and *L. kangdingensis*, and closely matches that of HWA. Oviposition rates were

significantly greater at 5, 10, and 15°C than at 20 and 25°C. Head capsule width significantly increased with incrwasing larval instar, with means of 19, 26, 35, and 44 mm, for 1st, 2nd, 3rd, and 4th instars respectively. Laboratory and field data were used to develop a phenology forecasting model to predict the occurrence of all developmental stages of *L. osakensis*. This model will allow land managers to predict accurately the optimal timing for *L. osakensis* larval sampling throughout its established range.

Introduction:

Populations of both eastern hemlock, *Tsuga canadensis* (L.), and Carolina hemlock, *Tsuga caroliniana* Engelmann are impacted 70 years after the first report of the accidental introduction (Souto, 1996) of one of North America's most impactful forest pest, the hemlock woolly adelgid (HWA), *Adleges tsugae* Annand (Hemiptera: Adelgidae). Both eastern and Carolina hemlocks are important components of eastern North American forests. Eastern hemlock occurs in the Appalachian Mountains including New Brunswick and west to the Great Lakes. Carolina hemlock has a much more limited distribution and grows in isolated locations in western North Carolina, South Carolina, Georgia, Tenneseessse, Tennessee, and southwest Virginia (Jetton et al., 2008). While not having the economic value that it once did (Ellison et al., 2014), eastern hemlock is an ecological keystone species because it is associated with riparian ecosystems where it serves as food and refuge to a large component of fauna and flora (Dilling et al., 2007; Ingwell et al., 2012). The loss of these trees across the landscape, caused by HWA, has had lasting effects on the floral composition; shifting from hemlock dominant or co-dominant stands to hardwood dominant stand (Ellison et al., 2005; Eschtruth et al., 2006). Moreover, the loss of these species has caused adjacent aquatic and terrestrial habitats to increase in temperature and understory vegetative competition with invasive plants (Eschtruth et al., 2006; Siderhurst et al., 2010).

HWA was only considered an occasional ornamental pest of hemlocks for the first 30 years following its discovery in the eastern US. However, in the 1980s, the range of HWA expanded dramatically and consequently started causing observable decline to hemlocks on a landscape level (Eschtruth et al., 2006; Ellison et al., 2005; Siderhurst et al.; 2010). With the exception of 1st instar crawlers and sexuparae, HWA are sessile. HWA's primary dispersal mechanism is during the crawler stage as phornots on wildlife, including birds and arthropods (McClure, 1990; Russo et al., 2016; Leppanen and Simberloff, 2018). HWA is currently established in at least 22 states in the US and in a few provinces in Canada (Canadian Food Inspection Agency [CFIA]), 2017; U.S. Department of Agriculture [USDA], 2017; Virginia Polytechnic Institute and State University, 2021). As global warming continues, HWA is projected to continue its northward dispersal into remaining un-infested areas of eastern hemlock (McAvoy et al., 2017; Kantola et al., 2019).

Within HWA's bivoltine lifecycle are the sistens (overwintering) and the progrediens (spring) generations. The overwintering generation occurs from June to late March and the spring generation from late March to June, depending on latitude (McClure, 1989; 1996; Gray and Salom, 1996). In Japan, HWA requires a primary host (tiger-tail spruce, *Picea torano* Voss. (Siebold ex K. Koch) (Pinales: Pinaceae)), and a secondary hemlock host (*Tsuga* spp.), to sustain alternating sexual and asexual generations. However, in HWA's introduced range of eastern North America, tiger-tail spruce is not present, resulting in a strict anholocyclic life cycle; with both generations occurring on their secondary hosts, eastern and Carolina hemlock (McClure, 1989; Gray and Salom, 1996).

Efforts to control HWA, using a classical biological control approach, began in the early 1990s and have continued to the present. Of the eight insect agents evaluated and approved for release, *Laricobius* spp. have received most attention. During quarantine studies, prior to the request for release, biological evaluations are conducted to determine an agent's host range. These studies are often, although not always, accompanied with life history, phylogeny, morphometrics, and temperature response studies. The goals of these additional studies are to assemble enough evidence to identify and adequately understand the potential range once a biological control agent is released and to identify any potential negative factors associated with the agent. The latter two, morphometrics and temperature response studies, supply researchers with important biological information such as the identification of larval stages and relationship between development and temperature including thermal requirements and minimum temperature thresholds. Temperature-dependent studies also aid in optimizing rearing conditions, predicting establishment, potential range, rates of dispersal, and help build phenological models to optimize sampling efficiency (Frazer and McGragor, 1992; Judd et al., 1993; 1994; Régnière, 1996; Régnière et al., 2014).

Laricobius nigrinus Fender (Coleoptera: Derodontidae) and *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) are the only two *Laricobius* spp. to have been released into eastern North America for the control of HWA (Mausel et al., 2010; Fisher et al., 2015). These two predator species have well documented biological traits that have made them among the most promising and impactful classical biological control agents against HWA since their introduction. The larvae and adults of both species are host-specific and consume the eggs, nymphs and adults of HWA. Additionally, *L. nigrinus* readily establishes in and outside of release sites and shares phenological synchrony with its host, HWA. The phenology of *Laricobius* spp. and HWA is such that when HWA breaks its summer dormancy, *Laricobius* spp. emerges from the soil and migrates to the canopy of hemlocks to re-associate with HWA (Mausel et al., 2008, Foley et al. 2021).

Under controlled constant temperature regimes, the relationship between insect developmental rate and temperature is usually non-linear when examined over the full range of temperatures that the species may be exposed to and many models have been proposed to describe this relationship (Chuine and Régnière, 2017). There have been multiple models proposed and used that try to describe this relationship. Predictive models can be used over large areas extrapolating temperature data from nearby weather stations, and provide valuable information for pest control professionals. The goals for this study were to model the development and survival of immature life stages and to determine adult fecundity rates as functions of temperature for *L. osakensis*. Comparisons are made with *L. nigrinus* and *Laricobius kangdingensis* Zilahi- Balogh and Jelínek (Coleoptera: Derodontidae) development. In the early 2000s, *L. kangdingensis* was investigated as a potential biological control agent for HWA, but a petition was never submitted for its release. Additionally, the head capsule width for each instar of *L. osakensis* is determined. The data from this study provide valuable information to HWA managers interested in determining when to sample for specific life stages of *L. osakensis*.

Material and Methods:

Effect of temperature on immature development and survival:

The larvae used for this experiment in 2018 and 2019 were the F3 and F4 progeny of *L. osakensis* adults collected in Osaka, Japan in 2015.. Prior to oviposition, adults were maintained as mating pairs (1♀:1♂) in individual 50 by 9 mm polyester Petri dishes (Fisherbrand™, Waltham, MA) and kept at the previous weeks' weekly mean outdoor temperature within an environmental chamber (I-35II, Percival Scientific INC., Perry, IA). Once oviposition began, individual eggs (<24 hrs old) were removed with their respective ovisac, randomly assigned a treatment temperature, and placed inside a petri dish containing moistened filter paper. The lid of the Petri dish was sealed with Parafilm to reduce the loss of moisture, and distilled water was added as a fine mist daily. Eggs within each Petri dish were inspected daily for hatch and each successive stadium. Because *Laricobius* spp., including *L. osakensis*, are active during the cooler months (October-to-April), six relatively low constant temperature treatments of 5, 8, 11, 15, 19, and 22°C were used at a photoperiod of 12:12 h (L:D). The total number of specimens tested (n) from the lowest to highest temperature was 38, 58, 60, 58, 62, and 75. HWA prey for the developing larvae were obtained from field sites near Blacksburg, VA. Each time food was brought from the field into the lab, it was visually inspected for the presence of wild *Laricobius* spp. eggs and/or larvae. Food was replaced within each petri dish every 1-2 d. To ensure a continuous food source for the developing larvae through the duration of this experiment, HWA-infested eastern hemlock branches were stored at the 5°C to slow development (Salom et al., 2002).

A dissecting microscope was used to examine all *Laricobius* spp. life stages. Egg hatch was indicated by presence of the empty chorion, and larval molts by exuviae. Once the developing larvae reached the final prepupal stage, ~2 mm of soil was added to each Petri dish to simulate entry into the soil (Lamb et al., 2005). The experiment ended when the adult emerged from the pupa. The number of days that each individual took to complete each life stage was recorded.

Effects of temperature on fecundity:

This experiment was conducted in 2021 using the F1 progeny of adults collected in Osaka, Japan in 2019. A single pre-mated adult was placed into individual 50 x 9 mm polyester Petri dish (Fisherbrand™) containing moistened filter paper. A hemlock twig with 15-20 HWA ovisacs with adults and eggs was then placed in the Petri dish and wrapped in parafilm. The filter paper in the petri dishes was moistened every 3-4 d. The HWA infested hemlock twig was replaced weekly and the number of *L. osakensis* eggs counted. The five temperature treatments used were; 5, 10, 15, 20, and 25°C at a photoperiod 12:12 h (L:D) and the sample sizes were 6, 9, 9, 12, and 17, respectively. The experiment ended once no eggs were found in a Petri dish for two consecutive weeks or until the adult died.

Field observations for inclusion in phenology model:

Field collections of *L. osakensis* were made at Hungry Mother State Park (HMSP) (36.8895° N, -81.5243° W), in Marion, VA from mid-December 2020 to late April 2021. During each sampling period, ~10 branches containing > 150 HWA ovisacs were clipped, brought back to the lab, and examined under a dissection microscope where the number and life stage present for both HWA and *L. osakensis* were recorded.

Phenology model:

Constant temperature observations of development time, survival, longevity and fecundity were used to calibrate the non-linear equations for each life stage using the maximum likelihood method of Régnière et al. (2012). Because models of varying complexity were compared, we used the corrected Akaike Information Criterion (AICc) to compare likelihoods (Cavanaugh, 1997) and chose the best model. The oviposition data collected at HMSP were used to fit a cumulative oviposition curve as a function of accumulated degree-days. An individual-based model was created to be run within the BioSIM/11software. BioSIM provides weather-driven process models with location-specific temperature data based on nearby weather stations. It adjusts temperature for differences in latitude, longitude and elevation between weather stations and simulation point (Régnière et al., 2014).

The optimal starting point for modeling *L. osakensis* phenology would be adult emergence from the soil in the fall. However, because the timing of emergence, the pre-oviposition period, and the initiation of oviposition are not well understood and were not modeled, we used December 1st as the starting date, which was prior to the first observation of *L. osakensis* egg presence in the field.

BioSIM/11 was used to run the *L. osakensis* model and to compare results with the HMSP field oviposition observations. A weather database for northeastern North America was created from four sources: GHCN-D (https://www.ncdc.noaa.gov/ghcn-daily-description), ISD-Lite (https://www.ncdc.noaa.gov/isd), GSOD (https://data.noaa.gov/dataset/global-surface-summary-of-the-day-gsod, and Environment Canada (http://climate.weather.gc.ca/index_e.html). From model output, BioSIM was used to generate an interpolated surface of the date of peak *L. osakensis* larval occurrence from 7,500 simulation points, using universal kriging with elevation as an external drift variable. The simulation points were randomly distributed over the map area, with elevations extracted from a digital elevation model at 30-second horizontal resolution (http://dds.cr.usgs.gov/srtm/version2_1/SRTM30/). This digital elevation model was also used by BioSIM as a source of external drift for the date interpolation procedure.

Head Capsule Measurements:

Laricobius osakensis larvae of a known life stage (L_1, L_2, L_3 or L_4) that died throughout the duration of the 2019 immature larval development study were preserved in 70% EtOH for head capsule measurements. The head capsule of specimens that made it to the adult stage was not measured. Head capsules were measured to the nearest 100th of a mm using a 3.5X-90X stereo zoom microscope.

Data analysis:

Effect of temperature on immature development:

Provided here is a brief overview of the methods described in detail in Régnière et al. (2012), as implemented in the BioSIM/11's Curve Calibration component (Saint-Amant, 2021a). This method assumes that development times have a lognormal distribution such that development duration (t, days) of a life stage for individual i is $t_i = \frac{1}{R(T,\boldsymbol{\rho})}\varepsilon$ where $R(T, \boldsymbol{\rho})$ is development rate as a function of temperature, and ε is an individual's relative development rate, a "trait" belonging to a log-normal distribution with mean 1 and standard deviation σ. Fast-developing individuals have $\varepsilon < 1$, slow individuals $\varepsilon > 1$. The values of parameter set $\boldsymbol{\rho}$ and the log-normal distribution parameter (σ) were estimated by maximum likelihood. Because the duration of *L. osakensis* larval stages can be very short (less than the observation period of one day), a temperature response was fitted to the entire larval development period (L_1 to L_4), and attributed a constant proportion of this total duration to each successive life stage based on the constant temperature observations.

BioSIM/11 offers the possibility of fitting one of 15 different thermal response equations with lower and upper thresholds. Because there were few observations near the lower and upper thermal limits, we used a regular-increment grid search method for those two parameters over a range of realistic values, estimating by maximum likelihood the remaining parameters of the set $\boldsymbol{\rho}$ (a variable number that depends on the equation being tested). The lower threshold was varied from 0 to 4°C, and the upper from 26 to 34°C, both in increments of 1°C. The equation and set of estimates yielding the lowest AICc was chosen as the best model separately for eggs, larvae, prepupae, and pupae.

Linear regressions of development on temperature for each life stage (including larval stages separately) were fit with the model $r = a + bT$, where r is average development rate, a and b are intercept and slope, respectively, and T is temperature (°C). The lower developmental threshold temperature is defined by $D_{th} = -a/b$, and the cumulative degree-day (C_{DD}) required to complete development above that threshold is given by $C_{DD} = 1/b$.

Effect of temperature on survival:

Survival responses were also fitted according to the method of Régnière et al. (2012). Given the observed number of survivors N_s out of the initial number of individuals of a given stage N_i at the onset in a given treatment, the expected number of survivors is $\eta_s = N_i \times S(T, \boldsymbol{\rho})$ where $S(T, \boldsymbol{\rho}) = s(T, \boldsymbol{\rho})^t$ and $s(T, \boldsymbol{\rho})$ is daily survival, itself as a function of temperature T with parameter set $\boldsymbol{\rho}$, and t is the average time to complete the life stage. The set of parameters $\boldsymbol{\rho}$ is estimated by maximum likelihood, under the assumption that the number of survivors is Poisson-distributed with mean $N_i \times S(T, \boldsymbol{\rho})$. This function was chosen by minimizing the AICc among a set of 16 equations offered by BioSIM/11 (Saint-Amant, 2012b) separately for each of the four life stages (egg, larva, prepupa and pupa).

Effects of temperature on adult longevity and fecundity:

The same method used for immature development time was used for adult longevity, with the distinction that adult longevity does not have an upper threshold temperature. Nineteen equations were tested with a lower threshold limit varying from 0 to 4°C in steps of 1°C, as was done for the other life stages.

The oviposition rate was determined by dividing the total number of eggs per replicate by the number of days between sampling periods and then used as the dependent variable. The effect of temperature on average oviposition rate was analyzed by ANOVA and means compared by Tukey's test (Tukey's HSD test, $P < 0.05$) (R version 3.6.1).

The approach of Régnière et al. (2012) was used to analyze and model fecundity implemented in BioSIM/11. This method assumes that a female oviposits a constant proportion of its remaining eggs each day. This proportion $O(T, \boldsymbol{\rho})$ varies as a function of temperature T and a set of parameters $\boldsymbol{\rho}$. The total fecundity of individuals (F_i) follows a lognormal distribution with mean F_O and standard deviation σ_F. The number of eggs laid each day by a female (F_t) at constant temperature is thus $F_t = F_i e^{-O(T,\boldsymbol{\rho})(t_t - t_0)}$, where t_t is the number of days after the first egg is laid by a female, and t_0 is the pre-oviposition period, number of days between emergence from the pupa and first oviposition. The set of parameters values $\boldsymbol{\rho}$, F_O and σ_F were estimated simultaneously by minimizing the maximum likelihood assuming the number of eggs laid in a given time interval by individual females is Poisson-distributed.

Field observations:

All statistical analyses were conducted using R version 3.6.1 and a $p < 0.05$ was considered the significance threshold. The linear regressions for egg and larval development equations were calculated using the *DevRate* package in R.

Phenology model:

Cumulative oviposition determined from the HMSP field egg and larval observations was fit to a logistic equation, $CEC = \frac{1}{1+e^{-(D-\mu)/\sigma}}$, where μ and σ are parameters to be estimated by least-squared non-linear regression, and D is double sine degree-day accumulation (Allen, 1976) after December first between lower (T_L) and upper (T_H) threshold values. The limited amount of data available (a single year in a single location) precluded accurate estimation of these thresholds. Instead we used the values $T_L = 2.1$°C and $T_H = 20.1$°C obtained in an earlier study of *L. nigrinus* (J.R.F). This degree-day function provided the initial model input (new eggs entering the simulation) as a function of time. The full simulation model is written in C^{++}. All code is open source, and can be found at:

https://github.com/RNCan/WeatherBasedSimulationFramework/tree/master/wbsModels/LaricobiusOsakensis.

Head capsule width measurements:

The statistical program R (version 3.6.1) was used for analysis of head width data. Data were checked for normality and then a linear model was used to determine differences in head capsule width between instars. Dyar's law (Dyar, 1890) assumes a geometric progression of head capsule width from one instar to the next. Dyar's ratio was calculated by dividing the post-moult head capsule width by the pre-moult width.

Results:

Effect of temperature on immature development and survival:

The development and survival observations reported in this study are summarized in Table 5.1. *Laricobius osakensis* completed development from egg to adult at only four of the six constant temperature treatments (11, 15, 19, and 22°C), and average development rate increased significantly with temperature in all life stages (Table 5.2; dotted lines in Fig. 5.1). The estimated lower-temperature threshold D_{th} for each life stage ranged from 1.8 to 7.7°C, and the cumulative degree-day requirement ranged from 32.6 to 211.6 above D_{th} (Table 5.2). The best model to describe the developmental responses to temperature, based here on the AICc, was that described by Régnière et al. (2012). The development functions provide very good descriptions of each life stage's developmental response to temperature, although the behavior near the estimated lower and upper thresholds (T_b and T_m) was not based on observations. The individual variability in development rates around the expected average value was very well described by the log-normal distribution in the egg, larval and prepupal stage. However, in pupae the observed variability was less than expected, because it was very low at the coolest temperature (Fig. 5.1).

Although the proportion of total larval development time spent in each instar varied slightly with temperature, the average values 0.206, 0.203, 0.227 and 0.364 were used to divide larval development into instars I to IV. In the model, a larva's physiological age ranges from 0 at hatch to 1 at pupation. Thus, when an individual larva's physiological age reached 0.206, it was considered to have molted to instar II, to instar III at age 0.409 and to instar IV at age 0.773.

Survival functions for eggs, larvae and pupae were selected on the basis of the AICc. However, poor results were obtained from applying this approach to prepupae, so we used the same model chosen for the other life stages for prepupae as well. Resulting functions, and parameter values, are given in Table 5.4 and illustrated in Fig. 5.2. Eggs developed successfully to the next life stage (1st instar) at all temperatures tested. Daily and overall egg survivorships were relatively high and did not vary much over the range of temperatures tested. Larvae did not develop at 5°C, and experienced a sharp decrease in daily survival at temperatures above 18°C, dropping from 95% at 18°C to 83% at 22°C (Fig. 5.2). Overall larval survival was 10% at 22°C temperature tested (Fig. 5.2). Prepupae did not survive at 5°C either. Daily survivorship was highest at 8°C (99%) and lowest at 18°C (96%). There was a slight increase in daily prepupal survival from 97% to 98% from 18 to 22°C, respectively. Daily and overall pupal survival responded similarly to temperature: there was no development at 5°C, and survival increased up to 18°C and then decreased.

Effects of temperature on adult longevity and fecundity:

A simple exponential model with lower threshold temperature (Logan et al. 1979) provided the lowest AICc for adult longevity (Table 5.5). Longevity dropped with temperature, from an average of 80 days at 5°C to 15 days at 25°C (Fig. 5.2). A Gaussian model (Taylor 1981) provided the best description of the relationship between temperature and the oviposition ratio, the proportion of remaining fecundity laid per day by individual females (Table 5.5; Fig. 5.3). Total fecundity also varied with temperature, as a result of the interaction between daily oviposition rate and longevity (Fig. 5.3). The model uses an average initial fecundity of $F_0 = 100.4$, assigned to each female as log-normally distributed random variable with mean 4.546 and standard deviation 0.355. The average daily oviposition rate varied with temperature, and was lowest at 25°C and highest at 10°C (Fig. 5.4).

Field Study:

Laricobius osakensis eggs were present from the start of sampling (29 December 2019) until the end (23 April 2020), with peak oviposition occurring in early March 2020 (Fig. 5.5A). The cumulative occurrence of *L. osakensi*s eggs in the field was well described ($R^2 = 0.98$) by the logistic function $CEC = \frac{1}{1+e^{-(D-\mu)/\sigma}}$, , as a function of the accumulation of degree-days from 1 December between the thresholds of 2.1 and 20.1°C, with estimates of $\mu = 105.1$ and $\sigma = 88.6$ (Fig. 5.5B). In late March, occurrence *L.*

osakensis larvae coincided with a dramatic decrease in the number of HWA eggs present (Fig. 5.5A). The simulation model closely reflected the observed larval abundance data (Fig. 5.5). The optimal time to sample *L. osakensis* larvae is 10 days before *L. osakensis* larvae have reached 50% of their density. This time period coincides with when *L. osakensis* adults have finished laying eggs (late March at Hungry Mother) and before larvae begin dropping from hemlock trees to the ground (late April at Hungry Mother). At this time, the maximum density of *L. osakensis* larvae per HWA occurs, and an accurate density ratio can be determined from sampling at this time. A 10-day sampling period based on the model was used to allow sufficient time to sample larvae and accommodate for any microclimatic variability (Fig. 5.5). An example of the model's full output of HWA phenology is illustrated in Fig. 5.6.

Head Capsule Measurement:

Frequency distributions of head capsule measurements separated out four non-overlapping peaks, corresponding to four larval instars (Table 5.6). We found a significant increase in larvae head capsule width for each successive larval instar of ($F_{3,88} = 653.4$, $P < 0.001$, Table 5.6). The head capsule width of insects follows a constant geometric progression as they develop from one instar to the next (Dyar, 1890). Dyar's ratios were relatively constant and ranged from 1.34 to 1.36 mm for all larval stages with the exception of the fourth instar which showed a slight decrease compared to the other three instars (Table 5.6).

Discussion:

This is the first paper that quantifies the effect of temperature on *Laricobius osakensis* development. Temperature-dependent developmental studies have been conducted for *L. nigrinus* (Zilahi-Balogh et at. 2012) and *L. kangdingensis* (Gatton et al., 2009). *L. nigrinus* was approved for released in the early 2000 (Zilahi-Balogh et al., 2003) and *L. kangdingensis* was never petitioned nor released. *L. osakensis* and *L. kangdingensis* share a sister clade and thus, are more closely related to one another than they are to *L. nigrinus* (Montgomery et al., 2010). *L. osakensis* did not complete development from egg to adult at two of the lower temperatures tested (5 and 8°C) (Table 5.1). Gatton et al. (2009) reported that *L. kangdingensis* failed to develop from egg to adult at 6 and 9°C. Zilahi-Balogh et al. (2003) also reported that *L. nigrinus* completed development at 6°C but not at 9°C. The lower developmental threshold from egg to adult for *L. osakensis* (3.6°C) was not only similar to both *L. nigrinus* (3.7°C) and *L. kangdingensis*, but also similar to that of their host, HWA (3.9°C) (Salom et al. 2002).

The upper developmental threshold for HWA progrediens was predicted to be between 22 and 27°C (Salom et al. 2002). Gatton et al. (2009) and Zilahi-Balogh et al. (2003) reported failure to develop from egg to adult in *L. kangdingensis* and *L. nigrinus* at temperatures of 18 and 21°C, respectively. *Laricobius osakensis* completed development from egg to adult at 22°C, the highest temperature tested in this study. This predator can develop and survive at higher constant temperatures than *L. nigrinus* and *L. kangdingensis*, and more closely matches the upper thermal threshold of HWA. Therefore, our results suggest that *L. osakensis* may be a better fit as a biological agent than *L. nigrinus* in the southern portion of HWA's invasive distribution. Nevertheless, further research is needed to more precisely estimate the upper thermal limits of *L. osakensis*.

The mean developmental time from egg to adult for *L. osakensis* from 11 to 22°C was on average 36% faster than for *L. nigrinus* at comparable temperatures (Zilahi-Balogh et al., 2003) (Table 5.1). *Laricobius nigrinus* only completed development at 12 and 15°C, and comparing these temperatures to those tested in this study (11 and 15°C), *L. osakensis* completed egg to adult development 39% quicker (Gatton et al. 2009; Table 5.1). Although there was no statistical difference between oviposition rates at 5 and 15°C, oviposition rates at 10°C were greater, with an average of 0.9 eggs per day (Fig 5.4). Based on oviposition rate and percent survivorship from these data, 10°C was optimal for oviposition for this species. For larval growth, 15°C was the optimal, whereas, 15 to 18°C was the optimal for the prepupal and pupal life stages, as measured by survivorship (Table. 5.1, Fig. 5.2). *L. osakensis* is more heat tolerant than *L. nigrinus* or *L. kangdingensis* as evidenced by their survivorship, fecundity, and rate of development (Tables 5.1 and 2; Figs 5.1, 5.2 and 5.3).

Over the past 10 yrs, temperature regimes used to rear *L. osakensis* at Virginia Tech have been largely based on those of *L. nigrinus* (Zilahi-Balogh et al., 2003; Salom et al. 2012; Foley et al. 2021). Salom et al. (2012) suggested that the techniques used to produce laboratory-reared *L. nigrinus* are also applicable to rearing other *Laricobius* species. Based on their apparent success in rearing >70,000 *L. osakensis* since 2011 (Foley et al. 2021), and based on the similar optimal thermal thresholds presented here for *L. osakensis*, Salom et al. (2012) was correct.

High rates of larval mortality were observed throughout the duration of this study. However, high rates of larvae mortality have consistently been noted for these species (Foley et al, 2021) and are attributed but not limited to moisture and handling time (Table 5.1, Fig. 5.2) (Zilahi-Balogh et al. 2003; Pers. Obs.). While each experimental unit was moistened regularly, saturation levels were not recorded. Efforts to minimize the amount of handling during daily inspection were made but likely played a role in total survivorship. Therefore, the degree of mortality for each respective life stage and temperature should be interpreted carefully.

The subterranean portion of the *Laricobius* spp. life cycle, which includes pupation, eclosion, and aestivation has been documented as a substantial morality factor for these species due to population mortality in excess of 60% (Foley at al. 2021a, 2021b). This study was no exception. None of the prepupae (n=13) tested at 8˚C completed development and only 55% of those tested at 11˚C (n=12) completed development (Table 5.1). However, at the highest temperatures tested in this study (15 to 22°C), subterranean survivorship was highest, which is consistent with the natural conditions of this system. The average daily high temperature for western Virginia (1980 – 2016) from mid to late April when most of these insects are dropping from the trees to their subterranean habitat is between 16 to 21°C (Rienecker et al., 2011).

Laricobius osakensis, like all other species of *Laricobius*, has four larval instars (Clark and Brown 1958; Franz 1958; Clark and Brown 1960; Zilahi-Balogh et al., 2003; Gatton et al. 2009). This is the first documentation of larval head capsule widths for *L. osakensis*. The mean head capsule widths for each *L. osakensis* larval instar were smaller than that of head capsule of *L. nigrinus* and *L. kangdingensis* (Zilahi-Balogh et al., 2003;

Gatton et al., 2009). However, the range of head capsule widths for each respective instar of *L. nigrinus*, *L. kangdingensis*, and *L. osakensis* overlaps. The ability to distinguish various *Laricobius* spp. based larval head capsule width is not possible (Montgomery et al., 2011).

In their respective native range in Japan *L. osakensis* and HWA eggs were present in December and January at two of the three field sites used to quantify the phenology (Vieira et al., 2013). In this study, *L. osakensis* eggs were present on the first sampling date (December) whereas HWA's eggs were not present until two months later (17 February). However, while *L. osakensis* eggs were present two months prior to the appearance of HWA eggs, no larvae were recorded at this time; suggesting a good phenological match in the timing of oviposition and the appearance of larvae (Figs. 5.5 and 5.6). During the 6th sampling period (25th February) there was a dramatic decrease in the total number of eggs from the previous and following sampling periods. It is unclear if this was an artifact of the sampling procedure or if this decrease was associated with egg mortality or recently eclosed larvae absconding from the HWA ovisac due to a lack of available eggs to consume. HMSP was the only location used in this study to quantify the phenology of the predator-prey this complex. While *L. osakensis* can be found at many of their original release sites (Mooneyham et al., 2016; Toland et al., 2018), there are a limited number of available field sites that are thought not be colonized by other *Laricobius* species. Colonization of a site by other *Laricobius* spp. occurs through the unintentional release of *L. nigrinus* or *L. rubidus* inadvertently reared for release (Toland et al., 2018; Foley et al., 2021a), through dispersal of *L. nigrinus* from nearby release sites (Foley et al., 2019; Jubb et al., 2021), or though the dispersal of *Laricobius rubidus*

(Coleoptera: Derodontidae) from adjacent sites where eastern white pine (*Pinus strobus* L.) are infested with pine bark adelgid (*Pineus strobi* Hartig) (Hemiptera: Adelgidae).

The model for larval density appeared to predict peak larval density well, based on field collections from HMSP (Fig. 5.5). However, sampling did not start prior to the beginning of oviposition by *L. osakensis*. Furthermore, sampling could not be continued past the 23rd of April, when larvae were still present in the trees. However, if continued our field observation would have likely continued to follow the model predictions closely. The most effective and least time-consuming method to confirm *Laricobius* spp. adult presence at any particular site is through beat sheet sampling (Mausel et al. 2010). However, this method does not work as well for *Laricobius* spp. larvae and can often result in false negatives (Mausel et al., 2010; Toland et al., 2018). The highest likelihood of confirming the presence and therefore establishment of *Laricobius* spp. at any particular site is to sample for larvae through branch clippings (Mausel et al., 2010; Toland et al., 2018; Jubb et al., 2021). Branch clipping sampling for larvae also gives land managers the opportunity HWA to determine the relative abundance of *Laricobius* spp. to HWA by counting the number of larvae to the number of ovisacs per branch. The development of this phenological forecast model will allow HWA land managers to more confidently and precisely predict when to sample for *L. osakensis* larvae.

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Table 5. 1. Percentage survivorship from the previous stage, duration (days), and rate of development of *Laricobius osakensis* for each respective life sage at six constant temperatures.

Life stage	Temperature (°C)	n	Survival (%)	Duration (d)		Rate (1/d)
				Mean ± SD	Median	Median
Egg	5	38	79	57.1±3.2	57	0.001
	8	58	86	26.1±1.8	26	0.0028
	11	60	87	13.7±1.7	13.5	0.0078
	15	58	100	10.3±1.6	10	0.0141
	19	62	89	7.1±0.8	7	0.016
	22	75	95	5.2±0.8	5	0.0328
Larva I	5	30	7	23.1±2.3	23	0.0045
	8	50	72	8.1±2.2	8	0.0385
	11	52	77	5.2±1.3	5	0.0643
	15	58	78	4.9±1.4	5	0.0693
	19	55	76	3.1±0.9	3	0.0983
	22	71	31	2.7±0.8	3	0.1213
Larva II	5	2		21.5±0.7	21.5	0.0015
	8	36	78	9.2±2.7	8.5	0.0341
	11	40	88	6.3±1.8	6	0.0847
	15	45	82	4.4±1.5	4	0.0942
	19	42	93	3.5±1.3	3	0.1212
	22	22	50	2±1.2	1.5	0.3222
Larva III	5	-	-	-	-	-
	8	28	71	14.1±3.4	14	0.0195
	11	35	86	6±2.1	6	0.0952
	15	37	92	4.5±1.7	5	0.0972
	19	39	69	3.6±1.2	4	0.0985
	22	11	73	2.1±0.8	2	0.2825
Larva IV	5	-	-	-	-	-
	8	20	65	17.2±4.0	16.5	0.0109
	11	30	73	11.5±3.7	12.5	0.0458
	15	34	76	7.8±1.7	8	0.033
	19	27	70	5.1±1.6	5	0.0789
	22	8	100	4±0.5	4	0.0365
Larva I – IV	5	-	-	-	-	-
	8	20	52	48.6±6.7	47	0.0938
	11	30	51	29.0±4.8	29.5	0.1534
	15	34	58	21.7±4.5	22	0.1938

	19	27	44	15.2±3.2	15	0.2792
	22	8	11	10.7±2.7	10.5	0.3958
Prepupa	5	-	-	-	-	-
	8	13	-	38.1±4.5	38	0.0046
	11	22	55	19.9±4.2	20	0.0114
	15	26	85	13.1±2.3	14	0.016
	19	19	84	10.3±1.6	10	0.0164
	22	8	75	8.3±2.4	8.5	0.0413
Pupa	5	-	-	-	-	-
	8	-	-	-	-	-
	11	12	55	29.3±1.7	30	0.0021
	15	22	84	21.3±1.3	21.5	0.003
	19	16	84	14.9±0.9	15	0.004
	22	6	75	7.8±1.2	8	0.0213
Egg to adult	5			-	-	-
	8			-	-	-
	11			91.8±16.4	93	0.1285
	15			66.4±12	67.5	0.1655
	19			47.5±8.3	47	0.2377
	22			32.0±7.7	32	0.3793

Table 5. 2. Parameters of linear regression (r = a +bT), minimum temperature threshold (-a/b), and cumulative degree days (C_{DD} = 1/b) to complete each stage based on development at constant temperatures (°C) of *Laricobius osakensis*.

	Parameters					Lower threshold	
Life stage	a	b	df	r^2	P	°C	C_{DD}
Egg	-0.044	0.0104	349	0.90	0.0x	4.2	95.9
Larva I	-0.035	0.0196	314	0.65	0.0x	1.8	50.9
Larva II	-0.161	0.0307	186	0.46	0.0x	5.2	32.6
Larva III	-0.114	0.0249	148	0.12	0.0x	4.6	40.1
Larva IV	-0.058	0.0140	118	0.61	0.0x	4.1	71.6
Larva $_{I-IV}$	-0.019	0.0047	118	0.76	0.0x	4.0	211.6
Prepupa	-0.024	0.0068	84	0.74	0.0x	3.5	147.5
Pupa	-0.054	0.0071	52	0.72	0.0x	7.7	141.7
Egg to adult	-0.010	0.0018	52	0.83	0.0x	5.5	556.7

Table 5. 3. Life stage developmental parameters, equation of Régnière et al. (2012).

Life stage	Ψ	K	T_b	T_m	ΔT_b	ΔT_m	σ	*AICc*
Egg	0.04035	0.08769	3	27	0.928639	0.5	0.135	1352.5
Larva	0.02027	0.08477	4	27	0.359761	0.5	0.184	697.7
Prepupa	0.10502	0.03293	4	33	49.9474	5.42	0.201	443.3
Pupa	0.009748	0.10923	0	33	0.100047	0.52	0.128	260.1

Equation: $R(T) = \psi\left\{e^{k(T-T_b)} - \left[\frac{T_m-T}{T_m-T_b} e^{-k\left(\frac{T-T_b}{\Delta T_b}\right)}\right] - \left[\frac{T-T_b}{T_m-T_b} e^{k(T_m-T_b)-\left(\frac{T_m-T}{\Delta T_m}\right)}\right]\right\}$

Table 5. 4. Survival equations and parameters,equation of Régnière et al., (2012).

Life stage	Parameters						Equation
Egg	k	TL	TH	ΔTL	ΔTH		$\frac{k}{e^{\left(1+e^{\frac{-T-T_L}{\Delta T_L}}\right)}\left(1+e^{\frac{-T_H-T}{\Delta T_H}}\right)}$
	5.246	3.1	33	0.3198	420.37		
Larva	kk1	kk2	k0	k1	k2	kk	$\frac{kk_1}{1+kk_2 e^{k_0+k_1 T+k_2 T^{kk}}}$
	0.97799	0.92843	-13.564	-0.0010343	0.3889	1.1101	
Prepupa	k	TL	TH	ΔTL	ΔTH		$1-e^{kk\left(1-e^{\frac{-T-T_L}{\Delta T_L}}\right)\left(1-e^{\frac{-T_H-T}{\Delta T_H}}\right)}$
	-93.507	-73.4	70.8	1.1997	1326.4		
Pupa	k	TL	TH	ΔT			$1-k\left(1-e^{\frac{-T-T_L}{\Delta T}}\right)\left(1-e^{\frac{-T_H-T}{\Delta T}}\right)$
	0.99524	3.5	28.6	1.896			

Table 5. 5. Adult longevity and fecundity equations and parameters of Régnière et al., (2012).

Stage	Parameters					Equation
	Ψ	k	T_b	ΔT	σ	Logan 1979
Adult longevity	0.028750	0.1066	4.0	99.985	0.401	$\psi e^{\left(k(T-T_b)-e^{k\frac{T-T_b}{\Delta T}}\right)}$
	Ψ	T_o	ΔT	F_o	σ	Taylor 1981
Oviposition	1.517823e-02	10.9	6.5351	100.4	0.355	$\psi e^{\frac{-1}{2}\left(\frac{T-T_O}{\Delta T}\right)^2}$

Table 5. 6. Mean (± SEM) head capsule width and Dyar's ratio (head capsule width / previous head capsule width) measurements of *Laricobius osakensis* larvae.

		Head capsule width (mm)		
Instar	*N*	Mean	Range	Dyar's ratio
I	40	0.19 ± 0.01	0.16 - 0.20	
II	15	0.26 ± 0.01	0.24 - 0.28	1.36
III	20	0.35 ± 0.04	0.20 - 0.40	1.36
IV	17	0.44 ± 0.02	0.40 - 0.48	1.34

Figure 5.1. Development of *Laricobius osakensis* at different constant temperatures in each life stage. Each row represents a different life stage: egg on top followed by larva, prepupa and pupa. The first column (a) of graphs represents non-linear equations predicting the development rate as a function of temperature. The second column (b) shows the density distribution as a function of relative development time. The third column (c) represents time (days) as a function of temperature. Gray zone in column one and two represent percentile [1 - 99].

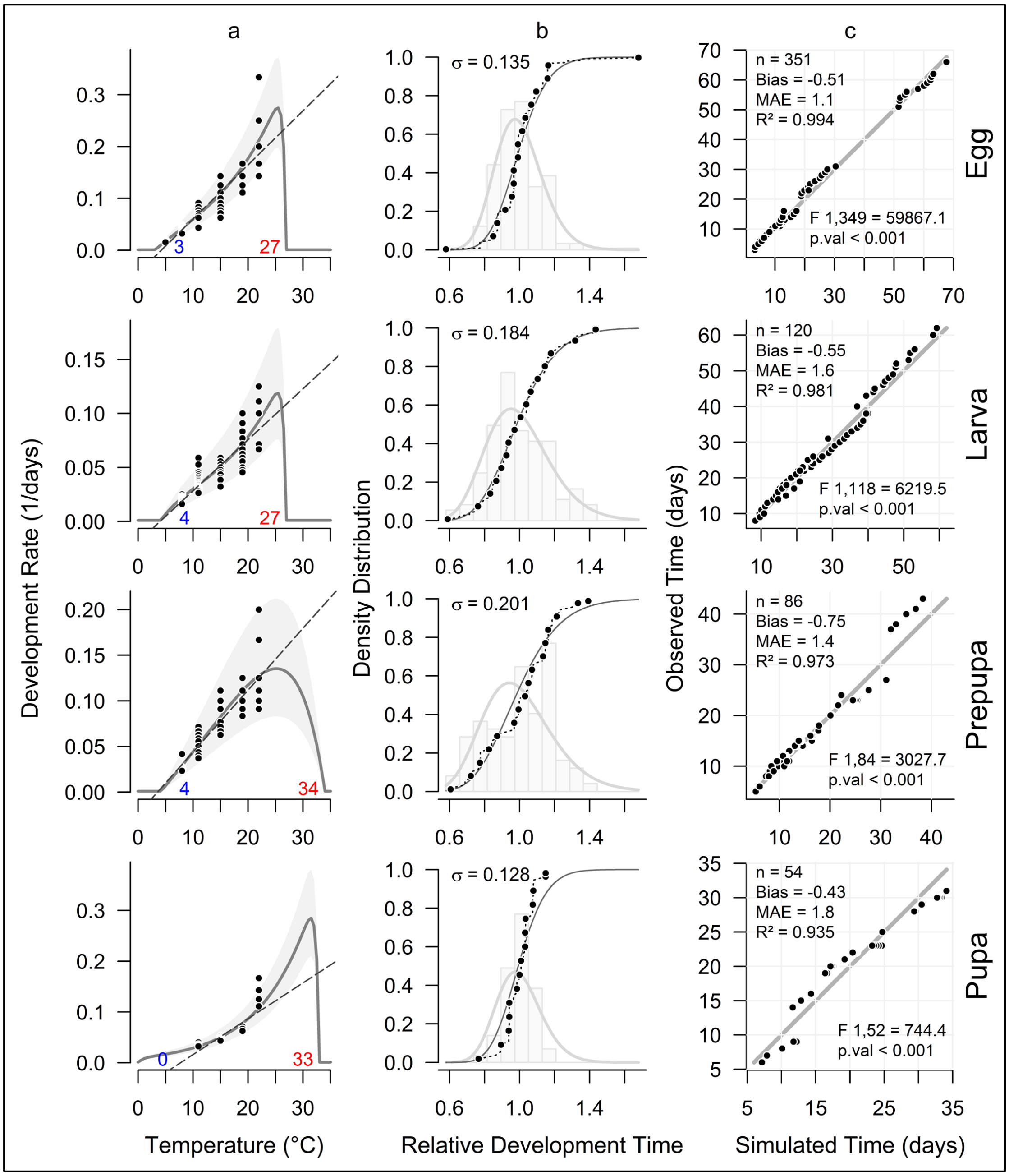


Figure 5.2. Survival of *Lariocbius osakensis* as a function of temperature in four life stages. The first row represents egg stage, followed by larva, prepupa, and pupa. The first column (a) shows non-linear equations predicting the daily survival (dark grey line) as a function of temperature with the underlying cumulative density distribution (grey). The second column (b) represents overall survival as a function of temperature, taking into account stage duration with the underlying cumulative density distribution (grey). The third column (c) represents overall survival as a function of temperature with the underlying cumulative density distribution (grey).

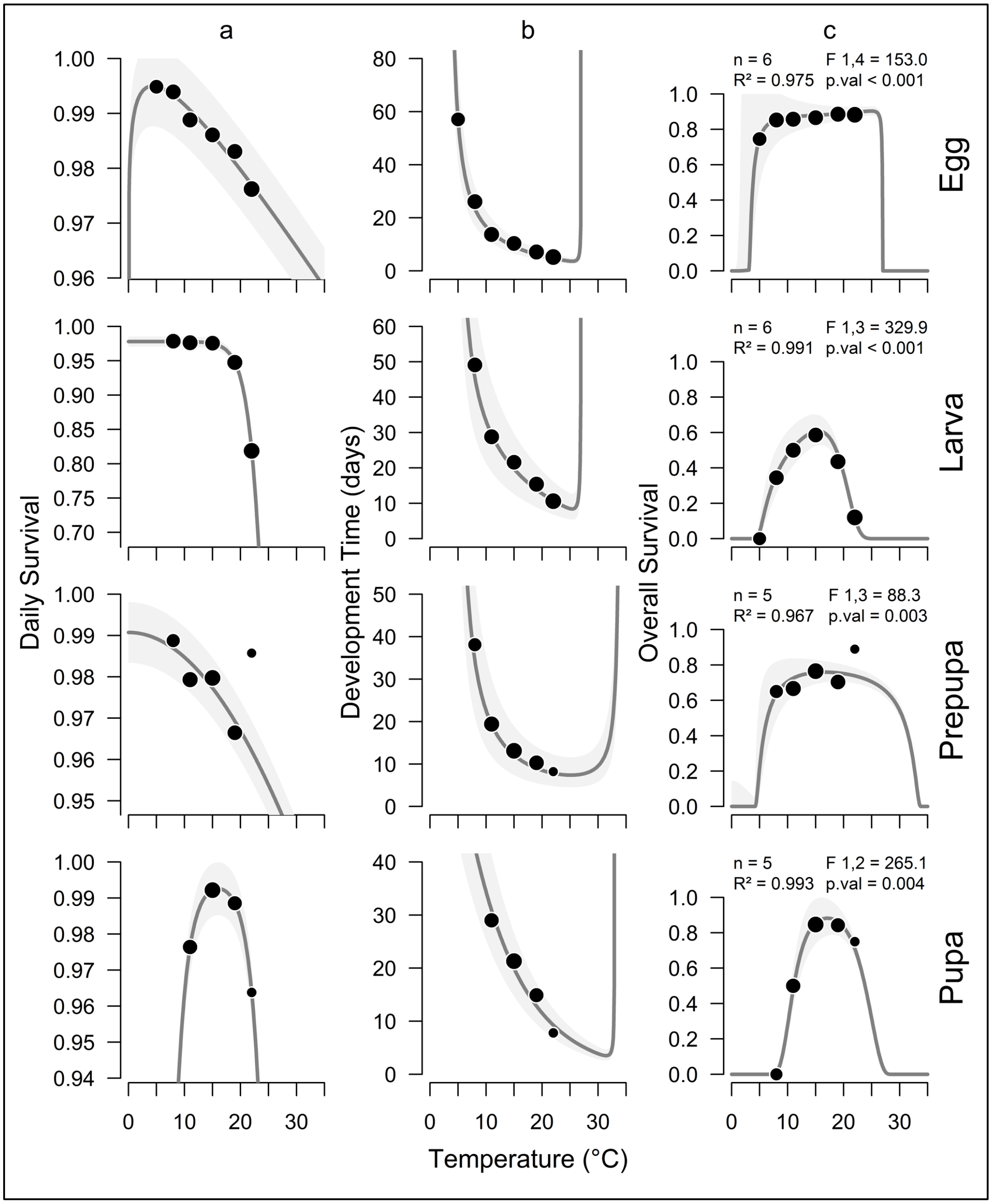


Figure 3. First row (left to right): *Laricobius osakensis* (a) Adult aging and (b) adult longevity as a function of temperature, (c) density distribution of relative longevity, and observed vs. simulated longevity comparison, respectively. Second row (left to right): (d) Oviposition ratio of remaining eggs rate as a function of temperature, (e) total fecundity as a function of temperature, (f) density distribution of relative fecundity, and observed vs. simulated brood comparison

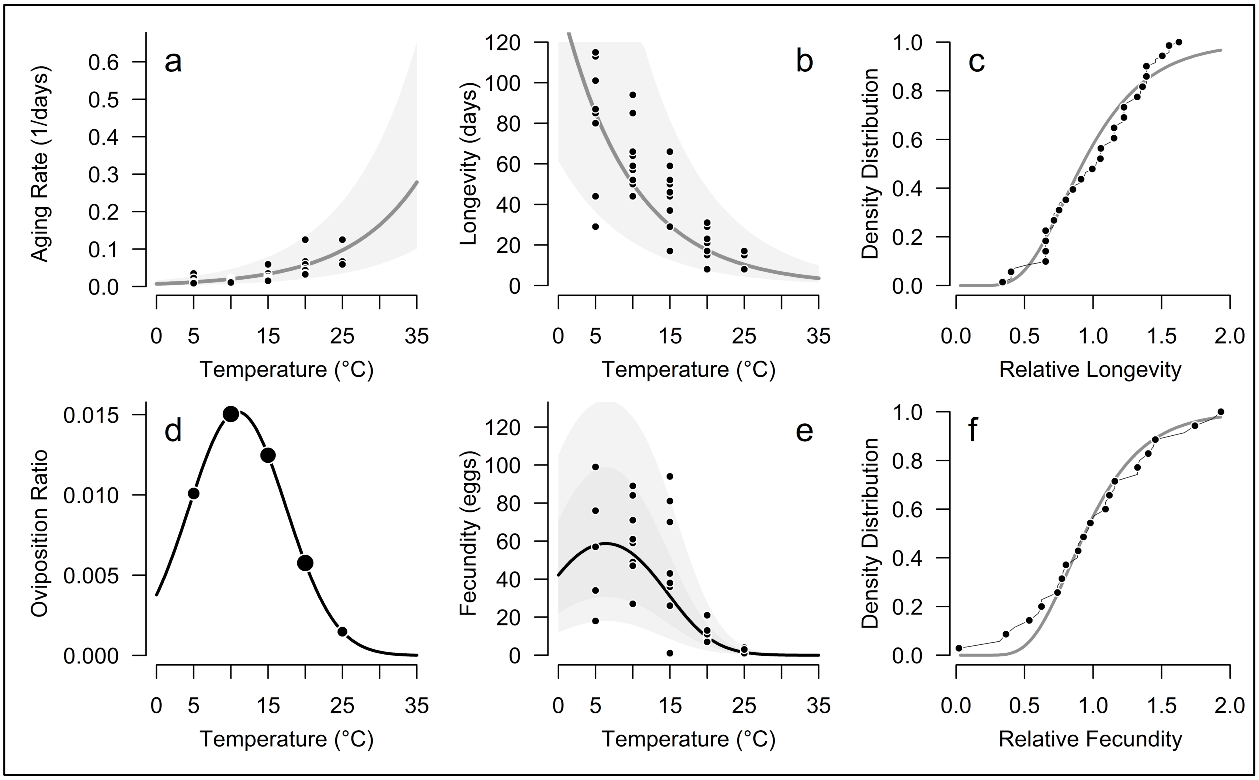


Figure 5.4. Oviposition rate for *Laricobius osakensis* at five constant temperatures. Boxes with the same letter are not significantly different at the 5% level (Tukey's HSD test). ANOVA (P < 0.01; F = 12.5; treatment df = 4; error df = 329).

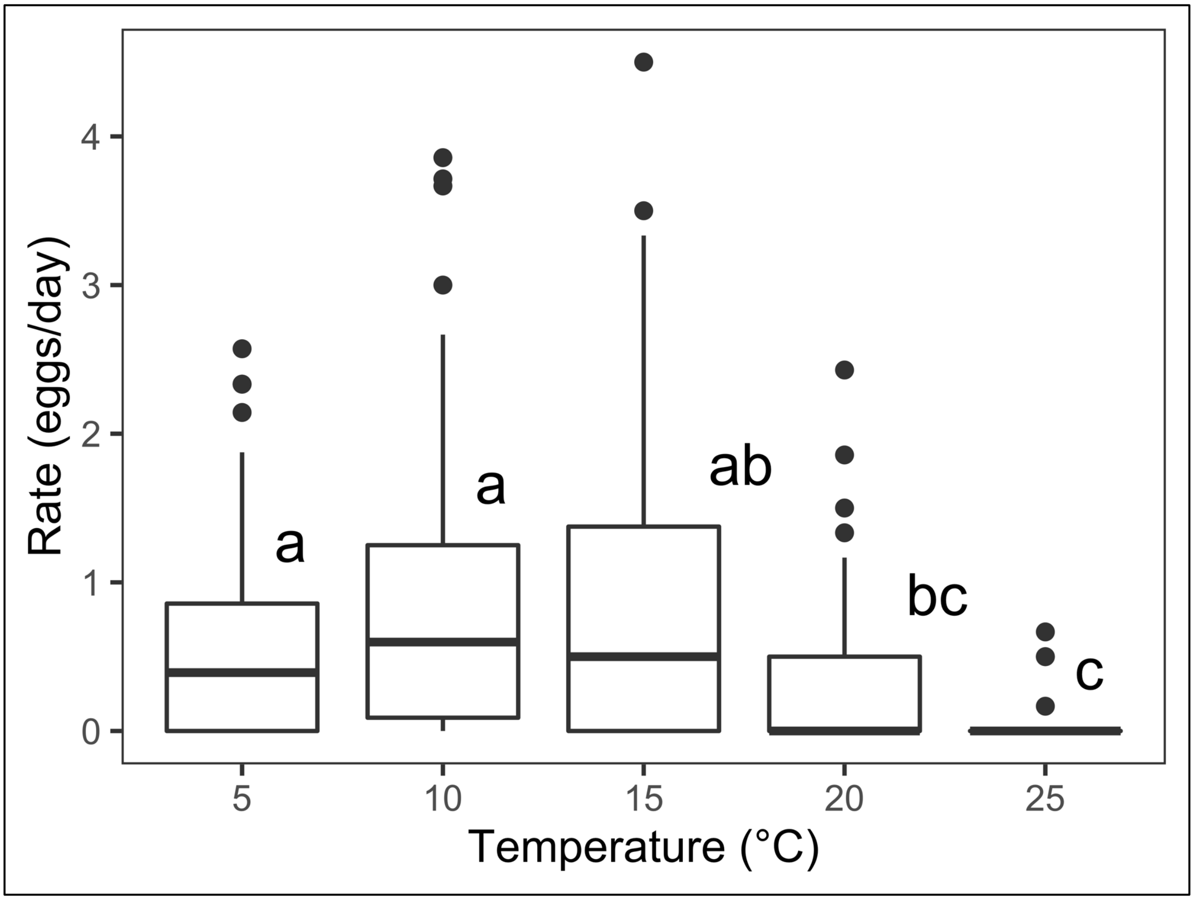


Figure 5.5. Observed and simulated abundance of eggs and larvae of *Laricobius osakensis* under field conditions at Hungry Mother State Park in 2020-2021. (a) number of individuals. (b) Cumulative.

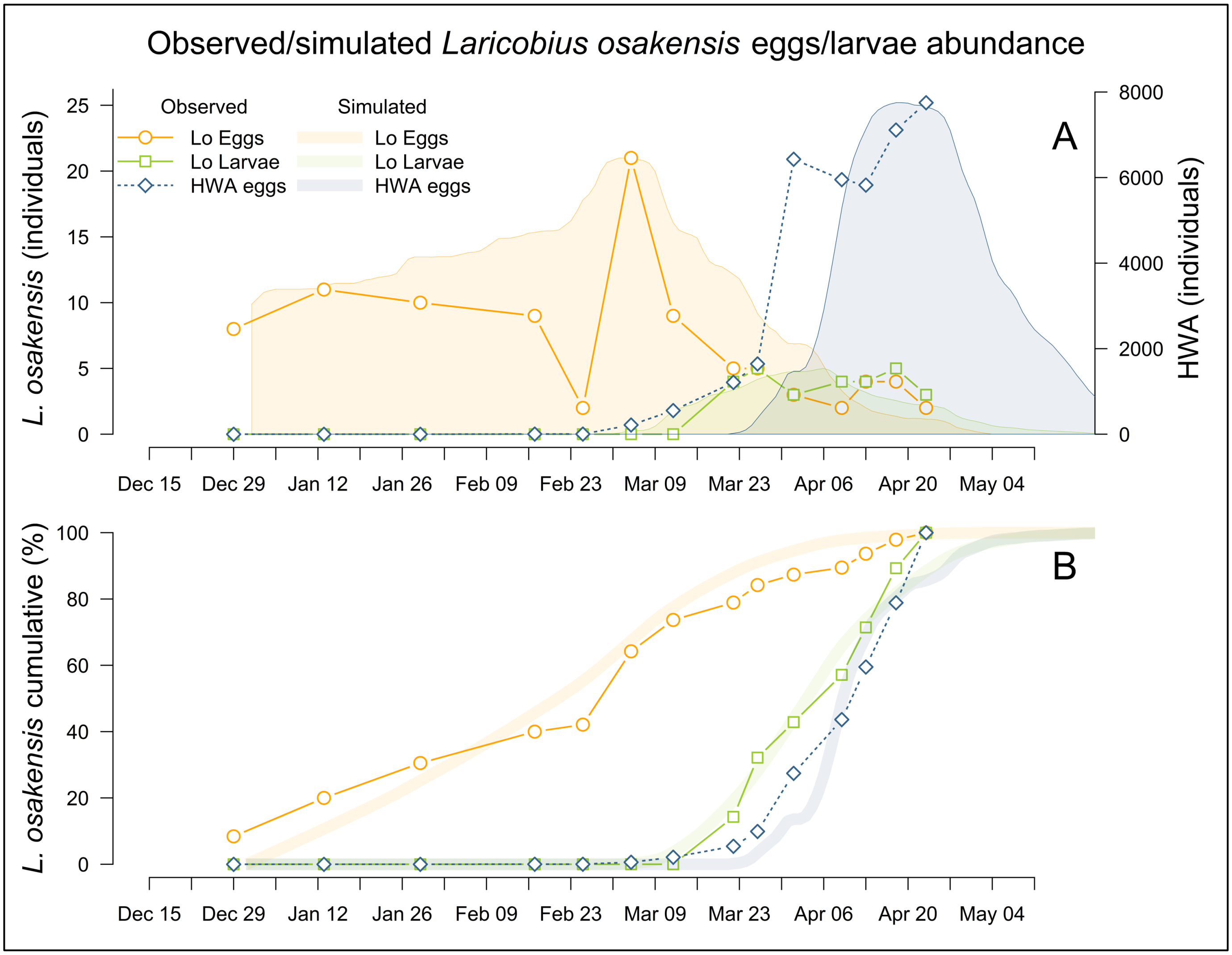
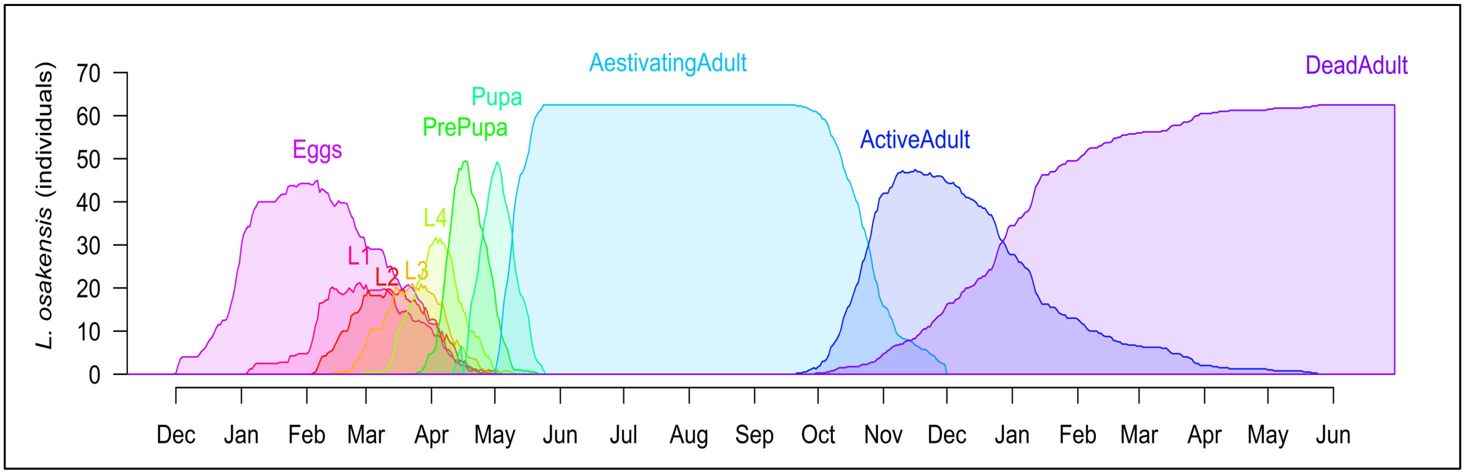


Figure 5.6. Number of *Laricobius osakensis* individuals in each successive life stage over time, as predicted by the model for Hungry Mother State Park from 2020-2021.



Conclusion:

In the seventy years since it was first identified in the eastern U.S, HWA has become one of North America's most injurious forest and urban tree pests. HWA impacts both Carolina and eastern hemlock. Since this initial invasion, multiple biological agents have been researched to help reduce this pest's impact on a landscape level, of which, *Laricobius* spp. have been arguably the most successful, well studied, and impactful. An often-used metric to classify a "successful" biological control agent is its dispersal and subsequent establishment success or failure. To that effect, *L. nigrinus* was documented to have established outside of release locations in multiple localities within the urban environment. One of the most widely used and effective tools for managing HWA infestations on a per tree basies is through the use of insecticides. However, in order to properly use insecticides as a treatment, homeowners and municipalities are required to have a basic knowledge on how to treat, when to treat, and where to treat. Upon discovering and learning about established biological control agents, in particular, *Laricobius* spp., on their property, the door opens to further conversations on what it means to manage and save their hemlocks. A conversation that usually ends with the property manager further understanding what it means to integrate *Laricobius* spp. as biological control agents with the use of preferential insecticide treatments. Thererfore, the urban establishment of these predator beetles is not just an added level of protection but a gateways for further conversations on how best to manage HWA-infested hemlocks on their property.

The findings from this research have contributed to our current understanding of the predator-prey complex shared between *Laricobius* spp. and HWA. While the specific research topics pursued were wide-ranging, they allowed for a deeper understanding of previously unknown or poorly understood biological attributes of these beneficial predators.

One of the main mechanisms that allowed for further hypothesis testing of this complex was through the formal documentation and analysis of the historical mass-production rearing data of *Laricobius* agents at Virginia Tech over the past a decade and a half. This historical analysis generated new avenues designed to increase production numbers and *Laricobius* spp. and thus density across the landscape by better understanding 1) the distribution and establishment of *Laricobius* spp. within urbanized localities, 2) the effect of laboratory and field rearing on *Laricobius* spp. subterranean survivorship and the timing of emergence in a field setting, and 3) the optimal thermal requirements and field sampling proficiency dates for *L. osakensis*.

Monitoring for this predator in urban habitats can help homeowners and municipalities determine how best to address the presence and potential impact of HWA-infested hemlocks.

The documentation and analysis of *Laricobius* spp. laboratory rearing practices highlighted the phenomena of early emergence and significant subterranean mortality. The field investigation into this confirmed that *Laricobius* spp. emergence is indeed not limited to the fall season and the degree of subterranean mortality observed in the lab is not an artifact of laboratory conditions to which beetles are exposed during the rearing process. Therefore, it is likely that the consistency of a relatively high rate of subterranean mortality and the timing of subterranean emergence are indicative of the natural mortality rates of these predatory beetles. This investigation also revealed that *Laricobius* beetles are subject to decreases in subterranean survivorship as moisture decreases and organic layer depth increases.

During quarantine studies prior to the request for release, biological evaluations are conducted to determine an agent's host range. These studies are often accompanied, although not always, with life history factors, phylogeny, morphometrics, and temperature developmental studies. The goals of these additional studies are to assemble enough evidence to identify and adequately understand the potential range of a relased biological control agent and to identify any potential negative factors associated with the agent. Temperature-dependent studies also aid in optimizing rearing conditions, predicting establishment, potential range, rates of dispersal, and help build phenological models to optimize sampling proficiency. The development rate of *L. osakensis* larvae was determined using six constant temperatures that spanned the spectrum of conditions *L. osakensis* was expected to tolerate. *L. osakensis* did not complete development from egg-to-adult at two of the lower temperatures tested (5 and 8°C), while it did complete development at the three highest temperatures (15, 19,and 22°C). Moreover, the rate of development was on average 38% quicker than for two other *Laricobius* agents from which temperature data were available. These temperature data also made it possible to forecast the phenology of *L. osakensis* larvae in order to confirm establishment at any given site. This is a valuable tool for researchers and land managers throughout the range of HWA infested hemlock.