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Research

Historic 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.

Key words: insect rearing, biological control, natural enemies, predators

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, 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 estivating 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, 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, Vieira et al. 2011, Salom et al. 2012), exhibit significant rates of predation (Jubb et al. 2020), 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 Fender (Coleoptera: Derodontidae) 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* 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, Fischer 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.

Methods and Materials

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 ecotypes 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 approval for release of *L. osakensis* in 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 (Fischer 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 Fig. 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 liters 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 (Fig. 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 (Fig. 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 prepupal stage, they drop from the branch into four-ounce Mason jars (Jarden Corporation, Rye, NY) attached to the bottom of the

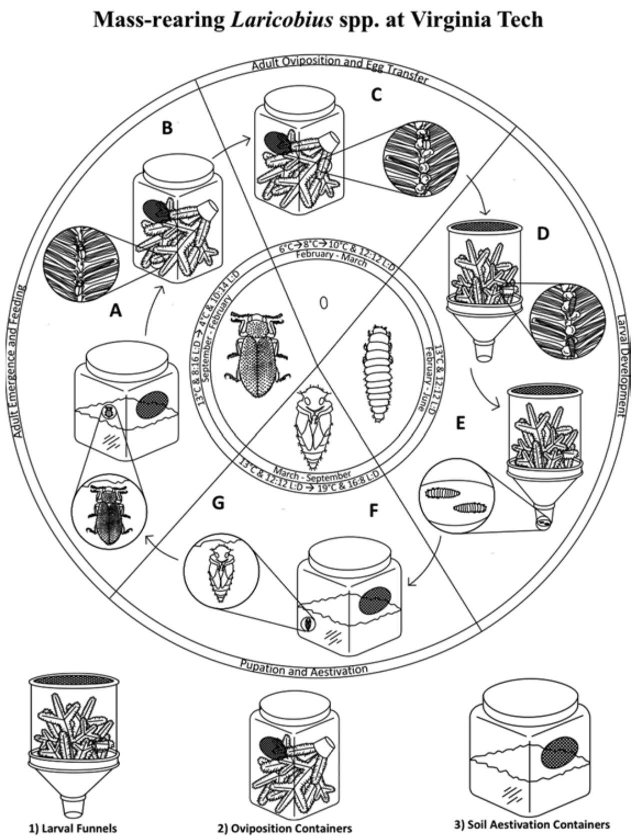

Fig. 1. Diagram of the specific rearing temperatures, shift in temperature treatments, and arenas used with respect to each distinct life stage (Center: egg, larva, pupa, and adult) for the production *Laricobius* spp. agents. There are three arenas used (bottom): 1) Larval funnels, 2) Oviposition containers, 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 P_1 reproductive adults for subsequent colony production and the rest are shipped to land mangers throughout the range of HWA infestations.

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 prepupae. If premature larvae (not yet prepupae) 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 prepupae located are removed, counted, and placed onto the soil in an estivation container with 5–7 cm of soil media composed of 2:1 peat moss:sand. Prior to adding prepupae, 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 estivation container, prepupae burrow into the soil and begin pupation. They are kept in estivation containers at a density of approximately 200 individuals per 820 cm^3 of soil (Fig. 1).

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

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 estivation break of HWA, beetles were given bouquets of hemlocks infested with first instar estivating HWA nymphs as a nutrient source (Fig. 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 estivating 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 wk 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 (Fig. 1). Host material and artificial diets are replaced every 2 wk. 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 yr; from 2004 to 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 1–3). Calendar dates reported for corresponding JD are for nonleap 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 1 and 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 1 and 3). Averages of each Pearson's correlations were calculated using a Fisher's *r*-to-*Z* transformation (Tables 1 and 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 nonparametric Kruskal–Wallis and Friedman tests. Kruskal–Wallis tests were conducted to determine whether 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 whether 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 1 and 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 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 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 17) to 190 (July 9) with a median of 128 (May 8) (Table 1 and Fig. 2). The average total number of days *L. osakensis* larvae dropped from 2010 to 2019 was 120 and ranged from JD 66 (March 7) to 186 (July 8) with a median of 116 (May 26).

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 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 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 1). The average correlation coefficient of survivorship versus first day underground for *L. nigrinus* is 64% larger than for *L. osakensis* (Table 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 3). The average subterranean survivorship, which includes both pupation and adult estivation, 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

Table 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	Minimum	25%	50%	75%	Maximum	Mean no. ± SD	Soil containers (n)	Coefficient (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*.
*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. 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

		Total	Soil containers	Julian date of adult emergence					
Year	Species	adults	(*n*)	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
	LO	2,391	86	163	294	303	310	351	300 ± 23.0
	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 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

				Survivorship vs. median days underground (Pearson's correlation)		
Year	Species	Subterranean survivorship (%)	Median days Underground[1]	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.

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 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 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 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 3). The average correlation coefficient of survivorship versus median days underground for *L. nigrinus* is 36% larger than for *L. osakensis* (Table 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 2) to 345 (December 11) and for *L. osakensis* was 158 (June 7) to 351 (December 17). The average median adult emergence JD for *L. nigrinus* ranged from 256 (September 13) to 318 (November 14) (Δ = 62 d) with mean and standard deviation of 296 (October 23) ± 18.4 from 2004 to 2019 (Table 2). The average median adult emergence JD for *L. osakensis* ranged 281 (October 8) to 313 (November 9) (Δ = 32 d) with mean and standard deviation of 297 (October 24) ± 20.8 from 2011 to 2019 (Table 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

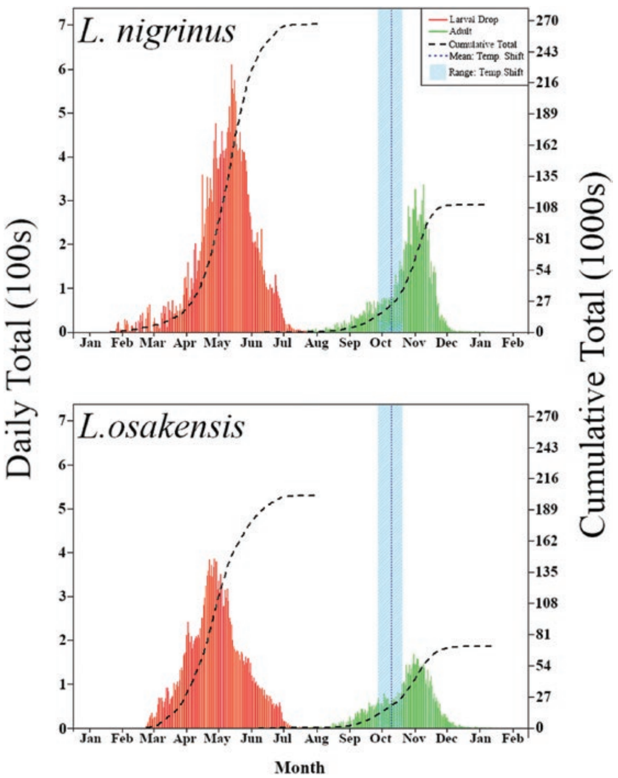

Fig. 2. Daily and cumulative larval drop (red) and subsequent adults emerged (green) from 2014 to 2019 for *L. nigrinus* (top) and *L. osakensis* (bottom). The blue dotted line (mean) and the surrounding light blue band shows the range in which the temperatures were changed from 19 to 13°C to stimulate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

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 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 second and third instars to the fourth prepupal 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 estivation.

Table 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**

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 and 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 (Fischer 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 estivation, when the insects are in their subterranean environment (Table 3). A second major challenge has been the early emergence of *Laricobius* spp. relative to their host, HWA (Table 2 and Fig. 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 mo), the insect pupates and enters into a period of presumed estivation. From 2004 until 2019, the average subterranean colony mortality for *L. nigrinus* and *L. osakensis* was 40 and 34%, respectively (Table 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, USA, 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 estivation) most susceptible to mortality, the effect of fourth instar larvae biomass on *Laricobius* spp. subterranean duration and survivorship, the effect of prepupa 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 d) is almost half compared to that of *L. nigrinus* (64 d). Based on these data, *L. osakensis* also remains in the soil longer than *L. nigrinus*.

When *Laricobius* spp. adults emerge before HWA breaks estivation, 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 (Fig. 1). The total number of adults for both species that emerged early from estivation from 2004 to 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 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 estivation in southwest Virginia. This reduction in laboratory temperature is usually initiated in early to mid-October around JD 274 (Fig. 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). 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 and Fig. 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 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 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 estivation 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 estivation container.

Challenge 3: Unintended Field Collections of *Laricobius* spp. Larvae

Laricobius nigrinus was first released from 2003 to 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 liters buckets, prior to bouquet construction, subsamples 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 fourth instar prepupal larvae are found in mason jars when the majority of the lab colony larvae are still developmentally younger (i.e., second 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 fourth instar prepupal *Laricobius* spp. larvae continued to be found in Mason jars, and therefore soil estivation 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. 2020). 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 prepupal 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 yr, 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 prepupae, 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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Author Contributions

JRF: Investigation; Writing – original draft; Writing – review & editing; Visualization; Conceptualization; Supervision; Funding acquisition; Methodology. CSJ: Writing – original draft; Writing – review & editing; Data curation; Methodology. ADS: Data curation; Formal analysis; Visualization; Writing; Conceptualization. DM: Writing – review & editing; Conceptualization. ALG: Writing – review & editing; Conceptualization. RB: Writing – original draft; Writing – review & editing; Conceptualization; Visualization. SMS: Funding acquisition; Supervision; Writing – original draft; Writing – review & editing; Conceptualization.

Conflicts of Interest

No conflicts of interest have been declared.

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