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Research

Biological Control - Parasitoids and Predators

Establishment and postrelease recovery of *Laricobius nigrinus* and *Laricobius osakensis* (Coleoptera: Derodontidae), released for biological control of *Adelges tsugae* (Hemiptera: Adelgidae), in the Northeastern United States

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Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is a major forest pest in the eastern United States responsible for killing millions of eastern hemlock, *Tsuga canadensis* (L.) Carrière and Carolina hemlock, *T. caroliniana* Engelmann. The US biological control program for HWA has largely invested in the rearing and release of *Laricobius nigrinus* Fender and more recently *L. osakensis* Montgomery and Shiyake. Though the establishment of *L. nigrinus* has been well-documented in the southern, mid-Atlantic, and coastal portions of the northeastern United States, documentation in interior areas of the northeastern United States is limited. Establishment of *L. osakensis* in the northeastern United States has not yet been documented. Release locations in the northeastern United States were surveyed for *L. nigrinus* and *L. osakensis* establishment to examine the relationship between establishment success and winter temperatures, as winter minimum temperatures likely limit the northern range of introduced *Laricobius* species. Our results suggest that *L. nigrinus* establishment is limited by winter minimum temperatures and that the probability of establishment declines as absolute minimum temperature declines. We found *L. nigrinus* established at sites in Maine, New York, and Pennsylvania, but did not recover any *L. nigrinus* in Massachusetts, New Hampshire, or Vermont. Similarly, we found *L. osakensis* established at sites in New York and Pennsylvania and recovered individuals in Maine, though further sampling is necessary to confirm presence of the F_3 generation. We also report the first field observation of reproduction of silver flies, *Leucotaraxis argenticollis* (Diptera: Chamaemyiidae), released predator of HWA, in the eastern United States.

Key words: establishment, hemlock, predation, winter mortality, adelgid

Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) has caused widespread mortality of eastern hemlock, *Tsuga canadensis* (L.) Carriere (Pinales: Pinaceae) and Carolina hemlock, *T. caroliniana* Engelmann, across their ranges in the eastern United States (Liebhold et al. 1995, Orwig et al. 2012). HWA has 2 parthenogenic generations per year, the overwintering sistens generation and the spring progrediens generation, and can produce over 100 eggs per adelgid (McClure 1991). High rates of HWA fecundity, a lack of resistance in native hemlock species, and no effective native natural enemies in the eastern United States (Montgomery and Lyon 1996, Wallace and Hain 2000) have allowed HWA populations to reach damaging levels on hemlock trees in the region. Hemlocks infested with HWA can die in as few as 4–6 yr in the southern portion of the range, and 10–20 yr in the colder northern portion of the range (McClure 1991, Eschtruth et al. 2013). Management of HWA has mainly focused on pesticide applications and biological control (Onken and Reardon 2011, Sumpter et al. 2018), however; insecticide use is not cost-effective at the forest scale and has non-target effects (Dilling et al. 2009), whereas biological control has potential to provide long-term, self-sustaining control of forest insect pests if successful (Debach and Rosen 1991).

The US HWA biological control program largely focuses on the release and establishment of specialist predators of HWA, with *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), releases beginning in 2003 (Mausel et al. 2010), and more recently, *L. osakensis* Montgomery and Skiyake releases beginning in 2012 (Toland et al. 2018). Over 500,000 *L. nigrinus* and 84,000 *L. osakensis* have been released in the eastern United States to date (Virginia Tech 2022). *Laricobius nigrinus* is native to northwestern North America and feeds on the western North American lineage of HWA whereas *L. osakensis* is native to southern Japan where it feeds on the Japanese lineage of HWA—the lineage invasive to the eastern United States (Havill et al. 2016). *Laricobius nigrinus* can hybridize with the native congener *L. rubidus* LeConte (Havill et al. 2012), whereas *L. osakensis* does not hybridize with *L. nigrinus* or *L. rubidus* (Fischer 2013, Fischer et al. 2015). Previously, Mausel et al. (2010) documented the establishment of *L. nigrinus* across a large portion of HWA's invaded range, however, many of the sites from that study were located in the southern United States and there have been few published efforts to document establishment and spread of *L. nigrinus* since then, with the majority of studies focusing on states south of New England (Foley et al. 2019, Jubb et al. 2021). Similarly, *L. osakensis* establishment has only been reported from south of New England (Toland et al. 2018), therefore, the ability for both species to establish in the northeastern United States has yet to be determined.

In New England, winters are much colder than in the southeastern United States (Skinner et al. 2003, Trotter and Shields 2009) which can result in higher levels of winter mortality of HWA (Paradis et al. 2008). High levels of winter mortality reduce the density of HWA ovisacs containing eggs on which *Laricobius* larvae feed. Mausel et al. (2010) suggested that *L. nigrinus* does not establish as easily in colder climates and likely requires larger releases for establishment to be successful. Following a rapid decline in winter temperatures associated with a 2011 polar vortex event in Massachusetts, *L. nigrinus* could not be recovered from sites where it had been previously established (JSE, personal observation). Though recovery attempts for introduced *Laricobius* species have continued, it has been over a decade since any published documentation of an established population of *Laricobius* species in New England, resulting in a knowledge gap in the literature. Understanding where and why *Laricobius* releases have been successful can help managers prioritize areas where *Laricobius* species are more likely to establish. Once release sites with established populations of *Laricobius* are identified, they can be used as source populations for releases elsewhere in the introduced range, reducing demand on mass-rearing facilities.

To address these needs, we visited *Laricobius* spp. release sites from 2020 to 2022 within the northeastern United States to document recovery (collection of F_1 or F_2 generation beetles) and establishment (collection of F_3 or later generation beetles) (Mausel et al. 2010). *Laricobius nigrinus* and *L. osakensis* are both univoltine and the generation of *Laricobius* released were considered the parental generation (P_1). Subsequent generations were considered F_1, F_2, and so on. By sampling HWA-infested hemlock trees at past release sites for *Laricobius* we aimed to (i) document recovery and/or establishment of *Laricobius* species within release sites and (ii) determine if and how winter temperatures and extreme cold events affect *Laricobius* establishment success.

Methods

Site Selection

In all years of study, we queried the HWA predator database (Virginia Tech 2022) to identify release sites for introduced *Laricobius* species. In 2020, we selected past *L. nigrinus* release sites in Massachusetts with detectable levels of HWA for sampling. In 2021, we expanded our search to all New England states with releases of 600 or more *L. nigrinus* and *L. osakensis*. Finally, in 2022, we sampled release sites in New York and Pennsylvania that received releases of 200 or more *L. nigrinus,* and any *L. osakensis*, and also resampled 4 sites in Massachusetts. The distribution of sites sampled in all 3 yr is shown in Fig. 1. Sites were distributed across a range of USDA Plant Hardiness Zones (PHZs) (https://planthardiness.ars.usda.gov/, USDA 2012), defined as a range of average annual extreme minimum winter temperatures, from zone 5a (−28.9 and −26.1 °C) to zone 7a (−17.8 and −15 °C). *Laricobius* release sizes, release years, and winter minimum temperatures at each site are listed in Table 1.

Laricobius Surveys

We timed collections of predator samples to coincide with peak progrediens egg abundance using phenology and degree-day models available at https://uspest.org/dd/model_app (Berry and Coop 2000) to estimate growing degree days (general purpose model, double sine method, lower threshold of 4°C, upper threshold of 54 °C, 1 January start date) and match them with growing degree days associated with HWA phenological events (50–95% progrediens egg abundance as well as 0–25% for the progrediens generation) described in Tobin and Turcotte (2018). These phenological events were chosen as the peak abundance of progrediens eggs, which coincides with peak abundance of *Laricobius* larvae (Zilahi-Balogh et al. 2003). Site visits for all years occurred between early April to mid-May.

To collect *Laricobius* adults, larvae, and prepupae, we beat-sheet sampled heavily HWA-infested hemlock branches and removed branch clippings to rear *Laricobius* in the laboratory. All branches sampled using both techniques were within arm's reach (0–2.5 m above the ground) except in Massachusetts sites in 2022 where pole pruners were used to sample the upper canopy (about 5.5–6 m above the ground). For beat-sheet sampling, we sampled 3 heavily infested 1-m-long branches on 5 trees by tapping branches with a wooden rod 10 times to knock predators onto a (60 cm × 60 cm) canvas square. We then used aspirators (Aspirator, Catolog #1135A, BioQuip, Ranch Dominquez, CA, USA) to collect predators from the canvas square into collection vials marked with site names and dates and then transferred in the laboratory to 1.5-ml microcentrifuge tubes (Thermo Fisher Scientific, Agawam, MA) with 95% ethanol and stored at − 80 °C for later DNA identification.

For branch sampling at each site, we collected one 30-cm-long terminal branchlet, heavily infested with HWA sistens ovisacs, from 5 trees by carefully clipping branchlets, so as to not dislodge predators, and placing them into individual 1-gallon zipper bags in the field to be transported back to the laboratory. In the laboratory, we removed samples from zipper bags, clipped the bottom 2 cm of each branchlet to reduce the risk of cavitation in the hemlock twig from disrupting water uptake and then placed them in water cups to keep samples hydrated. Samples in water cups were placed into rearing tubes (small Berlese funnel trap, Catalog #2845, BioQuip,

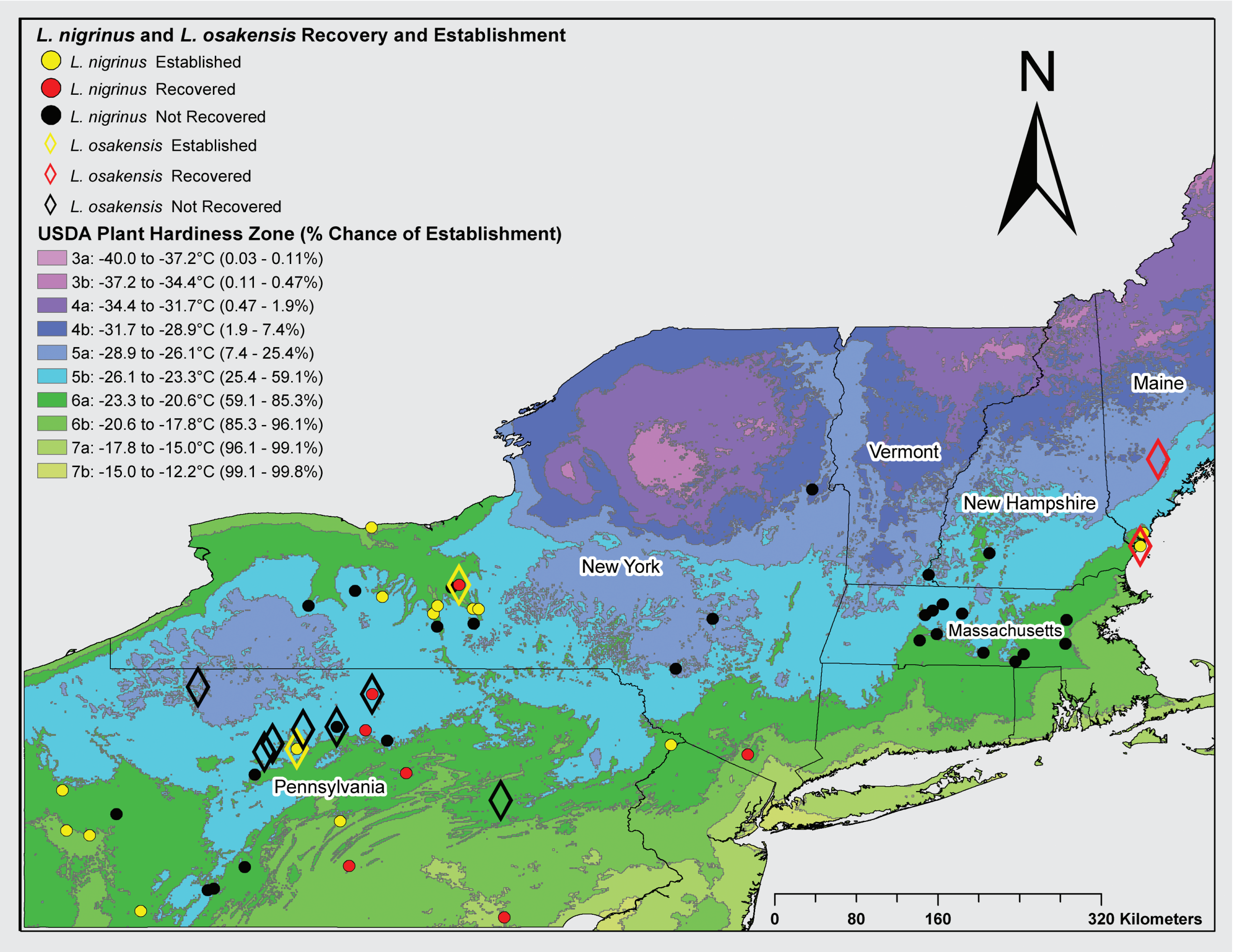

Fig. 1. Release locations with recoveries of *L. nigrinus* and *L. osakensis* sampled from 2020 to 2022 in Maine, Massachusetts, New Hampshire, New York, Pennsylvania, and Vermont with USDA PHZ. Percent chance of establishment in each USDA PHZ was calculated using logistic regression.

Rancho Dominquez, CA, USA) and stored at room temperature (21.1–23.9 °C) with ambient natural lighting. Rearing tubes were monitored weekly until *Laricobius* prepupae began to drop, and then were checked daily to transfer prepupal *Laricobius* that had dropped to vials with 95% ethanol to avoid samples desiccating or decomposing in the bottom of the rearing tubes.

In 2020 and 2021, we collected beat-sheet and rearing-tube samples. In 2022, due to the number of sites sampled, we limited collections to rearing-tube samples. In 2022, cooperators from New York and Pennsylvania collected hemlock clippings according to our protocols and shipped samples on ice overnight to our laboratory for processing, whereas samples from Massachusetts sites were collected and transported to the laboratory by car on the same day.

Laricobius Identification

Adult *L. nigrinus*, *L. osakensis*, and *L. rubidus* can be distinguished from each other using morphological features (except for hybrids of *L. nigrinus* and *L. rubidus*), however, identification of larval stages of these 3 species can only be accomplished with molecular techniques (Davis et al. 2011). To identify larval and prepupal *Laricobius*, we employed 2 types of DNA-based approaches. All samples collected in 2020 and 2021 were identified with DNA barcoding (described below), while samples collected in 2022 were identified based on restriction length polymorphisms (RFLPs) with a subset sequenced to verify results. For both approaches, whole genomic DNA was isolated from HWA predators using the Omega BIO-TEK Mag-Bind Blood & Tissue DNA HDQ 96 Kit (Omega BIO-TEK, Norcross, Georgia, USA) following the manufacturer's protocol, except for the following modification: To preserve larval cuticles as morphological voucher specimens, each sample was pierced using a 000-insect pin instead of homogenization prior to cell lysing. After cell lysing, larval cuticles were removed and stored in 95% ethanol. PCR amplification of the mitochondrial locus Cytochrome Oxidase I (COI) was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA), following the manufacturer's protocol with the primer pair LepF/LepR (Hebert et al. 2004), using the thermocycler protocol presented in Hebert et al. (2003). For samples collected in 2020 and 2021 the amplified fragments were cleaned using ExoSap digestion (Thermo Fisher Scientific, Agawam, MA) and sequencing was performed at the Keck DNA Sequencing Facility at Yale University. Forward and reverse sequence reads were then edited in Geneious R11.1.2 (Biomatters Ltd., Auckland, New Zealand), and consensus sequences were compared to published sequences in the NCBI GenBank database using the "blastn" search algorithm (Altschul et al. 1990).

Table 1. Winter minimum temperatures and release years and sizes at sites sampled in the Northeast from 2020 to 2022

							No. recovered, by year[a,b]					
					No. Released		2020		2021		2022	
State	City	Mean min temp (°C)	Abs min temp (°C)	Release years	Ln[c]	Lo[d]	Ln	Lo	Ln	Lo	Ln	Lo
MA	Amherst	–6.9	–26.3	2008, 2011	300	–	0	–	–	–	–	–
	Deerfield	–7.2	–26.9	2008, 2013	865	–	0	–	–	–	0	–
	Douglas	–5.7	–25.1	2005	300	–	0	–	–	–	–	–
	Easthampton	–6.6	–25.8	2004, 2008–2010, 2018	3,010	–	0	–	–	–	0	–
	Lincoln	–5	–23.6	2017, 2018	1,030	–	0	–	0	–	–	–
	Petersham	–7.7	–27.6	2013, 2020	1,710	–	0	–	0	–	–	–
	Sherborn	–5.3	–24.5	2007	300	–	0	–	–	–	–	–
	Sturbridge	–6.5	–25.8	2013	1,200	–	0	–	0	–	–	–
	Sunderland	–7.6	–27.3	2007–2010	993	–	0	–	–	–	0	–
	Sutton	–5.1	–20.9	2018	510	–	0	–	–	–	0	–
	Wendell	–8.2	–28.7	2007, 2008, 2010	367	–	0	–	–	–	–	–
ME	Frye Island	–8.9	–27.5	2016, 2017, 2019	–	2,000	–	–	–	126	–	–
	Kittery	–5.5	–24.1	*Ln*: 2006–2008 *Lo*: 2019–2021	900	1,320	–	–	**347**	40	–	–
	York	–6.9	–24.8	2007–2010	3,471	–	–	–	**314**	–	–	–
NH	Antrim	–7.5	–26.6	2014	798	–	–	–	0	–	–	–
NY	Aurora	–4.1	–19.5	*Ln*: 2019, 2020 *Lo*: 2019	1,055	133	–	–	–	–	14	**21**
	Bolton	–8.8	–26.5	2020	620	–	–	–	–	–	0	–
	Glenora	–5	–23.2	2009	200	–	–	–	–	–	**0****	–
	Hayt Corners	–5.6	–25.2	2013	800	–	–	–	–	–	0	–
	Lansing 1	–5.2	–24.7	2009	200	–	–	–	–	–	**16**	–
	Lansing 2	–6.5	–26.6	2009	300	–	–	–	–	–	**0****	–
	Lew Beach	–9	–29.1	2020	478	–	–	–	–	–	0	–
NY	Lodi	–5.1	–23.3	2009	300	–	–	–	–	–	**79**	–
	Naples	–6.3	–24.7	2016	444	–	–	–	–	–	**18**	–
	North Blenheim	–8.5	–29	2013	530	–	–	–	–	–	0	–
	Portageville	–7.2	–26.3	2013, 2018	746	–	–	–	–	–	0	–
	South Hill	–6.2	–24.6	2012	440	–	–	–	–	–	0	–
	Southfields	–3.9	–20.4	2018–2020	2,249	–	–	–	–	–	113	–
	Springwater	–8	–28.4	2013	450	–	–	–	–	–	0	–
	Watkins Glen	–5.6	–23	2013	450	–	–	–	–	–	0	–
	Webster	–4.5	–19.9	2019, 2020	973	–	–	–	–	–	**22*****	–
PA	Aristes	–5.7	–23.5	2013	–	500	–	–	–	–	–	0
	Benezette Twsp 1	–6.2	–25	2015	–	714	–	–	–	–	–	0
	Benezette Twsp 2	–6.3	–22.6	2020	–	250	–	–	–	–	–	0
	Blain	–3.4	–18.4	2019, 2020	1,229	–	–	–	–	–	30	–
	Buffalo Township	–4.5	–22.1	2020, 2021	929	–	–	–	–	–	0	–
	Cammal 1	–7	–26.2	2008, 2021	660	–	–	–	–	–	2	–
	Claysburg	–5	–23.8	2010	1,000	–	–	–	–	–	0	–
	Cross Forks	–7.7	–29.4	Ln: 2013 Lo: 2020	289	507	–	–	–	–	0	0
	Cummings Twsp	–7.1	–25.6	2007	500	–	–	–	–	–	0	–
	Drumore	–1.7	–13.3	2020	510	–	–	–	–	–	11	–
	Gibson Twsp	–6.2	–24.6	Ln 2009 Lo 2015	500	1,021	–	–	–	–	**1**	**3**
	Glenshaw	–2.8	–17.9	2019	500	–	–	–	–	–	**6**	–
	Grove Twsp 1	–7.3	–30.2	2014, 2015	–	1,000	–	–	–	–	–	0
	Huntingdon	–5.7	–23.3	2003	600	–	–	–	–	–	**0****	–
	Indian Lake 1	–5.5	–22	2021	772	–	–	–	–	–	0	–
	Indian Lake 3	–5	–21.3	2020, 2021	817	–	–	–	–	–	0	–
NH	Mead Twsp 2	–6.3	–23.7	2019	–	1,020	–	–	–	–	–	0
	Milford	–5.9	–26.2	2021	510	–	–	–	–	–	**44****	–
	Plymptonville	–5.4	–23.1	2016, 2017	946	–	–	–	–	–	0	–
	Portersville	–4.9	–23.2	2018	1,625	–	–	–	–	–	**2**	–
	Sewickley	–4	–22.4	2012, 2018	1,020	–	–	–	–	–	**6**	–
	Stewart Twsp 1	–4.9	–24.4	2013, 2017	4,026	–	–	–	–	–	**9**	–

Table 1. Continued

State	City	Mean min temp (°C)	Abs min temp (°C)	Release years	No. Released		No. recovered, by year[a,b]					
							2020		2021		2022	
					Ln[c]	Lo[d]	Ln	Lo	Ln	Lo	Ln	Lo
	Washington Twsp 2	−7.7	−26.3	2005, 2017, 2021	1,676	–	–	–	–	–	1	–
	Wellsboro	−6.5	−24.6	2021	–	500	–	–	–	–	4*	0
VT	Brattleboro	−7.7	−27.5	2009, 2012, 2017, 2019	1,245	–	–	–	0	–	–	–

[a] Bolded recoveries indicate establishment.

[b] Asterisks: * indicate those where the *Laricoibus* species recovered was not released; ** indicate those with established populations of introduced *Laricobius* based on records from the HWA Predator Database or state managers; *** indicate recoveries at sites where a spring 2021 release of adult *Laricobius* (P_1) occurred, therefore F_1 larvae would have been present that same year (2021), and in turn, F_3 larvae were present during 2022 collections.

[c] *Laricobius nigrinus*.

[d] *Laricobius osakensis*.

For samples collected in 2022, identifications were performed based on RFLP differences. Previously, Davis et al. (2011) developed an RFLP approach to differentiate *L. nigrinus* and *L. rubidus* based on the use of the restriction enzymes AseI and HpaII. Unfortunately, *in silico* analyses of published sequences in GenBank indicated that AseI cannot differentiate *L. nigrinus* from *L. osakensis* as both species have the same cut sites, and that the patterns based on HpaII might be too similar for *L. nigrinus* and *L. osakensis* to accurately differentiate these species (*L. nigrinus* is expected to have 2 bands one of ~320 bp and one of ~340 bp, *L. osakensis* is expected to have 2 bands of ~250 and ~410 bp). Therefore, we developed a novel assay based on 2 different restriction enzymes, SacI-HF and BstNI (New England Bio Labs, Ipswich, MA). PCR products for each sample were generated as per above, and then digested with the addition of 0.5 µL of SacI-HF at 37°C for 15 minutes. SacI-HF was then heat-inactivated by incubating the digested product at 65°C for 20 minutes. To this product, 0.5 µL of BstNI was added, and the PCR product digested at 60°C for 15 minutes. The resulting double-digested PCR product was then visualized on a 2% agarose gel in comparison to the New England Bio Labs 100 bp Quick-Load Ladder. Under this approach, fragments amplified from *L. nigrinus* are predicted to have 3 bands (~85, ~180, and ~390 bp), fragments amplified from *L. osakensis* are predicted to have 4 bands (~85, ~140, ~180, and ~255 bp), and fragments amplified from *L. rubidus* are predicted to have 2 bands (~220, and ~440 bp). A subset of samples (95 in total) were re-amplified and the PCR product was submitted for DNA sequencing as per above, to verify the accuracy of the RFLP assay.

Collection and Processing of Weather Data

Daily minimum temperatures from 2003 to 2022 were acquired from PRISM (PRISM Climate Group 2022). Weather data were downloaded and extracted using the R package "prism" (Hart and Bell 2015) and winter (December–March) temperature data were summarized using the R package "data.table" (Dowle and Srinivasan 2020). From these data, we calculated the mean minimum temperature and the absolute minimum temperature for each site between the first *Laricobius* spp. release and 2022. Temperature data collection and manipulation were conducted in R version 3.6.2 (R Core Team 2022).

Model Selection and Data Analysis

We assessed the effect of winter minimum temperatures (mean minimum winter temperature and absolute minimum temperature) on the establishment of *L. nigrinus* at field sites in Massachusetts, Maine, New Hampshire, New York, Pennsylvania, and Vermont. Establishment was defined as a recovery of F_3 generation *L. nigrinus* adults, larvae, or prepupae. Sites where F_3 generation *Laricobius* could not have been recovered or where F_3 and later or earlier generations could have both been collected (collections made too soon after release events) were not included in the analysis. A site in Pennsylvania which had many recoveries of *L. nigrinus* near the Delaware Water Gap National Recreation Area, located along the border of Pennsylvania and the northwestern corner of New Jersey, was also considered established based on recoveries of *L. nigrinus* in the HWA database which predated releases. The Delaware Water Gap region is one with several established *L. nigrinus* sites and releases dating back to 2003. Two sites in New York where we did not collect *L. nigrinus* were also considered established based on recovery records in the HWA Predator Database (Virginia Tech 2022). We could not analyze the effect of cold weather on *L. osakensis* establishment because we sampled too few sites with releases old enough to sample for F_3 or older beetles.

For model selection, we fit all combinations and interactions of winter minimum temperature, release size variables, years since first release, and years since last release variables as generalized linear models with a binomial distribution to analyze the effects of winter minimum temperatures, release sizes, and years since the first and/or last release on establishment of *L. nigrinus* in R version 4.2.2 (R Core Team 2022). We then created a list of all models and used Akaike's Information Criteria corrected for small samples sizes (AICc) (Akaike 1973) and Bayesian Information Criteria (BIC) (Schwarz 1978) to choose the best-fit models (Burnham and Anderson 2002). We defined the best-fit models as those which had a delta AICc and BIC score less than 2 while also considering one model with a value of 3.10 due to its utility in mapping the probability of *L. nigrinus* establishment in USDA PHZs. Logistic regression of establishment by absolute minimum winter temperature was plotted using ggplot2 (Wickham 2009) in RStudio (Posit Team 2022).

Results

Laricobius Recoveries, COI Identification, and Establishment

DNA barcode sequences from 343 samples of *Laricobius* were generated during this study. Two hundred and seventy sequences were of sufficient quality and length to be submitted to GenBank (Accession Numbers: OR000449-OR000719). Comparison of a subset of restriction-digested samples based on our double-digest approach indicated that 55 of 56 samples (98.2%) of *L. nigrinus,* 19 of 20 samples (95%) of *L. osakensis,* and all 5 *L. rubidus* samples were correctly identified with this approach. We recovered *Laricobius* adults, larvae, and prepupae in all survey years, and DNA analyses confirmed that while *Laricobius rubidus* was only collected in Massachusetts, all 3 *Laricobius* spp. were established and/or recovered at one or more sites in Maine, Pennsylvania, and New York. No *Laricobius* were recovered from New Hampshire or Vermont. *Laricobius nigrinus* and *L. osakensis* recoveries by year and site can be found in Table 1.

Laricobius nigrinus was recovered at 18 out of 49 release sites and at one *L. osakensis* release site during 2020–2022 sampling (Table 1, Fig. 1). We found that *L. nigrinus* was established at 15 sites (Table 1, Fig. 1); at 3 of these sites, beetles were not recovered, but previous recoveries of the F_3 generation and older were documented for these sites in the HWA Predator Database (Virginia Tech 2022). Six of the sites where we recovered *L. nigrinus* had recent releases and, therefore, we were not able to determine if the specimens collected were F_3 or older generation beetles, so we did not consider these sites to be established. *Laricobius osakensis* was recovered at 4 of 11 sites across the Northeast from 2020 to 2022 (Table 1, Fig. 1), and was found to be established at 2 of them.

Effect of Winter Temperatures on Establishment

Results from model selection indicated that 2 models, assessing the establishment of *L. nigrinus* by mean minimum winter temperature (model 1) and mean minimum winter temperature and average release size (model 2), were both considered to be the best-fit models (i.e., ΔAICc or ΔBIC <2) (Table S1). We also chose to use a model which had a ΔAICc and ΔBIC of 3.10 because it allowed us to assess establishment by absolute minimum winter temperatures (model 3), which is useful for making predictions of establishment success for USDA PHZs, which use the average absolute winter minimum temperatures to define zones. Results of models 1 and 2 were nearly identical, with the additive term for average release size having no significant effect on establishment (Table 2). For this reason, we chose to report the results of the reduced model (model 1) which found that mean minimum winter temperature since first release at each site significantly affected the establishment of *L. nigrinus* (model 1: $P = 0.006$, pseudo$R^2 = 0.239$, LL = −19.00, RMSE = 0.423) (Table 2). Absolute minimum winter temperature since first release had a similar effect on *L. nigrinus* establishment (model 3: $P = 0.015$, pseudo$R^2 = 0.177$, LL = −20.55, RMSE = 0.438) (Table 2). Results did not differ qualitatively between model 1 and 3 so we also present model 3 results (Fig. 2) which may be more applicable for managers since it captures the effect of extreme cold events that occur periodically and have been associated with significant mortality of HWA (Tobin et al. 2017). Model 3 also enables the prediction of establishment success for each USDA PHZ which are defined by absolute minimum winter temperature.

Effect of Size of Release Population

Model selection indicated that models fit with release size variables (models 4 and 5) did not fit as well as models including winter minimum temperature variables. Indeed, we found that models analyzing establishment by release size were not significant. We found that total release size (model 4) did not significantly affect establishment ($P = 0.411$, pseudo-$R^2 = 0.014$, LL = −24.63, RMSE = 0.486) and neither did average release size (model 5) ($P = 0.484$, pseudo-$R^2 = 0.011$, LL = −24.72, RMSE = 0.488) (Table 2).

Table 2. List of model parameter estimates and measures of model performance for candidate models

Model no.	Estimate [95% CI][a]	R^{2b}	LL	RMSE
1. *Estab*[c]		0.239	−19.00	0.423
(Intercept)	6.59 [2.27, 12.34] **			
MM[d]	**1.17 [0.45, 2.17]** **			
2. *Estab*		0.281	−17.95	0.409
(Intercept)	8.60 [3.34, 1.56e + 01] **			
MM	**1.29 [0.53, 2.32e + 00]** **			
AR[e]	−0.003 [−0.01, 9.90e − 04]			
3. *Estab*		0.177	−20.55	0.438
(Intercept)	12.40 [3.46, 24.63] *			
AM[f]	**0.52 [0.16, 1.01]** *			
4. *Estab*		0.014	−24.63	0.486
(Intercept)	−0.68 [−1.69, 0.27]			
TR[g]	0.0003 [−0.0004, 0.001]			
5. *Estab*		0.011	−24.72	0.488
(Intercept)	0.13 [−1.39, 1.82]			
AR	−0.001 [−0.01, 0.002]			

[a] Italicized font indicates response variables. Significant effects are bolded (excluding intercepts). Asterisks: ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.

[b] R^2 is an approximate measure of the proportion of variance explained by the model calculated as McFadden pseudoR^2 statistic.

[c] Establishment.

[d] Mean minimum winter temperature (°C).

[e] Average release size.

[f] Absolute minimum winter temperature (°C).

[g] Total released.

Discussion

From field sites across the northeastern United States, we were able to recover individuals of the introduced predators *Laricobius nigrinus*, and *L. osakensis*, and their native congener *L. rubidus*, the majority of which were recovered as larvae and prepupae from spring beat-sheet sampling and rearing from HWA-infested branch clippings. Results from our analyses demonstrate a significant effect of winter temperature (absolute minimum and mean minimum winter temperatures) on the establishment of *L. nigrinus* in the Northeast. These results suggest that the probability of *L. nigrinus* establishment decreases in colder climates, USDA PHZs 5b and lower (average absolute winter minimum temperatures of −23.3°C and lower; Fig. 2). Like their HWA prey, *L. nigrinus* is not as tolerant of extreme winter cold as the native *L. rubidus* (Toland et al. 2019). This means that in colder climates (USDA PHZ 5b and lower), *L. nigrinus* is likely subject to high winter mortality due to greater exposure to lower lethal temperatures and food scarcity due to increased HWA winter mortality. Based on these results, we recommend focusing releases of *L. nigrinus* in USDA PHZs 5b and above due to the increased probability of successful establishment.

Sites with *L. nigrinus* establishment ranged from USDA PHZs from 5b (−26.1 to −23.3°C) to 6b (−20.6 to −17.8°C) (Fig. 1). These results suggest *L. nigrinus* has the potential to establish across much of the interior of New England where USDA PHZ is currently 5b (−26.1 and −23.3°C) and above, which represents successful establishment in colder regions than previously predicted (Mausel et al. 2010), though populations densities in colder zones may remain lower than in warmer zones. Mean minimum temperatures and absolute minimum temperatures at sites with established *L. nigrinus* ranged from −2.8 to −6.9°C and −17.9 to −26.6°C (Table 1), respectively. These data suggest that *L. nigrinus* populations can persist through extreme cold temperatures known to drastically reduce

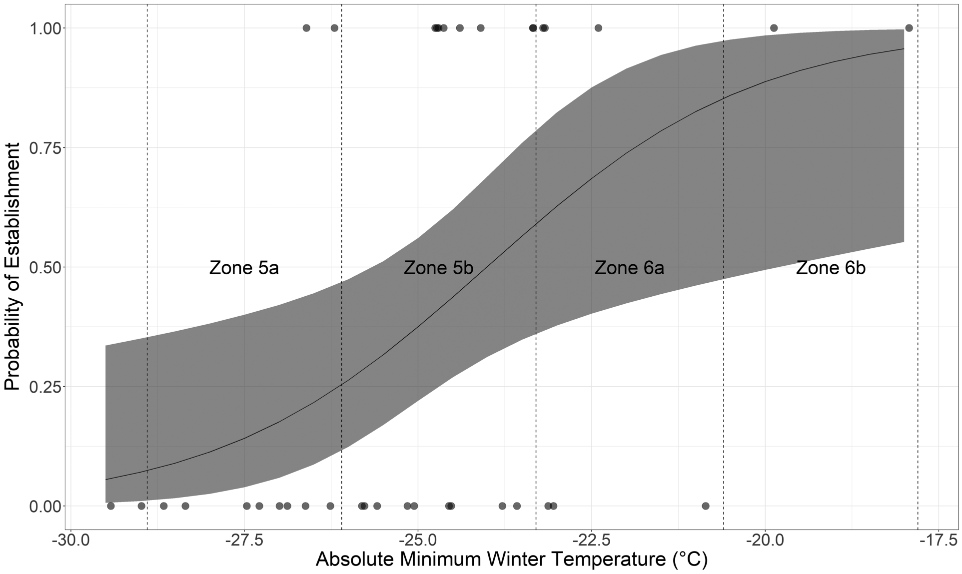

Fig. 2. Logistic regression results of model 3 ($P = 0.015$, pseudo$R^2 = 0.177$, LL = −20.55, RMSE = 0.438), showing the probability of *L. nigrinus* establishment by absolute minimum winter temperature (°C) in the northeastern United States Circles represent individual field sites, the solid line represents model fit, and the shading around the solid line represents 95% confidence intervals. The plot is broken into zones corresponding to USDA PHZ.

HWA population density and believed to reduce *Laricobius* densities (Toland et al. 2019).

Despite recoveries of *L. nigrinus* and *L. osakensis* at various sites, continued sampling will be needed to confirm establishment at sites where sampling was conducted prior to the F_3 generation. One of these sites, Harriman State Park in Southfields, NY, appears likely to have established *L. nigrinus* (larval collections were of either F_2, F_4, or both generations) but continued sampling will be needed to confirm the presence of F_3 or later generations. Similarly, our collection of 126 *L. osakensis* from Frye Island, ME in USDA PHZ 5a (−28.9 to −26.1 °C) was of the F_2 or F_5 generation, indicating that at least 2 winters had passed with mean minimum and absolute minimum temperatures of −8.9 and −27.5 °C, respectively (Table 1), suggesting that this species can survive in USDA PHZ as low as 5a (−28.9 to −26.1 °C).

One *Leucotaraxis argentocollis* larvae (another promising biological control agent for *A. tsugae*) was recovered at the site in Southfields, NY, and confirmed through genetic analysis to be the western lineage (Havill et al. 2018). In the lab and within branch enclosures in the field, western collected *Leucotaraxis* have been shown to feed and complete development on eastern lineage HWA (Motley et al. 2017) and lab-reared pupae have been shown to successfully overwinter at field sites across the eastern United States (Dietschler et al. 2023). While *Leucotaraxis aregenticollis* was not the target species of this study, the finding of a larva in the Harriman State Park (Southfields, NY) sample is significant to the *A. tsugae* biological control program. This represents the first collection of an immature *L. argenticollis* resulting from a free release. *Leucotaraxis* species were released at this location in 2015, 2021, and 2022 (1 month prior to sampling for this study; Virginia Tech 2022). At this time, we are unable to confirm the establishment of *Leucotaraxis* at this site due to the possibility of the collection being the progeny of parent flies released one month earlier. However, this confirms flies are able to reproduce in the wild from free releases of adult *L. argenticollis*.

The vast majority of *L. nigrinus* released in our study (at 40 out of 49 sites) were the laboratory-reared offspring of beetles collected from the region around Seattle, Washington (Virginia Tech 2022), which has winter temperatures that are far warmer than our sites (USDA PHZs 8a and 8b), with average annual minimum winter temperatures between −12.2 and −6.7 °C. Some of the *L. nigrinus* released at the remaining 9 sites were collected in parts of Idaho with USDA PHZs of 6b and 7a (−20.6 and −15 °C), which are also warmer than most of our study sites. At 6 of these 9 sites, we had no recoveries. At 7 of these 9 sites, *L. nigrinus* from the Seattle area were also released (Virginia Tech 2022). The molecular techniques used in this study to identify *L. nigrinus* are not capable of identifying the region (Washington or Idaho) from which the *L. nigrinus* originated. Thus, we were unable to analyze the effect of beetle origin on establishment.

One possible explanation for not collecting *Laricobius* spp. at more of our sites is the low HWA populations during sampling. HWA populations were very low at all Massachusetts sites from 2018 through 2020, which may have been due to a fungal epizootic event associated with abnormally high rainfall in 2018 (Chandler et al. 2022). In 2022, we observed many field sites in New York and Pennsylvania that appeared to be in the "bust" phase of HWA population cycles which was apparent due to many samples without new growth and signs of previously-high HWA levels (old, weathered ovisacs and sooty mold on distal growth). There were also reports of very high (>90%) overwintering mortality in both New York (ND, unpublished data) and Pennsylvania (T. Tomon PA DCNR, personal communications). Low densities of HWA can result in false negative recoveries due to *Laricobius* spp. populations being at undetectable levels (Davis et al. 2012). That said, it is possible that some of the sites we did not recover either *Laricobius* spp. could be false negatives.

Our recovery of *L. nigrinus* in Wellsboro, PA, a release site for *L. osakensis*, could be an example of natural dispersal or may be a result of contamination with *L. nigrinus* in the released cohort of *L. osakensis*. The *L. osakensis* released at Wellsboro, PA were sourced from a field collection at an established *L. osakensis* release site in Maryland (Virginia Tech 2022), where *L. nigrinus* are widely established. Therefore, it is possible that both *Laricobius* species were released at the Wellsboro, PA site. Similar contamination of *L. osakensis* releases has been previously observed in laboratory rearing (S. Salom & C. Jubb Virginia Tech, personal communication). Wild-collected HWA are used as a food source for laboratory colonies of *L. osakensis* and the wide establishment of *L. nigrinus* in the southern United States has complicated efforts to rear pure colonies of *L. osakensis* (Foley et al. 2021). However, it is also possible that the *L. nigrinus* dispersed into the Wellsboro, PA site, as they have been documented to disperse away from release sites (Foley et al. 2019). The nearest *L. nigrinus* release sites date back to 2008 and 2013 are 36–46 km away from Wellsboro, PA across an area of Pennsylvania predominantly within USDA PHZ 5b (−26.1 to −23.3 °C). Another recent recovery of an adult *L. nigrinus*, identified using a restriction enzyme profile, in Effingham, New Hampshire for another project (RSC, unpublished data), was within USDA PHZ 5a (−28.9 and −26.1 °C). The beetle recovered there dispersed from release sites in Maine or New Hampshire, the closest of which are about 60 km away. This recovery indicates that dispersal by *L. nigrinus* across long distances and an area largely within USDA PHZs 5b and 5a is possible and furthermore, is evidence for establishment in those USDA PHZs.

We sampled in years with low HWA populations which likely hindered our ability to detect introduced *Laricobius*. Therefore, absence of *Laricobius*, particularly *L. nigrinus* could be due to low or undetectable population levels. Our sampling regime may have limited our ability to recover *Laricobius* due to the height of branches collected and the volume of HWA-infested hemlock sampled. It has been documented that when HWA populations are low, about 86% *L. nigrinus* are found above 15 m in the hemlock canopy (Davis et al. 2012). We sampled branches within arm's reach (2.5 m) and with pole pruners (5.5–6 m), increasing the chances of false negative *Laricobius* recoveries. Samples collected from Massachusetts in 2022 were from heights of about 5.5–6 m above the ground using pole pruners and resulted in no detection of introduced *Laricobius*. Increasing the number of trees sampled or increasing the length and/or volume of branches clipped could have allowed us to recover more beetles. Future studies could use bucket trucks, tree climbers, or larger containers for rearing a larger volume of infested hemlock material like "Leuco-Lari Containers," developed by the USDA Forest Service and Cornell University, which can fit larger volumes of hemlock material (Mayfield et al. 2021). However, our study demonstrates that recoveries of introduced *Laricobius* can be made even with relatively small collections of HWA-infested branch material and in years when live HWA are scarce.

Molecular techniques were instrumental in the accurate identification of these larvae and prepupae to species since morphological identification to species in these life stages is not possible (Davis et al. 2011). Though large collections of larvae and prepupae likely indicate the collection of introduced *Laricobius* species, small collections from hemlock without molecular confirmation of species identification does not necessarily indicate recovery of introduced *Laricobius* species and could lead to false positives in recoveries at release sites. For this reason, we strongly suggest that collections of these life stages are identified using molecular techniques. We did not analyze the proportion of hybrid *L. nigrinus* and *L. rubidus* collected for this study, however, we have stored samples for future hybrid analysis.

For *Laricobius* released against HWA in the Northeast, particularly New England, establishment success was largely undocumented in the literature until now. Our study has found that the probability of *L. nigrinus* establishment decreases in colder climates (USDA PHZs 5b and lower). Indeed, much of interior New England and many colder sites in Pennsylvania and New York do not yet have established populations of introduced *Laricobius*. Based on our findings of established *L. nigrinus* in USDA PHZ 5b, it does not appear to be due to an inability of *Laricobius* to establish in these colder sites but is likely due to higher rates of mortality of *Laricobius* and HWA, its food source, in colder climates (USDA PHZs 5b and lower) making them more difficult to persist/and or to detect.

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Author Contributions

Ryan Crandall (Conceptualization-Equal, Data curation-Lead, Formal analysis-Lead, Investigation-Equal, Methodology-Equal, Validation-Lead, Visualization-Lead, Writing – original draft-Lead), Jennifer Chandler (Data curation-Supporting, Methodology-Supporting, Writing – review & editing-Equal), Nicholas Dietschler (Investigation-Equal, Writing – review & editing-Equal), Jeremy Andersen (Investigation-Equal, Methodology-Equal, Writing – review & editing-Equal), Joseph Elkinton (Conceptualization-Equal, Funding acquisition-Lead, Methodology-Equal, Resources-Lead, Supervision-Lead, Writing – review & editing-Equal)

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Supplementary Material

Supplementary material is available at *Environmental Entomology* online.

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