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Establishment of the predator *Laricobius nigrinus*, introduced as a biological control agent for hemlock woolly adelgid in Virginia, USA

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Abstract *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a predatory beetle native to western North America, has been released since 2003 for management of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), a non-native pest killing hemlocks in eastern North America. Over 420,000 *L. nigrinus* have been released in the eastern USA from field and lab-reared sources, 14,000 of which were deployed in the Commonwealth of Virginia, USA. In order to determine the establishment rates of *L. nigrinus* in Virginia, surveys were conducted in 2017 and 2018 at all release sites within this state. During the study, stand-level HWA densities were estimated, and hemlock tree health and predator–prey ratios were quantified. The identification of *Laricobius* spp. recoveries were made using microsatellite analysis. During the period of the study, *L. nigrinus* were found to have established at 82% of Virginia release sites and were the primary species recovered (80%). Both *Laricobius rubidus* (18%) and hybrids (2%) were also recovered. Stand-level HWA densities varied greatly over sites and years but showed a general decline in year two of the study. Establishment at such a high percentage of release sites suggests that the climate in Virginia is suitable for the predator and the insect is adaptable to the wide variety of site conditions where hemlocks typically grow.

Keywords *Laricobius nigrinus* · Derodontidae · Coleoptera · *Adelges tsugae* · Hemiptera · Adelgidae

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Introduction

Post-release assessment of natural enemy establishment is a critical component of classical biological control program evaluations. The ability of agents to colonize an introduced region can be a predictor for future success against the pest and can help guide management decisions (van Driesche and Bellows 1996). *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is a predatory beetle of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), a devastating pest causing widespread dieback and mortality of two native hemlock species in eastern forests: *Tsuga canadensis* (L.) Carriere (eastern hemlock), and *Tsuga caroliniana* Engelmann (Carolina hemlock) (Havill and Foottit 2007). *Laricobius nigrinus* has been released in the eastern USA since 2003 as a part of a large-scale biological control

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program implemented for the management of HWA. Although HWA is native to Asia and western North America, it was inadvertently introduced into the eastern USA from Japan (Havill et al. 2006). It has two anholocyclic generations per year (sistens and progrediens) (Havill and Foottit 2007; McClure 1989). *Laricobius nigrinus* is univoltine (Zilahi-Balogh et al. 2003). Both species are uniquely winter-active. During extensive testing to evaluate the suitability of *L. nigrinus* as a biological control agent, it was found to be phenologically synchronous with the sistens generation of HWA. This generation produces the eggs of the progrediens generation, which are the primary host of *L. nigrinus* larvae. *Laricobius nigrinus* is also highly specific to HWA and during evaluations, only completed development on this species (Zilahi-Balogh et al. 2002).

To date, over 420,000 *L. nigrinus* beetles have been released from field and lab-reared sources throughout the range of HWA infested eastern hemlock (Virginia Tech 2019). In Mausel et al. (2010) establishment of *L. nigrinus* was documented across a wide geographic range of 22 early release sites from Massachusetts to Georgia, suggesting that *L. nigrinus* was adaptable to areas outside of its native environment. Dispersal from original release sites has also been documented (Davis et al. 2012), and movement of this species out of forested release areas and into urban environments further highlights its potential for adaptation (Foley et al. 2019). Investigations evaluating the impact of this predator on the overwintering generation of HWA appear promising (Jubb et al. 2020). However, additional predators are likely necessary for effective management of this species, as the progrediens generation may rebound at some locations after predation of the preceding sistens generation by *L. nigrinus* (Crandall et al. 2020). During previous studies, a native species, *Laricobius rubidus* Leconte, was also collected from HWA-infested hemlock during sampling (Davis et al. 2012; Mausel et al. 2008, 2010). The primary host for this species is pine bark adelgid (PBA), *Pineus strobi* Hartig (Hemiptera: Adelgidae) on white pine, *Pinus stobus* L. (Clark and Brown 1960; Wantuch et al. 2019). However, it can feed and complete development on HWA (Zilahi-Balogh et al. 2005). *Laricobius nigrinus* and *L. rubidus* are closely related sister species that are capable of mating and producing hybrid progeny (Fischer et al. 2015; Havill et al. 2012). Observations by Fischer et al. (2015) showed both species preferred to remain on their primary hosts, while hybrids appeared more frequently on HWA.

As *Laricobius* spp. release efforts continue throughout the eastern USA, it is critical to engage in post-release monitoring efforts. State-wide evaluation of *Laricobius* spp. releases can provide an assessment of the effectiveness of biological control efforts and help guide future management efforts. Since the initial releases in 2003, roughly 14,000 *L. nigrinus* adults have been deployed at 26 locations in the Commonwealth of Virginia in the eastern USA (Virginia Tech 2019). Most releases were made within the Valley and Ridge and Blue Ridge physiographic provinces of Virginia with a small number of releases occurring in the Piedmont and Coastal Plain regions. Prior efforts to evaluate establishment in Virginia have been limited in scope (Heminger 2017; Mausel et al. 2010). In the study reported here, we sampled for *L. nigrinus* at all existing Virginia release sites to understand this beetle's ability to survive under a variety of abiotic conditions state-wide. During those efforts we also classified stand-level HWA densities, hemlock tree health, *Laricobius* spp. species composition, and predator–prey ratios at each site. The results from this study will help guide future releases of *L. nigrinus* in Virginia and offer insight to its ability to establish as well as impact HWA populations and hemlock tree health.

Materials and methods

Field sites

The HWA Predator Release and Monitoring Database (Virginia Tech 2019) was queried in the spring of 2017 to identify all known *L. nigrinus* release sites in the Commonwealth of Virginia, USA. A total of 26 sites were noted; however, five of those occurred within a one-kilometer radius in the Mountain Lake area and were therefore treated as one site during these investigations (Table 1). All beetle releases made in Virginia were of the coastal strain biotype, which was different from a more cold tolerant interior strain found in Idaho, Montana, and interior British Columbia (Davis et al. 2011; Havill et al. 2012; Mausel et al. 2011). Release sites were situated within four distinct USDA Plant Hardiness Zones: 6a (−23.3 to

Table 1 Virginia *L. nigrinus* release site names (listed alphabetically), latitude and longitude, USDA Plant Hardiness Zone of site location, original release dates, and number of predators released

Site name	Latitude (decimal degrees)	Longitude (decimal degrees)	Plant hardiness Zone[a]	*L. nigrinus* release date	No. released
Bear Creek	36.911486	−84. 401136	6b	2013	225
Big Cherry #1	36.827663	−82.702242	6b	2008	500
Big Cherry #2	36.832379	−82.702242	6b	2008	500
Burns Creek	36.924661	−82.536936	6b	2008	300
Channels S.F	36.828643	−81.962809	6b	2010	1000
Cherokee Flats	37.414479	−80.583234	6b	2014	400
Devil's Fork	36.820031	−82.630216	6b	2008	300
Dickey Creek	36.736894	−81.432461	6b	2005	75
Gullion Fork	36.995914	−81.27317	6b	2013	225
Highland	36.692104	−81.517071	6b	2004	1200
Hurricane	36.721789	−81.487527	6b	2003	300
James River	37.640505	−78.79973	7a	2005	300
Kentland Farm	37.208931	−80.589822	6b	2003	258
Lick Creek	37.01072	−81.427409	6a	2004	150
McCoy	37.214902	−80.6015	6b	2013/2014	150/267
Mountain Lake	37.368654	−80.536671	6a	2009/2010	42/1800
Nature Camp	37.875946	−79.214285	6b	2012	430
North Fork	37.443668	−80.515333	6b	2003	600
Pinnacle	36.961556	−82.053298	6b	2006	310
Poverty Creek	37.252649	−80.533711	6b	2009/2010/2014	150/1000/539
Rose Hill	36.682086	−83.364423	6b	2014	275
Sandy Point S.F	37.682968	−76.944674	7b	2010	2040

[a]Plant Hardiness Zones are based on average annual minimum temperature and were acquired from https://planthardiness.ars.usda.gov. 6a (−23.3 to −20.5 °C), 6b (−20.6 to −17.8 °C), 7a (−17.8 to −15.0 °C) and 7b (−15.0 to −12.2 °C)

−20.5 °C), 6b (−20.6 to −17.8 °C), 7a (−17.8 to −15.0 °C) and 7b (−15.0 to −12.2 °C), with the majority being within 6b (USDA 2012). Sites were visited from March to April in 2017 and 2018 for sampling and coincided with the period of peak HWA egg abundance, *L. nigrinus* oviposition, and just prior to *L. nigrinus* larval presence. The exact timing of each site visit varied depending on USDA Plant Hardiness Zone and local temperatures leading up to the sampling period. Colder temperatures delayed HWA and *L. nigrinus* ovipositional periods. For all locations, visits were planned so that sampling occurred on days where precipitation was not forecast, and temperatures were not below 0 °C, as *L. nigrinus* would likely not be active (Mausel et al. 2010; Zilahi-Balogh et al. 2003).

HWA density assessment

Approximately 20–30 branches were randomly sampled from the lower canopy (0–2 m above ground) at each site. Current years' growth on a 30 cm distal portion of each chosen branch was evaluated. The number of HWA cm^{-1} was approximated for each branch, and an overall mean density was then estimated to achieve a stand-level density assessment. HWA densities were categorized based on the following parameters:

1. No HWA = HWA not present.
2. Low HWA = An average of fewer than 1 HWA per 30 cm of current years' growth.
3. Moderate HWA = An average between 1–10 HWA per 30 cm current years' growth.
4. High HWA = An average greater than 10 HWA per 30 cm current years' growth.

Stand-level HWA density was not estimated at Big Cherry #2, or Sandy Point State Forest (S.F.) in 2017.

Tree health assessment

At each site, hemlock tree health was assessed using USDA Forest Service Forest Inventory and Analysis (FIA) crown condition parameters (crown density, transparency, dieback, and live crown ratio) (USDA Forest Service 2011). A percentage value in 5% increments was assigned for each parameter, and, collectively, these values served as a broad indicator of overall tree health. Ten trees were selected at each site per year, and assessments were performed. A mean percentage for each parameter was then calculated for each site. Evaluations were conducted by the same researcher in both years of the study so as to minimize subjective bias. Tree health was not evaluated at Big Cherry #2 in 2017, or in either year at Sandy Point S.F.

Laricobius spp. adult sampling (beat sheet method)

Adult *L. nigrinus* densities were quantified using beat sheet sampling techniques described in Jubb (2019) and Mausel et al. (2010). Sampling was performed for approximately 20–30 min on HWA infested branches in the lower canopy (0–2 m above ground) of hemlock trees throughout the site. Beat sheets were placed under selected HWA infested branches and the upper portion of the branch was then tapped approximately ten times using a 60 cm section of PVC pipe to dislodge adult *Laricobius* spp. present on the branch. All adults found on the sheet were collected using an aspirator, and then transferred to vials containing 95% ethanol for subsequent genetic analysis to determine species or hybrid designation. The total number of adults collected per site was recorded. Beat sheet sampling was not conducted in either year at Mountain Lake or Sandy Point S.F. due to lack of HWA.

Laricobius spp. larval sampling (branch clip method)

Laricobius nigrinus larval densities were quantified using branch clip sampling techniques described in Jubb (2019) and Mausel et al. (2010). Briefly, the distal 30 cm portion of approximately 20–25 HWA infested branches were selected and cut from the lower canopy of hemlock trees at each site. Branches were transported to the Virginia Tech Insectary and the number of HWA per branch was estimated. Branches were then placed into rearing funnels in conditions known to be appropriate for developing *Laricobius* spp. larvae (L:D 12:12, 13–15 °C) (Lamb et al. 2005; Salom et al. 2012). Larvae were permitted to feed and develop through four instars and were collected as pharate pre-pupae from collection jars attached at the base of funnels. Jars were checked daily for the presence of pre-pupae and, when recovered, were placed into vials filled with 95% ethanol for genetic analysis. The total number of larvae recovered from each site was quantified and predator–prey ratios were calculated by dividing the total number of larvae recovered per funnel by the total number of HWA present within that funnel for each site. Branches were not collected at Sandy Point State Forest (S.F.) in 2017 or in either year at Mountain Lake, due to the lack of HWA.

Genetic analysis of *Laricobius* spp. adult and larval recoveries

Microsatellite loci from adult and larval *Laricobius* spp. recoveries were evaluated to determine species (*L. nigrinus* or *L. rubidus*) or hybrid (*L. nigrinus* × *L. rubidus*) designation. DNA was extracted from adults and larvae, and six microsatellite loci were amplified using protocols described by Klein et al. (2010). Fragment analysis was performed using a 3730xl 96-Capillary Genetic Analyzer at the DNA Analysis Facility at Science Hill, New Haven, CT, USA. Genotypes were called using Geneious Prime 2019 (Biomatters, Inc, Foster City, CA, USA). Final species and hybrid designations were made using Structure 2.3.2 (Stanford University) and New Hybrids 1.1 (University of California) software programs. At sites where *Laricobius* spp. recoveries were high, a random subsample of approximately 30 individuals was selected for analysis.

Statistical analysis

FIA tree health parameters (crown density, transparency, dieback, and live crown) were pooled by year and analyzed to determine if there were differences between values in 2017 and 2018 using a paired t-test at a significance level of $\alpha = 0.05$. Tree health parameter response variables were tested for goodness-of-fit to a normal distribution using skewness and kurtosis values (Thode 2002). Dieback variables did not fit a normal distribution and were square-root-transformed to meet the assumptions of the test. Spearman's rank correlation analysis was used to determine the association between *Laricobius* spp. larval densities and HWA densities in funnels. Additionally, combined adult + larval recoveries were analyzed with stand-level HWA density and the four tree health parameters. All correlation analyses were run at a significance level of $\alpha = 0.05$. All statistical analyses were performed using JMP Pro 13.0 (SAS Institute 2018).

Results

HWA density assessment

HWA densities varied greatly across sites and between years. However, the data indicate a decline in densities at ten sites during the period of the study (Table 2). In 2017 the percentage of sites with no, low, moderate, or high HWA densities was 5, 36, 23, and 27, respectively. In 2018, the percentage of sites with no, low, moderate, or high HWA densities was 5, 59, 32, and 5, respectively.

Tree health assessment

Two tree health parameters were significantly different between the two years of the study and indicated that overall hemlock health may have improved in 2018 at *L. nigrinus* release sites in Virginia. Mean crown density in 2017 was 62% (range: 45.5–89.0), and in 2018 was 64% (range: 44.5–91.0), with no significant difference detected between the two years ($t = 1.27$, df = 423, $p = 0.2037$) (Fig. 1, Table 2). Mean transparency in 2017 was 38% (range: 9.5–57.5), and in 2018 was 32% (range: 5.0–56.5), with a significant decrease between the two years ($t = -3.71$, df = 441, $p = 0.0002$) (Fig. 1, Table 2). Mean dieback in 2017 was 34% (range: 8.5–60.0) and in 2018 was 25% (range: 5.0–49.5), with a significant decrease between the two years ($t = 5.97$, df = 448, $p < 0.0001$) (Fig. 1, Table 2). Mean live crown ratio in 2017 was 63% (range: 37.5–91.0) and in 2018 was 64% (range: 31.5–100.0), with no significant difference detected between the two years ($t = 0.68$, df = 449, $p = 0.4958$) (Fig. 1, Table 2).

Laricobius spp. adult sampling (beat sheet method)

A total of 44 *Laricobius* spp. adults were collected at eight out of 22 (36%) sites in the spring of 2017 (Table 3). Mean (± SE) number of adults collected per site was 2.1 ± 1.3 (range: 0–26) with Poverty Creek having the highest number of recoveries. A total of 186 adult *Laricobius* spp. were collected at 15 out of 22 (68%) sites in 2018 (Table 3). Mean number of adults collected per site was 8.5 ± 3.7 (range: 0–80) with the Kentland Farm site having the greatest number of recoveries during the study. No adult recoveries were made at Mountain Lake, Rose Hill, and Sandy Point S.F. throughout the study.

Laricobius spp. larval sampling (branch clip method)

A total of 961 *Laricobius* spp. larvae were recovered at 15 out of 22 (68%) sites in 2017 (Table 3). Mean number of larvae collected per site was 45.8 ± 17.2 (range: 0–343). Mean predator–prey ratio for all sites was 0.03 ± 0.01 (range: 0–0.14) (Table 3). Kentland Farm had the greatest number of larval recoveries and the highest predator–prey ratio. A total of 1,503 *Laricobius* spp. larvae were recovered at 19 out of 22 (86%) sites in 2018 (Table 3). Mean number of larvae collected per site was 68.9 ± 14.5 (range: 0–211) with Highland having the highest number of recoveries. Mean predator–prey ratio for all sites pooled was 0.02 ± 0.01 (range: 0–0.10) with Kentland Farm having the greatest predator–prey ratio. No larval recoveries were made at Mountain Lake, Rose Hill, or Sandy Point S.F. during the period of the study.

There was no significant association between the number *Laricobius* spp. larvae and the number of HWA in funnels. There was a significant positive relationship between combined *Laricobius* spp. adults + larvae and stand-level HWA density, and a

Table 2 HWA stand-level density, mean diameter at breast height (DBH), and mean percent ± SE FIA tree health parameters (crown density, transparency, dieback, and live crown ratio) for Virginia release sites (listed alphabetically) in 2017 and 2018

Site	Year	Stand HWA density[a]	Mean DBH (cm)	Mean % crown density	Mean % transparency	Mean % dieback	Mean % live crown ratio
Bear Creek	2017	Moderate	5.9 ± 1.0	50.5 ± 4.5	37.0 ± 2.7	34.5 ± 2.5	44.0 ± 4.2
	2018	Low	11.3 ± 2.9	62.5 ± 3.8	33.0 ± 5.1	24.0 ± 3.5	41.5 ± 5.2
Big Cherry #1	2017	Low	5.4 ± 1.0	52.5 ± 5.1	48.0 ± 4.1	29.5 ± 5.0	71.0 ± 8.5
	2018	Moderate	3.1 ± 0.4	57.0 ± 3.2	43.5 ± 4.0	28.5 ± 5.7	60.0 ± 5.3
Big Cherry #2	2017	–	–	–	–	–	–
	2018	Moderate	3.9 ± 0.3	72.5 ± 3.7	22.5 ± 3.2	16.0 ± 5.5	92.0 ± 2.2
Burns Creek	2017	Moderate	13.5 ± 2.5	45.5 ± 6.0	57.5 ± 3.4	39.5 ± 2.9	37.5 ± 4.6
	2018	Low	10.2 ± 1.7	59.5 ± 5.6	36.0 ± 6.2	30.0 ± 2.9	31.5 ± 2.7
Channels S.F	2017	High	6.4 ± 3.0	49.0 ± 3.4	55.5 ± 5.5	60.0 ± 6.8	48.0 ± 6.2
	2018	Low	11.6 ± 3.3	52.0 ± 3.1	41.5 ± 5.0	35.5 ± 5.0	56.5 ± 3.5
Cherokee Flats	2017	Low	4.6 ± 0.8	53.0 ± 7.1	39.0 ± 4.3	34.0 ± 4.7	61.0 ± 8.5
	2018	Low	6.2 ± 0.4	59.0 ± 1.9	36.5 ± 3.2	28.0 ± 3.7	63.0 ± 6.7
Devil's Fork	2017	High	9.7 ± 2.7	53.5 ± 5.8	47.5 ± 5.8	52.0 ± 5.6	51.5 ± 7.0
	2018	Low	5.9 ± 1.5	44.5 ± 7.1	56.5 ± 5.0	49.5 ± 7.7	55.0 ± 5.1
Dickey Creek	2017	High	6.5 ± 1.5	52.0 ± 4.7	46.5 ± 4.4	48.5 ± 5.9	52.5 ± 7.0
	2018	Moderate	6.8 ± 1.3	50.5 ± 5.4	38.0 ± 2.2	36.5 ± 5.9	58.0 ± 4.6
Gullion Fork	2017	High	8.3 ± 2.8	46.0 ± 4.9	41.5 ± 4.7	28.0 ± 4.4	50.0 ± 5.5
	2018	Low	11.1 ± 2.0	52.0 ± 3.0	31.0 ± 1.6	25.5 ± 4.6	44.0 ± 4.8
Highland	2017	Low	12.0 ± 3.3	85.5 ± 2.9	19.0 ± 2.8	12.0 ± 2.7	81.0 ± 4.3
	2018	Moderate	8.0 ± 1.7	85.0 ± 2.6	14.0 ± 2.8	8.0 ± 2.2	63.5 ± 8.6
Hurricane	2017	Low	8.0 ± 1.2	55.5 ± 4.5	42.5 ± 3.1	39.0 ± 2.4	58.5 ± 4.5
	2018	Low	6.6 ± 0.9	65.0 ± 3.6	31.0 ± 2.6	34.0 ± 6.5	73.5 ± 5.4
James River S.P	2017	High	4.3 ± 0.6	69.0 ± 4.2	36.5 ± 4.9	36.0 ± 4.3	70.0 ± 3.8
	2018	Moderate	6.9 ± 1.6	63.5 ± 3.1	28.5 ± 1.8	38.5 ± 6.7	71.5 ± 2.2
Kentland Farm	2017	Low	5.2 ± 0.6	89.0 ± 4.0	9.5 ± 3.2	8.5 ± 2.5	91.0 ± 3.0
	2018	High	4.4 ± 0.4	91.0 ± 0.8	5.0 ± 0.0	5.0 ± 0.00	100 ± 0.0
Lick Creek	2017	Moderate	9. 2 ± 1.7	56.0 ± 3.5	50.5 ± 1.8	34.5 ± 3.9	51.0 ± 4.0
	2018	Low	12.9 ± 1.0	62.0 ± 2.2	42.0 ± 3.5	30.0 ± 4.6	59.0 ± 5.9
McCoy	2017	Low	4.0 ± 0.4	66.0 ± 3.8	27.5 ± 2.8	30.0 ± 4.0	65.0 ± 3.7
	2018	Low	2.6 ± 0.4	58.5 ± 2.3	38.5 ± 3.2	28.7 ± 3.6	62.2 ± 3.1
Mountain Lake	2017	None	10.8 ± 1.0	69.0 ± 2.5	35.0 ± 2.6	36.5 ± 2.4	68.3 ± 2.4
	2018	None	10.9 ± 1.1	68.5 ± 1.8	29.0 ± 2.8	12.7 ± 3.1	72.0 ± 2.4
Nature Camp	2017	Moderate	5.3 ± 1.1	67.0 ± 3.5	30.0 ± 3.3	21 ± 2.45	69.0 ± 3.5
	2018	Moderate	7.0 ± 1.2	69.0 ± 2.0	26.0 ± 1.9	12.5 ± 2.01	72.0 ± 2.7
North Fork	2017	Low	9.5 ± 0.6	67.0 ± 3.7	32.5 ± 3.1	32 ± 1.86	69.0 ± 2.4
	2018	Low	7.0 ± 0.6	60.5 ± 2.2	43.0 ± 4.7	37 ± 4.90	51.0 ± 4.2
Pinnacle	2017	Low	7.5 ± 1.4	75.5 ± 1.8	23.5 ± 3.1	20.5 ± 2.41	71.0 ± 2.4
	2018	Low	5.7 ± 0.7	74.5 ± 2.4	26.0 ± 1.8	9.5 ± 2.52	67.5 ± 2.9
Poverty Creek	2017	High	7.4 ± 1.3	64.0 ± 7.0	45.0 ± 6.6	37 ± 4.23	63.5 ± 7.3
	2018	Moderate	3.0 ± 0.6	59.0 ± 4.0	33.5 ± 3.8	32 ± 3.35	70.0 ± 3.3
Rose Hill	2017	Moderate	9.0 ± 1.9	69.0 ± 5.3	36.0 ± 4.0	35 ± 4.08	74.5 ± 5.6
	2018	Low	11.2 ± 1.7	75.0 ± 2.1	29.5 ± 3.9	22 ± 3.74	58.5 ± 5.8

Table 2 continued

Site	Year	Stand HWA density[a]	Mean DBH (cm)	Mean % crown density	Mean % transparency	Mean % dieback	Mean % live crown ratio
Sandy Point S.F	2017	–	–	–	–	–	–
	2018	Low	–	–	–	–	–

[a]Stand-level HWA density categories:

None = HWA not present

Low = average less than 1 HWA per 30 cm of current years' growth

Moderate = average between 1–10 HWA per 30 cm current years' growth

High = average greater than 10 HWA per 30 cm current years' growth

– Data not collected at site

significant negative relationship with crown transparency (Table 4). No significant associations were noted between *Laricobius* spp. adults + larvae and crown density, dieback, and live crown ratio (Table 4).

Genetic analysis of *Laricobius spp*. adult and larval recoveries

Laricobius nigrinus was the primary species recovered from Virginia release sites. *Laricobius rubidus* and hybrids of *L. nigrinus* and *L. rubidus* were also recovered. Recoveries of *L. nigrinus* were made at 82% of release sites and as such, establishment of this species at those locations can be confirmed (Fig. 2, Table 5). In both years combined, the mean percent *L. nigrinus* recovered at release sites was 80% followed by 18% *L. rubidus*, and 2% hybrids. In 2017, the mean percent *L. nigrinus* at release sites was 71%, followed by 27% *L. rubidus,* and 2% hybrids. In 2018, the mean percent *L. nigrinus* at release sites was 85% followed by 12% *L. rubidus* and 3% hybrids. No recoveries of *L. nigrinus* were made at Nature Camp, Mountain Lake, Rose Hill, or Sandy Point S.F. in either year. Only *L. rubidus* was recovered from Nature Camp during the period of the study. At three sites, Cherokee Flats, Devil's Fork, and North Fork, the adult and larval recovery numbers across both years were < 10 ($n = 4$, 3, 9, respectively). All other sites had recovery numbers > 10 across both years (Table 5). Some collected *Laricobius* individuals were not identifiable due to unsuccessful DNA extraction, or issues with loci amplification as a result of poor sample quality.

Discussion

Continued evaluation of biological control agents after release is important in order to justify and guide future work with those particular agents (Stiling 1990). Our study was the first comprehensive state-wide evaluation of establishment in the eastern USA the results of which indicate that *L. nigrinus* has colonized successfully a majority of release sites in Virginia. *Laricobius nigrinus* has been able to persist long-term at many of these locations, and based on original release dates, F_{15} generation individuals were recovered. They were able to establish within three of the four USDA Plant Hardiness Zones in which they where released, in addition to multiple physiographic regions. These regions are unique in many facets such as elevation, soil characteristics, and overall climate (Virginia Department of Conservation and Recreation 2019), which highlights the adaptability of *L. nigrinus* to different abiotic conditions. This species was also able to persist at sites where stand-level HWA densities were low, an important attribute of successful biological control agents.

Laricobius nigrinus was not recovered at four release sites during the period of this study. Several factors may have negatively affected their ability to establish at these locations. At two of the four sites where establishment was not confirmed (Mountain Lake and Sandy Point S.F.), HWA populations were either not found or were extremely low during the two-year period of the study. The Mountain Lake sites are situated in Giles, County VA at an elevation of 1100–1200 m. Low winter temperatures frequently experienced at these elevations are a likely cause of

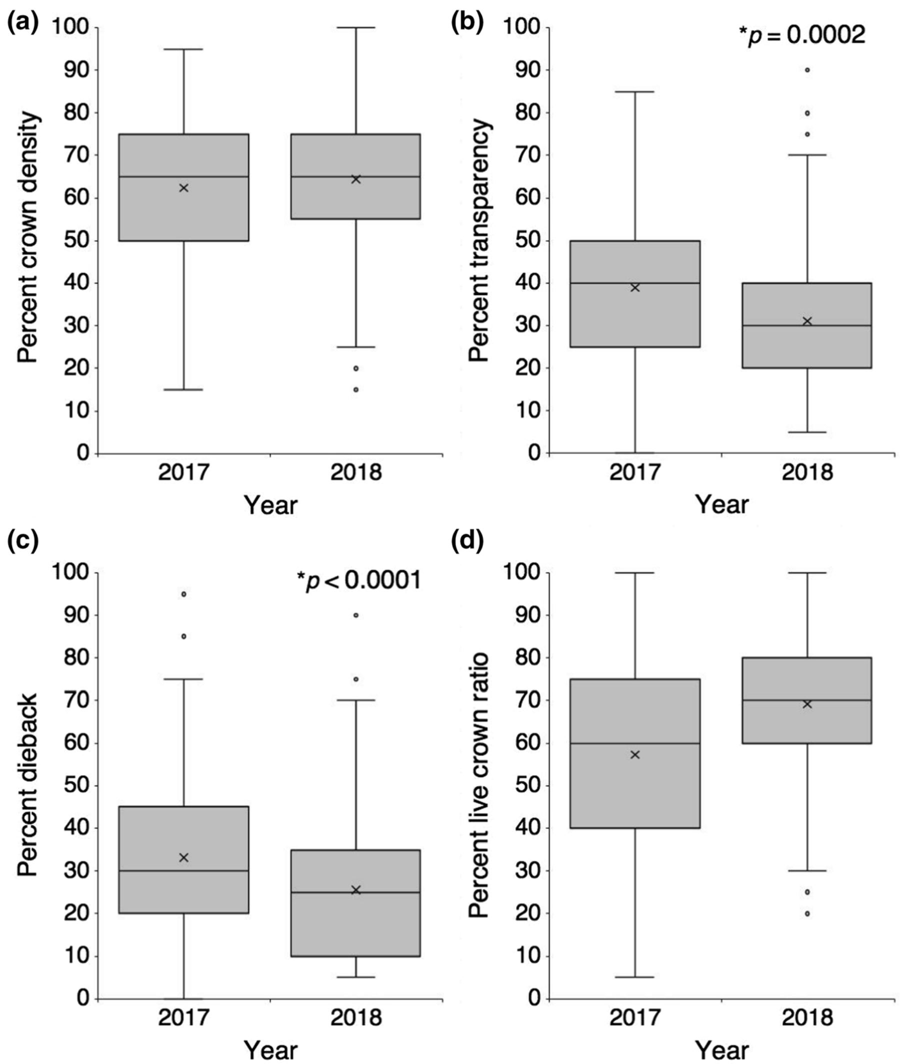

Fig. 1 Box plots depicting 2017 and 2018 mean percent FIA tree health parameters: **a** crown density, **b** transparency, **c** dieback, and **d** live crown ratio from *L. nigrinus* release sites in Virginia. Significant differences in parameter values between the two years was tested using a paired t-test. *Significance at α = 0.05, x = parameter mean, shaded boxes show interquartile range with median indicated by horizontal line, box whiskers extend to minimum and maximum values, circles indicate outliers

mortality for HWA (McAvoy et al. 2017; Paradis et al. 2008; Tobin et al. 2017; Trotter and Shields 2009) and *L. nigrinus* populations. Conversely, summer temperatures may also increase HWA mortality (Mech et al. 2018) and could therefore be a potential cause for low HWA populations at Sandy Point S.F., which is located in Zone 7b. Other factors that may have influenced the ability of *L. nigrinus* to successfully colonize include release size (Mausel et al. 2010) and the timing of the release relative to the life cycle of both *L. nigrinus* and HWA. If releases are made too late, *L. nigrinus* may not have sufficient prey on which to feed and develop.

A lack of recoveries at these sites, however, does not necessarily indicate a lack of establishment. Populations of *L. nigrinus* may have been too low to detect. There were sampling limitations in this study that could have been the cause for low or no recoveries. Both adult and larval sampling was limited to the lower canopy of hemlock trees at most sites due to ease of accessibility specifically with beat sheet sampling. Beat sheet sampling often provides false

Table 3 The number of *Laricobius* spp. adults recovered from beat sheet sampling, the number of larval recoveries from branch clip sampling, and the predator–prey ratio (larvae per HWA) in foliage funneled for each Virginia release site (listed alphabetically) in 2017 and 2018

Site	2017			2018		
	Adults	Larvae	Larvae per HWA	Adults	Larvae	Larvae per HWA
Bear Creek	2	76	0.026	0	24	0.025
Big Cherry #1	0	5	0.002	3	23	0.012
Big Cherry #2	0	0	–	2	24	0.007
Burns Creek	0	0	–	1	27	0.019
Channels S.F	0	34	0.005	4	209	0.066
Cherokee Flats	1	2	0.005	0	1	0.000
Devil's Fork	0	0	–	0	10	0.023
Dickey Creek	0	105	0.021	11	127	0.044
Gullion Fork	0	80	0.023	4	52	0.017
Highland	0	25	0.007	15	211	0.034
Hurricane	0	4	0.010	6	101	0.033
James River S.P	2	33	0.010	12	97	0.012
Kentland Farm	8	343	0.144	80	188	0.096
Lick Creek	0	20	0.005	5	59	0.021
McCoy	3	81	0.096	12	96	0.058
Mountain Lake	–	–	–	–	–	–
Nature Camp	1	21	0.003	4	83	0.016
North Fork	1	2	0.004	0	5	0.001
Pinnacle	0	0	0	3	58	0.021
Poverty Creek	26	130	0.074	24	108	0.022
Rose Hill	0	0	–	0	0	0.000
Sandy Point S.F	–	–	–	0	0	0.000

–Data not collected at site

Table 4 Spearman's rank correlation results for multiple factors tested at a significance level of α = 0.05. Highlighted rows indicate significant correlations

Factor 1	Factor 2	Spearman's ρ	P-Value
Laricobius spp. larvae	No. HWA funneled	0.1556	0.3796
***Laricobius* spp. adults + larvae**	**Stand HWA density**	**0.3708**	**0.0309**
Laricobius spp. adults + larvae	Crown density	0.2061	0.2423
***Laricobius* spp. adults + larvae**	**Transparency**	**−0.3407**	**0.0486**
Laricobius spp. adults + larvae	Dieback	−0.1454	0.4121
Laricobius spp. adults + larvae	Live crown ratio	0.2345	0.1819

negatives due to habits of *L. nigrinus* adults (Mausel et al. 2010) and, in this study, recoveries of this life stage were much lower when compared to larval recoveries (Table 4). Additionally, Davis et al. (2012) suggested that *L. nigrinus* adults disperse vertically (> 15 m) within trees following their release, which may have confounded our ability to locate individuals using the two described sampling methods. Further

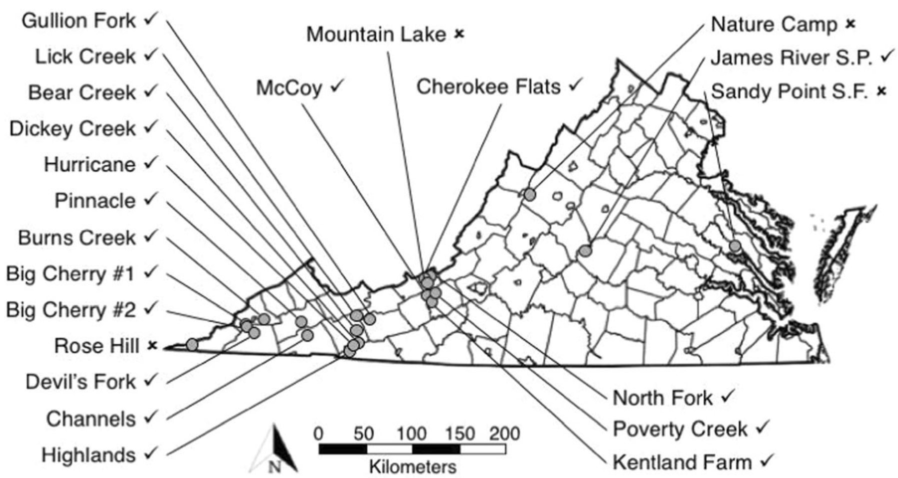

Fig. 2 Combined results of 2017 and 2018 establishment evaluations for *L. nigrinus* at release sites in the Commonwealth of Virginia, USA. '✔'—Indicates sites where *L. nigrinus* was recovered; '×'—Indicates sites where no *L. nigrinus* recoveries were made

Table 5 The number of samples successfully identified using microsatellite loci analysis, and percent *Laricobius* recoveries identified as *L. nigrinus*, *L. rubidus*, and hybrids for each Virginia release site (listed alphabetically) in 2017 and 2018

	2017				2018			
Site	n	% *L. nigrinus*	% *L. rubidus*	% Hybrids	n	% *L. nigrinus*	% *L. rubidus*	% Hybrids
Bear Creek	26	100	0	0	24	100	0	0
Big Cherry #1	5	100	0	0	24	4	88	8
Big Cherry #2	*	*	*	*	25	100	0	0
Burns Creek	*	*	*	*	28	100	0	0
Channels S.F	29	72	28	0	32	88	0	13
Cherokee Flats	3	0	100	0	1	100	0	0
Devil's Fork	*	*	*	*	9	100	0	0
Dickey Creek	30	0	100	0	35	94	3	3
Gullion Fork	28	96	4	0	30	100	0	0
Highland	23	100	0	0	37	100	0	0
Hurricane	7	43	57	0	33	91	3	6
James River S.P	27	100	0	0	33	100	0	0
Kentland Farm	33	100	0	0	48	100	0	0
Lick Creek	18	17	78	6	59	91	2	7
McCoy	58	76	17	7	69	77	19	4
Mountain Lake	–	–	–	–	–	–	–	–
Nature Camp	20	0	100	0	30	0	100	0
North Fork	3	33	67	0	0	0	0	0
Pinnacle	*	*	*	*	31	100	0	0
Poverty Creek	41	88	10	2	38	87	8	5
Rose Hill	*	*	*	*	*	*	*	*
Sandy Point S.F	–	–	–	–	*	*	*	*

–Data not collected at site

*Recoveries not made at site

investigations of *L. nigrinus* establishment at sites where they were not initially recovered should therefore include sampling in a variety of canopy positions within trees and should be primarily focused on branch clip sampling for larvae.

Data analyses for two tree health parameters, transparency and dieback, indicated an increase in overall health of hemlocks between the two years of the study. There was also a significant negative association between *Laricobius* spp. adult and larval recoveries when compared to the tree health parameter, transparency. This suggests that as transparency decreased, *Laricobius* spp. densities increased in 2018. Although this type of relationship is a positive finding for a biological control program, we cannot confidently make assertions about the relationships of *Laricobius* spp. densities and tree health using the parameters of this study, as it is difficult to identify trends from a two-year evaluation of the data. In order to have a more consistent and thorough analysis of tree health at Virginia release sites, and an understanding of how *Laricobius* spp. populations interact with these parameters, sites should be revisited periodically over time to capture important trends.

Genetic analysis of recovered *Laricobius* spp. suggests that *L. nigrinus* was the species most frequently recovered from Virginia release sites. The percentage of hybrid recoveries in this study (3%) were lower than in previous studies (11–28%) (Arsenault et al. 2015; Fischer et al. 2015; Havill et al. 2012; Mayfield et al. 2015; Wiggins et al. 2016). However, our data were more comparable with a recent study where only 2% of all *Laricobius* larvae and adults recovered were hybrids (Jubb et al. 2020). While it is possible that hybridization rates have decreased over time at release sites, microsatellite analysis using the six selected loci indicated in Klein et al. (2010) is limited to detecting only earlier generation hybrids. Offspring of prior hybrids could have backcrossed with either species and may have been identified as *L. nigrinus* or *L. rubidus* by the software programs used in the analysis (Havill et al. 2012). Increasing the number of loci used in microsatellite analysis could improve our ability to detect hybrids and understand how populations of each species are changing spatially and temporally at release sites.

Predator–prey ratios in this study were lower than those indicated in a study conducted in the native range of *L. nigrinus* (Mausel et al. 2017). The disparity between the findings in the East and West were not explained by the parameters of this study and are therefore not well understood. Many factors such as climate, tree health, lack of tree host resistance and natural enemy complex likely affect predator–prey populations in the East. There was a significant positive relationship between adult + larval densities when compared to HWA stand-level densities, which is consistent with results from previous studies (Mausel et al. 2008; Toland et al. 2018) and suggests a strong relationship between predator and prey. However, further investigations are necessary to have a more thorough understanding of the dynamics involved with *L. nigrinus* and HWA populations in eastern North America.

Our study provides information that is critical to steering future management of this predator and its prey and also serves as a template for land managers to conduct post-release monitoring of *Laricobius* spp. populations in their respective regions. We have confirmed *L. nigrinus* establishment at 82% of sites in Virginia. Continued monitoring of HWA and *L. nigrinus* populations at non-recovery sites is important to determine establishment status, as populations of both species may have been too low to detect during the period of our study. As predator populations continue to build at some Virginia sites, future work could include collection and redistribution of *L. nigrinus* to other suitable release areas in the eastern USA Future releases must be made at sites with adequate HWA populations to support the release size and must be made during the appropriate time period in order to have a higher probability of establishment. The results of this study further support previous findings that *L. nigrinus* is adaptable to climatic and other environmental conditions outside of its native range (Mausel et al. 2010) and can persist in these environments in spite of low prey populations. These are qualities of potentially successful biological control agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no known conflicts of interest.

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