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First Report on Establishment of *Laricobius osakensis* (Coleoptera: Derodontidae), a Biological Control Agent for Hemlock Woolly Adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in the Eastern U.S.

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Abstract: The hemlock woolly adelgid (HWA) is an invasive insect species native to Japan causing significant hemlock mortality in the eastern United States. *Laricobius* spp. have been targeted as biological control agents because they are adelgid specialists. *Laricobius osakensis* Montgomery and Shiyake is native to the same region of Japan from which the strain of HWA found in the eastern United States originated. Studies in Japan found that it is phenologically synchronous with HWA. Following approval to release *L. osakensis* from quarantine in 2010, approximately 32,000 were released at a total of 61 sites starting in 2012. In winter of 2014 and 2015, periods of extreme cold temperatures throughout the eastern USA, as well as the polar vortex, resulted in extensive mortality to HWA, which likely delayed the establishment of *L. osakensis*. The ability of the beetle to survive and establish in the eastern United States is reported here. In the first year of this study (2015–2016), limited numbers of *L. osakensis* were recovered, as HWA populations were still rebounding. In the second year (2016–2017), 147 *L. osakensis* were collected at 5 of 9 sites sampled, coinciding with rebounding HWA populations. Larval recovery was much greater than adult recovery throughout the study. HWA density was directly correlated with warmer plant hardiness zones and recovery of *Laricobius* beetles was significantly correlated with HWA density. Our results suggest that *L. osakensis* is successfully establishing at several of the sampled release sites and that the best predictor of its presence at a site is the HWA density.

Keywords: classical biological control; monitoring; *Adelges tsugae*; *Laricobius osakensis*; *Tsuga canadensis*

1. Introduction

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), an insect pest native to Asia and the western United States, is causing significant mortality to eastern and Carolina hemlock trees in the eastern United States [1]. Hemlock trees are a long-lived, late successional, climax species in eastern hardwood forests, making the impact that HWA has on this tree species of critical environmental concern [2,3].

Biological control has the potential to contribute in a sustainable manner to the management of HWA and the improvement of health of hemlocks in the eastern United States. All life stages of *A. tsugae* except the crawler stage are sedentary, and thus, the extended amount of time spent on trees makes them easily accessible to predators. Beetles in the genus *Laricobius* Rosenhauer have been identified for biological control because they are specialists of Adelgidae [4]. *Laricobius rubidus* LeConte

is native to the eastern United States, and feeds on pine bark adelgid, *Pineus strobi* (Hartig) [5], though it can also feed and develop on HWA [6]. *Laricobius nigrinus* Fender is a native predator of HWA in western North America [7], and was first introduced in the eastern United States as a biological control agent in 2003 [7,8]. To date, *L. nigrinus* has successfully established and spread throughout the eastern United States [7,9].

Laricobius osakensis Montgomery and Shiyake, was discovered in Japan in 2005 from sampling *Tsuga sieboldii* (Carriere) [10]. It has the potential to be a promising biological control agent because it is a natural predator of the HWA strain present in the eastern United States [1,11]. Both the adults and larvae of *L. osakensis* feed on and are highly host-specific to HWA. The predator prefers to feed and oviposit on HWA over other adelgid and closely related non-adelgid species in laboratory tests, posing little risk to native fauna [12]. It has also been shown in laboratory studies that *L. osakensis* has no negative impact on *L. nigrinus* or *L. rubidus*, when co-occurring on HWA infested hemlock branches [13]. Additionally, *L. osakensis* cannot produce viable eggs from attempted mating with either species, even though the latter two species can successfully hybridize [14].

Releases of *L. osakensis* were initiated in the fall of 2012, following the Finding of No Significant Impact (FONSI) in 2010 by the USDA APHIS. There have been releases at 61 sites since 2012 [15]. Previous collections of *L. osakensis* from the original release sites recovered 224 beetles in the 2012–2013 field season at a Mountain Lake site, near Pembroke, VA, but only two beetles were recovered in the 2013–2014 field season [16]. Twelve beetles were recovered in the 2012–2013 field season at the Carnifex, WV site, with only five recovered in the 2013–2014 field season. One beetle was found in the 2013–2014 field season at a site in Goshen, VA. The decreases in recovery appeared to be correlated with HWA numbers declining because of an extended period of extreme below average temperatures caused by the first polar vortex event [16].

As releases continue, it is important to determine the success of *L. osakensis* at establishing, its dispersal potential, and impact on HWA populations. These data may provide information to help predict the long-term spread of the predator and provide insights into the type of site or habitat that may best be suitable for successful establishment of the insect. Based on the close association between both *L. osakensis* and HWA in their native habitat, we hypothesize (i) that this biological control agent will be able to establish on HWA in the eastern United States; (ii) that there is a greater likelihood of *Laricobius* recovery at higher HWA densities; (iii) that colder plant hardiness zones would decrease the likelihood of recovering *Laricobius* beetles; (iv) that larger release numbers of *L. osakensis* would result in a greater likelihood of recovering *L. osakensis*; and (v) and that each year post release would lead to an increase in the distance of *L. osakensis* dispersal. We tested this hypothesis by sampling for the presence and density of *L. osakensis* at ten sites, from Pennsylvania to North Carolina, where the predator was released between 2012 and 2016, as well as considering tree health, plant hardiness zone, and the impacts from the polar vortex.

2. Materials and Methods

2.1. Field Sites

Ten sites were monitored in this study with a northern extent of Trouts Run, PA and a southern extent of Great Smoky Mountain National Park, TN (Figure 1). Tree health measures were made at seven local sites in VA and WV (Table 1). These local sites, except for Shenandoah National Park, were sampled for *Laricobius* monthly from October 2015–April 2016 and October 2016–April 2017, while the remaining sites were sampled once in the fall and spring by collaborators (Table 2). Beetle releases at the study sites took place from 2012 to 2015. Numbers released ranged from 500 to 2000. Plant hardiness zone (based on average annual extreme minimum temperature from 1976–2005) ranged from 5b (15 to -10°C) to 7a (0 to 5°C) [17].

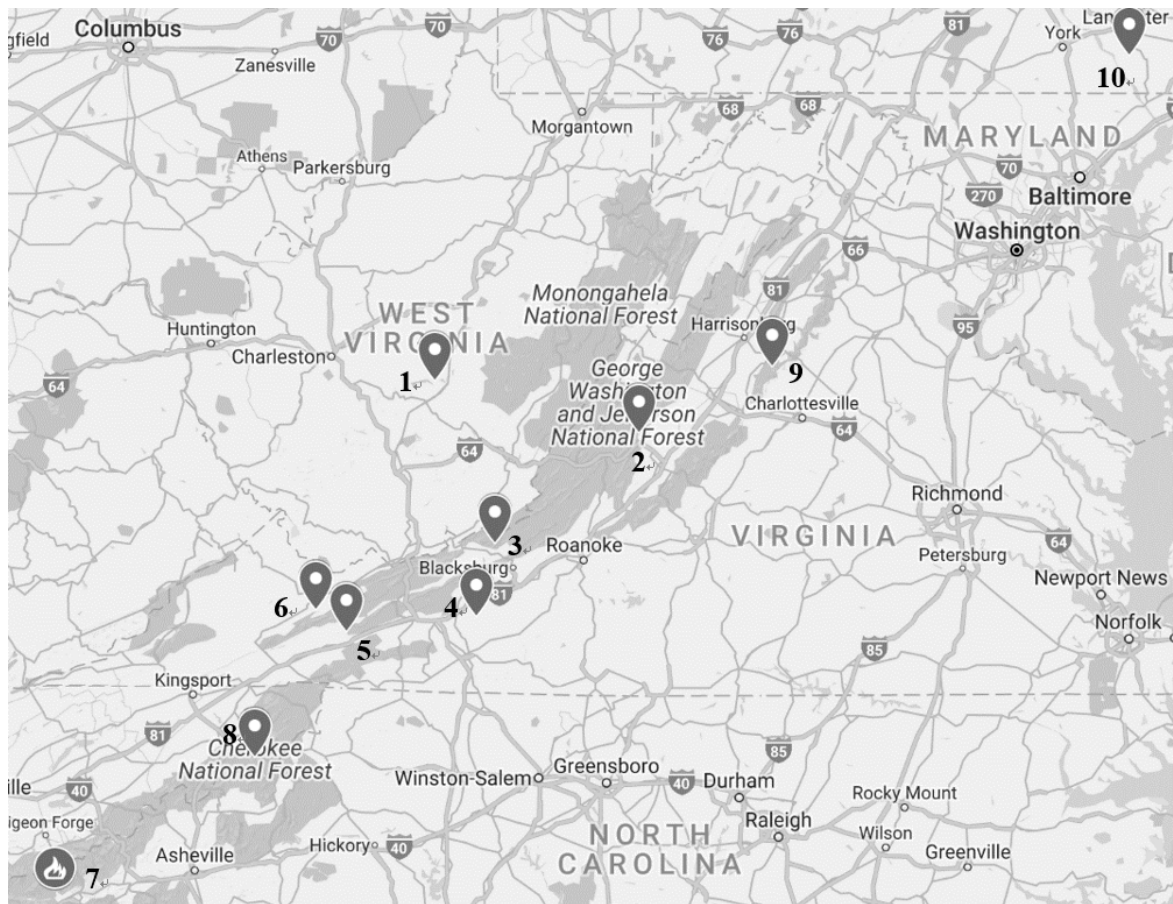


Figure 1. Map of locations of *Laricobius osakensis* release sites used in the study. Pins are numbered by field site. 1. Carnifex, WV; 2. Goshen, VA; 3. Mountain Lake, VA; 4. Powhatan Boy Scout Camp, VA; 5. Hungry Mother State Park, VA; 6. Saltville, VA; 7. Great Smoky Mountain National Park, TN; 8. Cherokee National Forest, TN; 9. Shenandoah National Park, VA; 10. Trout Run, PA.

Table 1. The local *Laricobius osakensis* release sites in Virginia and West Virginia, post-release sampling year, and average health index measures (\pm SD) of hemlock trees.

Site	Year	Average % Live Crown Ratio	Average % Live Branches	Average % Tips Alive	Average % New Growth	Average % Crown Density	Tree Health Index (THI) ^{1,2}
Mountain Lake (VA)	2015	88.8 \pm 10.3	78.8 \pm 16.5	78.8 \pm 16.5	39.4 \pm 14.8	87.5 \pm 2.9	74.7 \pm 20.3 AB
Carnifex, WV	2015	90.0 \pm 10.0	85.0 \pm 8.7	86.7 \pm 15.3	86.7 \pm 15.3	90.0 \pm 10.0	87.7 \pm 2.2 A
	2016	95.0 \pm 7.1	90.0 \pm 14.1	90.0 \pm 14.1	86.7 \pm 15.3	95.0 \pm 7.1	91.3 \pm 3.6 a
Saltville (VA)	2015	63.8 \pm 11.9	65.0 \pm 12.0	66.9 \pm 11.6	48.8 \pm 3.5	50.0 \pm 16.0	58.9 \pm 8.8 B
	2016	60 \pm 10.7	66.25 \pm 13.0	68.75 \pm 15.5	37.5 \pm 3.5	50 \pm 19.1	56.5 \pm 12.9 b
Goshen (VA)	2015	55.6 \pm 15.9	62.5 \pm 16.9	66.3 \pm 16.0	56.3 \pm 30.7	55.6 \pm 12.7	59.3 \pm 4.9 B
	2016	72.0 \pm 10.9	66.0 \pm 8.9	72.0 \pm 4.5	20.0 \pm 0.0	68.0 \pm 8.4	59.6 \pm 22.3 b
Hungry Mother State Park (VA) (1st/2nd)	2015	64.3 \pm 11.3	61.4 \pm 10.7	55.7 \pm 7.9	41.4 \pm 10.7	54.3 \pm 18.1	55.4 \pm 8.8 B
	2016	65.7 \pm 18.1	62.9 \pm 17.0	57.1 \pm 7.6	10.0 \pm 0.0	57.1 \pm 24.3	50.6 \pm 23.0 b
Powhatan Boy Scout Camp (VA)	2015	22.9 \pm 12.2	36.4 \pm 17.0	33.6 \pm 19.1	3.9 \pm 2.8	17.0 \pm 9.5	22.8 \pm 13.2 C
	2016	35.0 \pm 22.0	41.3 \pm 18.7	40.0 \pm 15.6	7.5 \pm 10.3	31.0 \pm 16.4	31.0 \pm 13.7 c
Shenandoah National Park (VA)	2015	84.0 \pm 15.2	77.0 \pm 8.4	72.0 \pm 13.0	52.0 \pm 17.9	64.0 \pm 11.4	69.8 \pm 12.3 AB
	2016	64.0 \pm 20.7	74.0 \pm 13.4	62.0 \pm 4.5	20.0 \pm 0.0	54.0 \pm 24.1	54.8 \pm 20.7 b

¹ Tree Health Index is the mean \pm S.D. of all five health measurements (%) for each site. ² Statistical means separated by year are indicated by uppercase letters for 2015, and lowercase letters for 2016. Means followed by the same letters are not significantly different at $p \leq 0.05$.

Table 2. Field collections of *Laricobius* spp., including information on site location, plant hardiness zone of the field site, year of beetle release, number of beetles released, and *Adelges tsugae* (HWA) density (adelgids/cm) at time of sampling.

Site	Year of Release	Number Released	Plant Hardiness Zone ²	Field Season	HWA Density (adelgid/cm)	<i>Laricobius</i> Adults (Percent Estimated <i>L. osakensis</i>) ³	<i>Laricobius osakensis</i> Larvae	<i>Laricobius nigrinus</i> Larvae	<i>Laricobius rubidus</i> Larvae
Mountain Lake (VA)	2012	500	5b	2015–2017	~0	0	0	0	0
Carnifex (WV)	2012	500	6a	2015–2017	~0	0	0	0	0
Saltville (VA)	2013	2000	5b	2015–2017	~0	0	0	0	0
Goshen (VA)	2013	2000	6b	2015–2016	1.4 ± 1.0	0	0	0	7
				2016–2017	1.1 ± 0.6	0	21	2	38
Hungry Mother (VA) ⁴	2014/2015	617/300	6b	2015–2016	1.7 ± 0.8	3 (80%)	8	2	0
				2016–2017	1.6 ± 0.8	12 (13%)	12	62	16
Powhatan Boy Scout Camp (VA)	2014	1000	6b	2015–2016	1.0 ± 0.4	0	2	0	26
				2016–2017	1.2 ± 0.6	3 (19%)	11	39	8
Shenandoah National Park (VA) ¹	2015	500	6b	2015–2016	1.9 ± 0.7	0	2	3	3
				2016–2017	1.8 ± 0.5	1	0	0	0
Great Smoky Mountain National Park (TN)	2014	921	6b	2015–2016	1.3 ± 0.3	0	2	0	0
Trouts Run (PA) ¹	2015	1021	6a	2016–2017	1.6 ± 0.4	41 (100%)	46	0	0
South Cherokee National Forest (TN)	2014	781	7a	2015–2016	1.9 ± 0.6	0	0	0	5
				2016–2017	1.8 ± 0.6	0	0	0	0

¹ Released beetles were a mix of three *Laricobius* spp. with ~34% being *L. osakensis*. ² Plant hardiness zone temperatures; Average annual extreme minimum temperature 1976–2005; 5b: −15 to −10 °C, 6a: −10 to −5 °C, 6b: −5 to 0 °C, 7a: 0 to 5 °C. (<http://planthardiness.ars.usda.gov>). ³ Estimated percent of *L. osakensis* adults based on identified larval recoveries.

⁴ Augmented release of *L. osakensis* at Hungry Mother.

2.2. Evaluating Tree Health and HWA Density

Tree health data were collected in 2015 and 2016 from release trees at all sites in VA and WV. The percentages of live crown ratio, live branches, tips alive, new foliage, and crown density were recorded, and the average of these five measures was calculated to provide an overall tree health index (THI) from 0–100%, with 100 representing a completely healthy tree [18]. Hemlock woolly adelgid density was recorded from all 10 sites on each tree sampled for *L. osakensis*. Four branches were randomly selected from the tree, and ten branchlets/twigs were selected from each of the branches to determine the number that had HWA. The number of HWA and the length of the branchlet was recorded and averaged to determine the mean number of HWA/cm.

2.3. *Laricobius* Sampling

2.3.1. Beat Sheet Sampling

Presence of *Laricobius* adults at the six local sites mentioned above was tracked monthly from October to May for two years (2015–2017) using beat sheet sampling at the plot center of release and surrounding trees. The beat sheet was made of a 71 cm² canvas sheet secured to an X-frame made of PVC piping. Sampling was carried out by placing the sheet under lower canopy branches with high HWA populations, and tapping ten times with a 1 m long walking stick. The sheets were then examined to identify predators that were dislodged from branches. If predators were found, they were returned to the hemlock tree unharmed. The tree number (when applicable), the number of *Laricobius* adults recovered, and site conditions were then recorded. Beat sheet sampling only took place on days where temperatures were above 0 °C, and when it was not windy, snowing, or raining, as these are the worst conditions to find *Laricobius* species [6]. Beat sheet sampling is less effective in the winter, due to colder temperatures, than in the spring and fall, often producing false negatives in recovery numbers [6,7].

2.3.2. Branch Clip Sampling

To further assess the presence of *Laricobius* beetles at a site, branch clip sampling was found to be a more accurate method than beat sheet sampling for adults [7]. Branch clippings were collected from nine of 10 sites for each of two years (only one year at Trouts Run, PA), either in March or April, depending on when HWA oviposition was at its peak [15]. *Laricobius osakensis* eggs and larvae were sampled by selecting branches up to 2.5 m from the ground with HWA ovisacs between 2–3 mm in diameter, which indicates peak egg abundance [6]. Branch sections of 20–30 cm were clipped using hand pruning shears. Samples were placed into individually labeled bags, taken back to the Virginia Tech Insectary in Blacksburg, VA, and placed into rearing funnels [19]. Once larvae completed development, pre-pupae began to drop into mason jars attached to the rearing funnels. The funnels were inspected several times a week and the number of larvae were recorded for the branch clippings from each tree [19,20]. Any larvae found throughout this process were placed in a vial of 99% ethanol. Genetic testing based on the mitochondrial cytochrome oxidase I (COI) gene was used for species identification since *Laricobius* larvae are morphologically indistinguishable among species [15,21], and larvae of the native *L. rubidus* may be present at these sites. A partial cytochrome oxidase subunit I (COI) was amplified for *Laricobius* larvae recovered over the field season. The amplification of the COI gene was performed according to the protocols described by Davis et al. (2011) and Fischer et al. (2014) [21,22].

2.4. *Laricobius* Dispersal

Dispersal of *L. osakensis* was assessed at sites where releases occurred at least two years prior to the study and where HWA was present at Hungry Mother State Park, Powhatan Boy Scout Camp, and Goshen. Beat sheet sampling was carried out on release trees in the plot center, and at approximately 30, 90, and 180 m from the perimeter of the plot center in each cardinal direction (where

applicable). In the spring, branches infested with HWA were clipped from trees approximately 30, 90, and 180 m from the plot center in each cardinal direction. The branches were labeled by distance from the release tree and brought to the Virginia Tech Insectary, Blacksburg, VA. Any larvae collected were put in ethanol and saved for genetic testing. The exact distances and directions from the release trees varied by site since trees were not always available at 180 m from the release tree.

2.5. Statistical Analysis

THI's were compared using a standard least squares model and a Tukey's test to determine significance of differences ($p < 0.05$). The main factor was field site, and the analyses were done separately for the two years of the study, fall 2015 and fall 2016.

The association between the number of *L. osakensis* larvae and selected study site variables (HWA density, tree health index, number of beetles released, and plant hardiness zone) and the association between *Laricobius* spp. larvae and adults and HWA density were measured using nonparametric Spearman's Rank correlation analysis. Correlation analysis was also used to determine the association between HWA density with tree health and plant hardiness zone.

Nominal logistic regression was used to develop a predictive model of *Laricobius* beetle presence in relation to each of the previously defined study site variables. The statistical significance of the predictor variable in the derived model was evaluated by the Wald χ^2 statistic [23]. The Odds ratio was calculated to quantify the odds of the beetle being present for each unit change in the predictor variable [23–25]. In addition, we used the area under the receiver operating characteristic (ROC) curve, AUC, to assess the predictive ability of the model [23]. AUC values of 0.5–0.7, 0.7–0.9, >0.9 indicate poor, good, and excellent discrimination ability of the model, respectively [23]. All analyses were carried out using JMP Pro version 12.1 (SAS, Cary, NC, USA).

Because of a lack of distance-related collections, no statistical analysis was performed on the dispersal data.

3. Results

3.1. Evaluating Tree Health Index and HWA Density

Tree health varied among sites, but remained relatively consistent at individual sites between years (Table 1). The Mountain Lake and Carnifex field sites had the highest tree health index (THI) >74. Virtually no HWA were observed at the two sites during the sampling periods (Table 2). Carnifex had a significantly greater THI than all sites except Mountain Lake and Shenandoah National Park in 2015 and above all sites sampled in 2016 (Table 1). Although the Saltville and Carnifex sites had no HWA (Table 2), THI was significantly lower (<60) at Saltville compared with Carnifex (Table 1). The Powhatan Boy Scout site had the lowest THI (<32), which was significantly lower than all sites in both years (Table 1). Some of the trees at this site were dying or had very few live branches, and some were cut back for trail maintenance. For sites that were infested with HWA during the two-year sampling period, densities changed little between years, ranging between 1.0–1.9 HWA/cm (Table 2).

3.2. Recovery of *L. osakensis*

Three *Laricobius* spp. adults were collected during the first field season (Fall 2015–Spring 2016), all at Hungry Mother State Park (Table 2). Since *Laricobius* beetles caught in the field were returned to trees, the species was not identified. An estimated percentage of *L. osakensis* adults were based off of the number of *L. osakensis* larvae found at each site from the branch clip sampling (Table 2). During the 2016–2017 field season, a total of 57 adult beetles were collected. Twelve were found at the Hungry Mother State Park site, three at the Powhatan Boy Scout Camp, 41 at Trout's Run, and one at Shenandoah National Park. All of the *Laricobius* adults recovered were returned to the trees unharmed since adult recoveries were low. As in the first field season, the percentage of *L. osakensis* from adult

recoveries were estimated based on the number of *L. osakensis* larvae collected from branch samples later in the spring.

In the first year of larval sampling (Spring 2016), a total of 71 larvae were collected, of which 60 yielded a definitive identification using genetic analysis. From the larvae that were identified, 14 were *L. osakensis* with eight of these found at the Hungry Mother release site, two at Shenandoah National Park, two at the Powhatan Boy Scout Camp, and two in Great Smoky Mountain National Park (Table 2). No *L. osakensis* larvae were collected from Goshen or South Cherokee National Forest. The remainder of the 60 positively identified larvae consisted of 41 *L. rubidus* and 5 *L. nigrinus* across varying sites.

In the second year of larval collections (Spring 2017), a total of 318 larvae were collected. Conclusive genetic analysis identification was achieved on 255 of them. Of these larvae, 90 were found of be *L. osakensis*, with 46 from Trout's Run, 11 from Powhatan Boy Scout Camp, 12 from Hungry Mother State Park, and 21 from Goshen (Table 2). No *L. osakensis* were recovered from other sites where larval branch clippings were collected, including Shenandoah National Park, and South Cherokee National Forest. Larval collection in the second year also yielded a total of 103 *L. nigrinus* and 62 *L. rubidus* from varying release sites (Table 2).

The results of the Spearman's correlation analysis indicated a significant positive correlation between the number of *Laricobius* spp. adults and larvae recovered and HWA density, but no significant correlation was found between *L. osakensis* larvae and HWA density. This suggests that there was too short a period of time from when the beetles were released, or that they were collected at too few sites across all sample sites. There was a negative correlation between number of *L. osakensis* larvae and THI, and a positive correlation between HWA density and plant hardiness zone (Table 3).

Table 3. Results of a nonparametric Spearman's rank correlation analysis between *L. osakensis* or HWA density and selected sampling site variables.

Factors	Spearman ρ ¹	p-Value
<i>Laricobius</i> spp. larvae and adults & HWA density	0.48	0.042
<i>L. osakensis</i> larvae & HWA density	0.29	0.247
<i>L. osakensis</i> larvae & THI	−0.54	0.045
<i>L. osakensis</i> larvae & Plant hardiness zone	0.21	0.412
<i>L. osakensis</i> larvae & Number of beetles released	0.19	0.447
HWA density & Plant hardiness zone	0.83	0.001
HWA density & THI	−0.50	0.066

¹ A negative spearman ρ value suggests a negative correlation (e.g., A decline THI, is correlated with an increase in *L. osakensis* larvae populations). A positive spearman ρ value suggests a positive correlation (e.g., an increase in HWA density is correlated with an increase in *Laricobius* spp. populations).

The nominal logistic regression analysis showed that the density of HWA at a site had a significant effect on whether *Laricobius* spp. beetles were recovered ($\chi^2 = 13.4562$, $p = 0.0002$). The AUC for this model was 0.89, indicating a very good predictive ability of the model. The odds of recovering beetles is predicted to increase by a factor of 26 for each unit increase in HWA density (HWA/cm). The analysis also showed that plant hardiness zone had a significant impact on beetle recovery ($\chi^2 = 5.57108$, $p = 0.0183$). The AUC for this model was 0.80, indicating a good predictive ability for this model. The odds of finding beetles increased by a factor of 1.29 for each unit of increase in plant hardiness (odds increased by 29% from a colder to a warmer plant hardiness zone (i.e., 5b to 6a). All other relationships analyzed were insignificant ($p \leq 0.05$).

3.3. Dispersal Study

Sampling for dispersal in 2016 yielded no *L. osakensis*. In 2017, 19 *L. osakensis* larvae were found on sample trees. At Powhatan Boy Scout Camp (2014 release), Goshen (2013 release), and Hungry Mother State Park (2014/2015 release), the furthest distances that larvae were collected were at 30,

30, and 90 meters, respectively, outside the perimeter of the release center. Since the recoveries were somewhat limited on a per site basis, there were not enough data to adequately quantify dispersal rate.

4. Discussion

Beat sheet sampling produced low numbers of *Laricobius* spp. beetles throughout both years of sampling, with only 60 beetles (41 of which were collected at Trout's Run) collected despite monthly field visits. Studies have shown that beat sheet sampling of small insects is difficult and can produce false negatives [7,26]. Beat sheet sampling of *Laricobius* beetles is also less effective in the winter [6], which could partially explain the lack of recoveries in this study. It should also be noted that the Great Smoky Mountain National Park release site was only monitored for one year because of a forest fire that destroyed many of the trees at the site.

The branch clipping sampling method for *Laricobius* larvae was much more successful at finding the target predator. The low number of recoveries in the first year was likely due to the colder than normal winter temperatures in the 2014 and 2015 seasons, which resulted in a significant decrease in adelgid density throughout the range of HWA in the eastern U.S. [27]. The low adelgid populations made it difficult to collect *L. osakensis*, since other than their occurrence as pupae and aestivating adults, this beetle can only be found directly associated with HWA [11]. With increased winter temperatures the following year, the adelgid populations rebounded. As a result, the numbers of *L. osakensis* collected increased along with an increase in HWA density. The increased *L. osakensis* populations suggests that the beetle is establishing at some of the release sites.

A significant negative correlation between tree health and *L. osakensis* larval recoveries is explainable given a negative correlative trend between HWA density and tree health was also observed. When the health of the tree rebounds, availability of feeding sites increases for the adelgids [28]. The relationship of tree health and adelgid populations is a density-dependent reciprocal feedback loop, with observed lag effects, often resulting in tree health decline over time [18]. With the lag effect in HWA and predator numbers as they relate to tree health [18], additional years of monitoring are needed to capture the lag effects we suspect are at work. Extreme cold temperatures associated with the polar vortex reduced HWA densities at our study sites [27], which gave trees an opportunity to rebound from pest attack.

Our analysis showed a strong significant relationship between HWA density and *Laricobius* beetles recovered, suggesting that if HWA populations remain high following predator releases, the likelihood of predators establishing increases. In turn, if populations of HWA decline following release of the predator, it will be much more difficult for them to establish. An extreme case of this is found when looking at our results from Mountain Lake, Carnifex, and Saltville. HWA did not rebound following the polar vortex and no *Laricobius* beetles were collected at these sites following the crash in HWA, even though they were found early on when HWA was present [16]. If there are insufficient densities of HWA, then *L. osakensis* is less likely to thrive. While low host availability was likely a strong factor in the low recovery numbers of *L. osakensis*, an alternative explanation could be undercrowding of *Laricobius* beetles. The sites we had in this study that had low HWA density, and as a result low *Laricobius* recovery, could be a result of the Allee effect (undercrowding resulting in a decline in population growth) [29,30]. Perhaps a way to mitigate this effect in the future would be to have smaller, but more frequent, releases, especially in sites still recovering from the polar vortex [30,31], or to identify a population threshold of adelgids at sites prior to release.

Plant hardiness zone was significantly correlated with the recovery of *Laricobius* beetles. We found a 29% increase in odds of finding beetles with each increase in plant hardiness zone (i.e., 5b to 6a), suggesting that warmer plant hardiness zones are more suitable for *Laricobius* spp. Sites in the warmer hardiness zones did not get the same extreme winter temperatures during the polar vortex [27], which led to a smaller decline in HWA density and in *Laricobius* spp. populations. This finding is limited, as our analysis dealt with plant hardiness zones 5b–7a, and HWA is not found south of Northern Georgia (7a/7b) [17].

The significant correlation between plant hardiness zone and HWA density indicated that trees in colder plant hardiness zones had lower densities of HWA. Trees from colder plant hardiness zones, such as at the Carnifex, Mountain Lake, and Saltville sites have had almost no HWA since the polar vortex occurred, suggesting that the HWA populations are struggling to rebound at these sites due to cold temperatures, allowing the trees to experience sustained growth for longer periods of time, leading to healthier trees overall (Table 1). Trees at the Saltville site, however, were not as healthy as the trees in the Carnifex and Mountain Lake sites, despite their lack of HWA. This could be attributed to the lag response in health that occurs when a tree is infested with HWA, or we could speculate that the lower THI is due to site conditions unrelated to climate [18].

Rearing, releasing, and assessing *Laricobius* beetles as biological control agents comes with some complications. In the Virginia Tech Insectary where *L. osakensis* is reared for releases, it was found that some of the 2015 *L. osakensis* releases were accidentally contaminated with *L. nigrinus* and *L. rubidus* (Table 2). Occasionally, when HWA were collected for feeding the predators at the Insectary, other *Laricobius* spp. were unknowingly present in the infested foliage. This can happen despite efforts to remove any incoming predators from the branches. In the future, it is likely that releases of predators are going to have a mixed composition of *L. osakensis*, *L. nigrinus*, and *L. rubidus*, despite efforts to purify colonies. The composition of *Laricobius* spp. is generally unknown until genetic testing is done on the larvae following rearing. Therefore, the 2015 beetle releases were probably of mixed species composition. Specifically, contaminated *L. osakensis* releases during the 2015 field season came from combination of the F3 and F5 cohorts. The F3 Parents (99 total) consisted of *L. osakensis* (23%), *L. nigrinus* (57%), *L. rubidus* (6%), and undetermined (13%). The F5 Parents (220 total beetles) consisted of *L. osakensis* (40%), *L. nigrinus* (46%), *L. rubidus* (5%), and unknown (8%). In total, the parents of the beetles released were only ~34% *L. osakensis*. The beetles recovered in the 2015–2016 field season from sites where these releases were made was 18% *L. osakensis*. This level of contamination will likely impact the results of future field monitoring data at those sites. The Goshen and Boy Scout Camp release sites had two and 39 *L. nigrinus* collected, respectively. Since there was no documented contamination at these release sites, this suggests that (1) *L. nigrinus* dispersed from other release locations, (2) there were undocumented releases at sites nearby, or (3) previous *L. osakensis* colonies had been unknowingly contaminated, as the colonies had not been monitored for contamination until 2015. The closest site to the Goshen release site where *L. nigrinus* was recorded was ~33 km away [15], and the release was made in 2012. Similarly, the closest site to the Boy Scout Camp at which *L. nigrinus* was observed was ~35 km away, where beetles were released in both 2010 and 2014 [15]. Due to the fact that *L. rubidus* is native to the field sites, it is not surprising to have this beetle species appear in our samples. Our field sites commonly had white pine (*Pinus strobus* L.) as a component of the forest, with pine bark adelgid as the preferred host for *L. rubidus* [32,33].

Because of prey overlap and similar life cycles, we predicted that the dispersal rates of *L. osakensis* and *L. nigrinus* would be similar. A previous study found that *L. nigrinus* dispersed at a rate of 30.6–39.2 m/year [34]. However, we were unable to obtain sufficient data on the dispersal of *L. osakensis* to make a comparison. In addition, some of the field sites lacked hemlock trees with HWA at the prescribed sample distances or there were issues with accessibility due to main roads and rivers. This study focused on local-scale dispersal of *L. osakensis*, and perhaps future studies after further establishment can monitor their dispersal over longer distances. Continued efforts of sampling at release sites are needed to better characterize *L. osakensis* dispersal abilities.

5. Conclusions

Laricobius osakensis appears to be established at Hungry Mother State Park, Goshen, and Powhatan Boy Scout Camp release sites. The most important factor in finding *L. osakensis* and determining its establishment is HWA density. The significant relationship between *Laricobius* recovery and tree health are a direct result of HWA density. The significant relationship between plant hardiness zone and *Laricobius* recovery is also a factor of how much HWA is present at these sites, especially after the

polar vortex. Some of the earlier release sites, including Mountain Lake, Saltville, and Carnifex had very low adelgid numbers, which explained the lack of *L. osakensis* recoveries. Continued sampling at all of the release sites is necessary to verify establishment. Our objective is to ensure successful establishment and spread of *L. osakensis* to control HWA and to build up predator populations for harvesting and redistributing on a consistent basis. The goal is to find sites to use as field insectaries, which will provide more natural conditions for rearing predators, as beetles will be better adapted to the environment, healthier, and more robust than lab reared beetles [7].

Author Contributions: S.S., K.M., and A.T. contributed to overall methodology and conceptualization of the study; The investigation was conducted by A.T. and K.M.; C.B. and A.T. conducted the formal analyses; A.T. was responsible for original draft preparation; S.M. was responsible for funding acquisition and work supervision. All authors contributed to the review and editing of manuscript.

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