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The Impact of Predation of *Laricobius nigrinus* (Coleoptera: Derodontidae) on *Adelges tsugae* (Hemiptera: Adelgidae) and *Tsuga canadensis* (Pinales: Pinaceae) Tree Health

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Abstract: The hemlock woolly adelgid (HWA), *Adelges tsugae*, has threatened the sustainable management of *Tsuga canadensis* in the eastern United States. Biological control efforts have led to the establishment of *Laricobius nigrinus*, a specialist predator of HWA. Although *L. nigrinus* has a significant impact on HWA populations, its effect on the health of HWA's host is unknown. In 2020, 14 eastern hemlocks at one site in Virginia were selected to determine whether predation of *L. nigrinus* at different densities on HWA populations had an effect on tree health. *Laricobius nigrinus* predation significantly impacted the HWA sistens generation, resulting in significantly more new shoots produced on treatment branches with the greatest density of *L. nigrinus* adults. Final HWA density was lowest on treatment branches with *L. nigrinus*, followed by the negative control, and the treatment without *L. nigrinus*. In June, the photosynthetic rate was significantly greater for the negative control, followed by *L. nigrinus* treatments. There were no statistical differences among measured tree physiological parameters in July and October, indicating a temporary effect from *L. nigrinus* predation on hemlock tree physiology.

Keywords: classical biological control; hemlock woolly adelgid; eastern hemlock; photosynthesis; *Laricobius nigrinus*

Citation: Preston, C.E.; Arneson, A.; Seiler, J.R.; Salom, S.M. The Impact of Predation of *Laricobius nigrinus* (Coleoptera: Derodontidae) on *Adelges tsugae* (Hemiptera: Adelgidae) and *Tsuga canadensis* (Pinales: Pinaceae) Tree Health. *Forests* **2023**, *14*, 698. https://doi.org/10.3390/f14040698

Academic Editors: Salvatore Moricca and Tiziana Panzavolta

Received: 1 March 2023
Revised: 20 March 2023
Accepted: 27 March 2023
Published: 29 March 2023

1. Introduction

Eastern hemlock, *Tsuga canadensis* (L.) Carrière (Pinales: Pinaceae) is a forest foundation species found throughout the eastern United States, with populations extending from southern Canada to northern Georgia and into Michigan and Wisconsin [1]. As a long-lived shade-tolerant tree species, it provides a unique habitat for 120 vertebrate and 300 invertebrate species [1]. In the early 1950s, the hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), was detected in Richmond, Virginia, and has since spread and killed hemlocks throughout the eastern US, threatening these unique forest ecosystems [2]. The presence of the hemlock woolly adelgid (HWA) within these systems has resulted in ecological alterations such as an increase in light exposure and increases in soil and water temperatures, due to thinning canopies and tree mortality of the eastern hemlock [1]. Such alterations have devastated flora and fauna that depend on these unique habitats [1,3–7].

The hemlock woolly adelgid is a small aphid-like insect in the order Hemiptera and in the family Adelgidae [8]. Its native range includes the Pacific Northwest and eastern Asia (Japan, China, and Taiwan) [9]. The source of the HWA invasion into the eastern US was confirmed to be from southern Japan, likely from nursery stock [2]. In the eastern US, HWA has two wingless asexual generations per year, known as the sistens and the progrediens [10]. All generations go through six developmental stages; the egg stage, four nymphal instars, and the adult stage [10]. In Virginia, sistens are present from June to mid-April and progrediens are present from March to mid-June [11,12]. After settling at the base of a hemlock needle, sistens and progrediens nymphs and adults use straw-like mouthparts, known as stylet bundles, to bore through woody tissues and feed on xylem ray parenchyma cells which store nutrients for the tree [13,14]. From these cells they ingest starches and sugars utilizing at least four trophically related enzymes that could cause systemic host responses [13].

Eastern hemlock responds in several ways to HWA feeding. Physical symptoms to HWA feeding include chlorosis, premature needle drop, branch dieback, and reduction in new growth [15]. Physiological responses vary. In early-stage HWA infestations, one study indicated that photosynthesis and xylem hydraulic conductivity increased [16]. However, other studies have found a reduction in photosynthesis [17,18]. Later-stage HWA infestations were observed to cause a reduction in photosynthesis [19], and a reduction in hydraulic conductivity, carbon assimilation, and stomatal conductance [20]. Water use efficiency has been observed to increase, likely due to an increase in water stress [20]. Additional effects include abnormal wood formation within the xylem [20], missing and partial ring formation [21], and the formation of false rings [22]. Tree mortality has been known to occur from four to 10 years regardless of tree age [23,24].

Several management strategies have been deployed to protect and conserve eastern hemlocks. In the forest setting, chemical treatment of individual trees has been an important conservation tool, but is impractical as a long-term, stand-alone solution. Therefore, a classical biological control program using predators has also been pursued. Currently two species of *Laricobius* have shown great promise as biological control agents. One of these, *Laricobius nigrinus* Fender, (Coleoptera: Derodontidae), native to western North America, is a specialist predator of HWA and only completes development on this pest species [25,26]. In 2003, *L. nigrinus* was approved for release and has successfully established and continues to disperse throughout the eastern US [27–30]. Using exclusion cages over branches on HWA-infested hemlocks at nine sites (from NJ to GA) where *L. nigrinus* was established, it was shown that the predator significantly impacted sistens populations over a four-year period [31]. However, the relationship of *L. nigrinus* adelgid predation on hemlock tree health was not determined. Studies that focused on the effectiveness of an integrated pest management approach combining chemical and classical biological control, evaluated tree health by measuring the amount of new shoot growth produced and determined crown health following the USDA Forest Service Forest Inventory and Analysis crown condition parameters. The results indicated a decline in tree health over time [32,33]; however, *L. nigrinus* was not determined to be established at the field sites [33]. The effectiveness of *L. nigrinus* on hemlock tree health was evaluated in a study using tree-ring analysis; ring-width declined after HWA arrived at the research site with no recovery of *L. nigrinus* following its release [34]. These studies evaluated tree health via external observation providing little insight into tree physiology.

This study focused on the tritrophic relationship between the predator (*L. nigrinus*), the prey (HWA), and the plant host (eastern hemlock). To address this, the study sought to determine if different densities of *L. nigrinus* feeding on HWA would have an effect on the net photosynthetic rate of eastern hemlocks at the branch level, after impacting the HWA population. Additional assessments include the evaluation of HWA sistens ovisac disturbance, new growth production, and the final HWA population density at the end of the study. The study was designed to indicate whether *L. nigrinus* feeding on HWA could affect hemlock growth and physiology over the short term (1 year).

2. Materials and Methods

2.1. Field Site Set Up

A forest site near of Wytheville, VA, named Gullion Fork, was selected for this study (GPS coordinates: 37°00′25.8″ N 81°15′41.9″ W). Fourteen eastern hemlock trees were selected based on overall tree health and HWA density. Tree health assessments were evaluated by one person using the following USDA Forest Service Forest Inventory and Analysis (FIA) crown condition categories: crown density, transparency, dieback, and live crown ratio [35]. Diameter at breast height (DBH) and tree classifications were also recorded for each tree (Table 1). Trees were selected in the fall of 2020, soon after HWA sistens broke aestivation. Four 1-m branches were selected from each tree. One branch with HWA populations < 1 adelgid per cm on current-year shoot growth was assigned as the negative control treatment (Con) (Figure 1). Three branches with HWA densities > 1 adelgid/cm on current-year growth were randomly assigned one of the following treatments: No *L. nigrinus* (NL), four *L. nigrinus* (L4), and eight *L. nigrinus* (L8). From the 1-m branches, two 20–30 cm branchlets from each branch were marked with green twist ties and were assigned either experiment 1 or experiment 2. Once treatments and branchlets were assigned, branches were tapped approximately ten times with a wooden dowel to dislodge any predators of HWA that were present. Bug dorm cages L 100 cm × W 66 cm (MegaView Science Co., Ltd., Taichung, Taiwan) were then applied over each branch and secured over a piece of insulated pipe foam 7.5 cm long by 1.25 cm wide and secured with zip ties. Three pairs of Onset HOBO data loggers (Onset Computer Corporation, Bourne, MA, USA) were placed on three study trees distributed throughout the field site. For each pair, one data logger was placed inside of a mesh cage and the other was placed on a branch nearby outside of the mesh cage. Each data logger was set to log temperature every 60 min. Average monthly temperatures at the site were obtained using data collected by the data loggers. The National Oceanic and Atmospheric Administration (NOAA) National Centers for Environmental Information database was used to obtain monthly precipitation measurements from station USC00449301 in Wytheville, VA (GPS coordinates: 36°56′11.2″ N 81°03′42.7″ W) (Table 2). Average monthly temperatures inside and outside mesh cages were obtained by data loggers on site (Table 3).

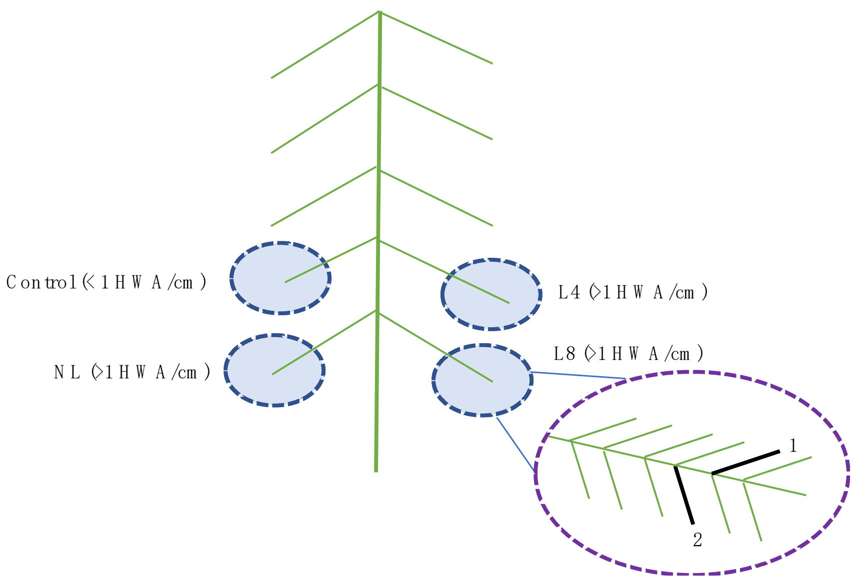

Figure 1. Tree set-up diagram. Green lines represent an example of an eastern hemlock tree with branches selected for the study. Small blue dotted circles represent mesh cages applied over treatment branches. Each cage was assigned one of four treatments: Control, NL, L4, or L8. The control branches had an HWA density < 1 HWA/cm, the other three treatments had an HWA density > 1 HWA/cm. The large purple circle represents a close up of a treatment branch. Bold black lines represent branchlets off of the main branch that were selected for experiment 1 and 2.

Table 1. Tree health assessment values, class classifications, and DBH measurements for each tree used at Gullion Fork near Wytheville, VA. Crown measures were based on a scale of 0–100.

Tree #	Class	DBH (cm)	Crown Density	Live Crown Ratio	Transparency	Foliage Density	Dieback
12	Suppressed	11.7	85	90	15	85	5
13	Suppressed	9.9	90	90	5	95	5
14	Intermediate	25.9	80	80	15	80	15
15	Suppressed	10.7	80	95	15	85	5
16	Suppressed	14.5	80	90	15	85	5
17	Intermediate	25.7	95	95	5	95	5
18	Suppressed	14	75	90	15	75	10
19	Suppressed	12.2	80	95	10	85	10
20	Suppressed	5.1	85	80	10	90	10
21	Suppressed	13.5	75	90	20	70	10
22	Suppressed	13.5	85	95	10	85	5
23	Suppressed	24.9	75	80	20	75	15
24	Suppressed	14.2	75	80	20	75	10
25	Suppressed	13.7	90	95	5	95	5

Table 2. Mean monthly temperatures recorded outside of the mesh cages at Gullion Fork near Wytheville, VA. Temperatures were recorded every 60 min using dataloggers on site. Precipitation data were obtained from the NOAA database.

		Temperature (°C)			Precipitation (mm)
Month	Sampling Date [a]	Mean	Minimum	Maximum	Mean
January		0.66	−11.90	29.70	3.28
February		1.51	−11.90	29.50	3.95
March		7.53	−10.20	36.20	4.09
April		10.70	−8.58	38.30	2.75
May		14.20	−1.57	37.20	1.39
June	14 June 2021	18.70	3.58	30.20	0.17
July	30 July 2021	19.90	9.28	30.00	3.97
August		20.30	9.96	30.90	3.25
September		15.70	1.22	28.30	3.08
October	1 October 21	13.30	3.68	25.00	3.79
November		5.72	−5.37	27.00	3.50
December		1.07	−12.20	26.90	2.62

[a] Represents the dates when photosynthesis, stomatal conductance, and transpiration were measured on treatment branches.

Table 3. Mean (+/− SE) temperatures inside and outside mesh cages from December 2020 to May 2021. Cages were removed after May. Group represents letters indicating statistical differences between mean temperatures determined by Tukey's HSD test. Different letters for outside and inside temperatures within a month indicate statistical significance ($p < 0.05$).

Year	Month	Location	Mean Temperature (°C)	SE (±)	Group
2020	December	Inside	1.36	0.204	AB
		Outside	0.78	0.204	BC
2021	January	Inside	0.89	0.204	AB
		Outside	0.42	0.204	A
2021	February	Inside	1.74	0.21	C
		Outside	1.28	0.21	BC
2021	March	Inside	7.77	0.204	D
		Outside	7.3	0.204	D
2021	April	Inside	11.02	0.206	E
		Outside	10.45	0.206	E
2021	May *	Inside	13.47	0.233	F
		Outside	14.65	0.204	G

* $p < 0.0001$.

2.2. Laricobius nigrinus Rearing

We used established rearing protocols for optimized lab rearing of *L. nigrinus* [36,37]. In April 2020, *L. nigrinus* prepupae were collected from hemlock branch clippings taken throughout Blacksburg, VA. Soil containers (710 mL) with ~8 cm of soil composed of 2:1 peat moss:sand and a soil moisture content ranging between 30% and 35% were prepared to hold prepupae. Weights of each soil container were recorded before prepupae were added. Approximately 75 prepupae were added to each soil container. Soil containers were held in an environmental chamber set at 13 °C with a 12L:12D photoperiod. Weights of the soil containers were checked weekly, and distilled water was applied to maintain the recorded container weight. Fungi found developing on the soil surface were removed using sterilized forceps. After 6–8 weeks, the environmental chamber temperature and photoperiod were changed to 19 °C and 16L: 8D. In mid-October, when HWA sistens were observed to have broken aestivation in Blacksburg, VA, temperature and photoperiod parameters were changed back to 13 °C and 12L:12D. At this time, daily checks occurred to collect any emerging *L. nigrinus* adults.

All emerged adults were collected and placed into rearing containers containing field collected HWA-infested hemlock branches. Each container held 25 adults. Foliage was replaced every two weeks. In November, the environmental chamber settings were changed to 10 °C with a photoperiod of 10L:14D. In December, the environmental chamber settings were changed again to 4 °C with a photoperiod of 10L:14D and remained at this setting until February. At this time, the environmental chamber settings were adjusted to 10 °C with a photoperiod of 12L:12D. In March, individual *L. nigrinus* adults were placed in 50 mm × 9 mm petri dishes with moistened filter paper and clippings of infested hemlock branches. These were held in the environmental chamber for 72 h. Clippings were then checked for *L. nigrinus* eggs. If eggs were present, that individual was assigned female, and if no eggs were present that individual was assigned male. Once all adults were identified male or female, groups of 4 consisting of 2 males and 2 females and groups of 8 consisting of 4 males and 4 females were placed in separate petri dishes with moistened filter paper and held in the environmental chamber until time of release.

To transport the beetles to the field site, petri dishes were placed in a Styrofoam cooler with an ice pack. At the site, in February, beetles set in groups of 4 were released into cages of treatment branches assigned L4 and beetles set in groups of 8 were released into cages of treatment branches assigned L8. Beetles were left inside of the cages for 2 months. Cages were removed 2 months after beetles were released into them.

2.3. Experiment 1—L. nigrinus Impact on HWA Sistens Densities

In April 2021, one of the two 20–30 cm branchlets was cut from all treatments and controls and taken to the lab for evaluation. During this time, HWA sistens had produced most of their eggs and *L. nigrinus* larvae were present. Branches were placed in water saturated Instant Deluxe Floral Foam (Smithers-Oasis North America, Kent, OH) wrapped in Parafilm M (Beemis N.A., Neemah, WI, USA). Each branch was placed on a sheet of metal grating which sat on the lid of a 118 mL Verones mason canning jar. Grafix 0.0127 cm Clear Dura-Lar Film (Grafix, Maple Heights, OH) was then cut 25 cm tall and taped as an open-ended cylinder to the width of the mason jar lid, creating a plastic covering surrounding the branch. This construction represents a smaller version of the *L. nigrinus* rearing funnels used by Salom et al., 2012 [36]. Funnels holding the branches were labeled and placed into an environmental chamber set at 13 °C with a photoperiod of 12L:12D. Mason jars were checked daily for *L. nigrinus* larvae until larval drop ceased for five consecutive days. *Laricobius nigrinus* larvae were collected and recorded for each branch. Any negative control and NL branches found with *L. nigrinus* larvae were removed from the study. In Blacksburg, VA, a study concluded that all *Laricobius* spp. larvae collected within the area were 98% *L. nigrinus* and 2% *Laricobius rubidus* LeConte (Coleoptera: Derodontidae) [28]. Therefore, we assume the percentage of *Laricobius* spp. larvae collected from treatment branches to be similar with the majority of larvae being *L. nigrinus*.

After larval drop concluded, each branch was evaluated for HWA sistens ovisac disturbance and HWA sistens winter mortality on 1-year old shoot growth. This was recorded based on the proportion of live intact HWA ovisacs, dead intact HWA ovisacs, and disturbed HWA ovisacs. Disturbed HWA ovisacs had a shredded appearance and remaining HWA eggs and the adelgid were sometimes exposed. Both live and dead HWA ovisacs were circular in shape, but live HWA within the ovisac produced a dark red hemolymph when prodded, whereas dead HWA were either dried out and shriveled or produced a brown substance when prodded. HWA ovisac disturbance has been used by several studies to determine *L. nigrinus* impact to the HWA population at the branch level [11,30,31,38,39]. Therefore, HWA sistens ovisac disturbance was used in this study as a way to assess the effect of *L. nigrinus* on the HWA sistens population.

2.4. Experiment 2—*L. nigrinus* Impacts on Tree Growth and Next Generation HWA Sistens Population Density

In May 2021, the second 20–30 cm branchlet for each treatment and control branch was selected. This experiment focused on tree physiology and population density of the next generation of HWA sistens. For this experiment, a 3 × 3 cm section of 1–2-year-old growth was prepped to fit the head gasket of the LI-6800 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE). Prep consisted of selecting 1-year-old growth on each branchlet, removing 1 cm sections of needles on both sides of the selection, and wiping down each selection with 70% ETOH to kill and remove any HWA present.

The first set of net gas exchange measurements were taken one month later in June. Only 10 of the 14 trees were selected due to time constraints. All measurements were taken between 9:00 AM and 5:00 PM. Measurements included light saturated photosynthesis (A) (μmol m^{-2} sec^{-1}), transpiration rate (E) (mmol m^{-2} sec^{-1}), and stomatal conductance (gsw) (μmol m^{-2} sec^{-1}). Chamber conditions were set as follows: reference CO_2 was set to 400 ppm, quantum flux saturated at 800 μmol m^{-2} sec^{-1}, stomata ratio was set to 0, flow rate was set to 200 μmol s^{-1}, relative humidity was set to 50%, and temperature was set near ambient. Before each measurement, the number of needles was recorded for each section. After the chamber was clamped over each section, measurements were taken once stability of A and gsw was reached. This typically occurred between 10 and 20 min. Three gas exchange measurements 3 sec apart were taken to determine an average. After measurements were completed, each section was clipped and taken to the lab in Ziplock bags. Pictures of each branch section were uploaded to GIMP (GNU Image Manipulation Program) version 2.10.18 imaging software to determine the actual leaf area of each section of needles that was present within the chamber when the measurements were taken. After the actual leaf area was determined for each branch section, measurements taken by the LI-6800 Portable Photosynthesis System were adjusted to account for the actual leaf area present within the chamber.

The second and third net gas exchange measurements were taken on shoot growth produced later in the growing season; July and October, respectively. In July, sections of new shoot growth present on the second 20–30 cm branchlet were prepped similarly to the sections prepped in May. In October, measurements were taken on the same new growth sections that were prepped in July. Chamber conditions were set to the same parameters used in June. The number of needles present on each section was recorded before each measurement was taken. In October, these sections were then clipped and stored in Ziplock bags to be taken to the lab. Leaf area was determined as described for June measurements.

There were cases when the number of needles present on new shoot growth branch sections changed between measurements taken in July and October. If only a few needles were missing, the average area of needles present was used to determine the average area per needle which was then added to the total leaf area to account for the missing needles that were present when the measurement in July was taken. If more than half of the needles were missing on a branch section, then these measurements were removed from the analysis. Aestivating sistens were found on most of the new growth sections after taking the last set of measurements. This was recorded along with the length of new shoot growth to be included in the final HWA density and the total production of new shoot growth analyses.

What remained of the second 20–30 cm branchlet was taken to the lab and used to determine the final HWA density and the total production of new growth for all 14 trees and for each treatment and control branch. To determine the final HWA density and the total production of shoot growth, the number of aestivating sistens on all new shoot growth, the number of new shoots produced, and the total length of new shoot growth was measured. Final HWA density was determined as the number of aestivating sistens divided by the total length of new shoot growth. Branches that did not produce new shoot growth were excluded from analyses focused on HWA density on new shoot growth.

2.5. Statistical Analysis

To determine if the temperature inside and outside the mesh cages was different, a linear mixed-effects model was used. Data logger location, month, and the interaction of data logger location and month were fixed effects with tree as a random effect. Pairwise comparisons were interpreted using Tukey's HSD *p*-value adjustment to control family-wise error rate.

Statistical analyses for experiment 1 analyzed the following responses: HWA sistens ovisac disturbance, HWA sistens winter mortality, and *L. nigrinus* larval recovery. This experiment was aimed at understanding how *L. nigrinus* impacts HWA sistens densities. Therefore, negative control branches, which were selected based on the presence of inherently low HWA densities (<1 HWA/cm), were excluded from these analyses. A generalized linear mixed-effects model with a negative binomial distribution was fit to HWA sistens ovisac disturbance, HWA sistens winter mortality, and *L. nigrinus* larval recovery data. The initial number of sistens was included as a covariate in the cases of HWA sistens ovisac disturbance and HWA sistens winter mortality. In the case of the *L. nigrinus* larval recovery model, the initial new shoot length was included as an offset variable to standardize the response over differential surface area and initial HWA density was included as a covariate. The replicate effect was included as a random intercept and treatment was included as a fixed effect for all analyses. Tukey's HSD *p*-value adjustment was used when comparing treatment means.

Statistical analyses for experiment 2 focused on external tree measurements and tree physiology. External tree health measurements included the number of new shoots produced, the probability of new shoot growth present, the amount of new shoot growth produced, and the density of the next generation of HWA sistens present on new shoot growth. Tree physiology measurements that were analyzed were the photosynthetic rate, transpiration rate, and stomatal conductance. The negative control group was included in these analyses to allow for comparison of tree health measurements from the positive control and treatment groups to a control that was theoretically functioning as though HWA were not present or as though the damaging threshold of HWA was not reached. A generalized linear mixed-effects model with a negative binomial distribution was used to model the number of new shoots produced, and the initial number of new shoots was included as a covariate. The probability of new shoot growth and the length of new shoot growth when it occurred was modeled using a zero-inflated linear mixed model. The zero-inflated model includes a logistic regression model that models the probability of no new shoot growth occurring (called the zero model hereafter) and a linear mixed model, called the length model, that models the length of new shoots in cases where they were present. This contextualizes cases of no new shoot growth as being produced by a systematic process, rather than just random sampling. Both the linear and zero models included only the treatment as a fixed effect and the replicate as a random effect. A linear mixed-effects model was used for the final HWA sistens density analysis. The initial HWA sistens density was included as a covariate and a logarithmic transformation was applied to the response to correct for heteroscedasticity of the residuals. Branches that did not produce new growth were removed from this analysis. Tree physiological measurements were taken at different times of the year and on 1-year old growth and current year growth. Measurements taken in June on 1-year old growth were analyzed separately from measurements taken in July and October, which were on current year new growth. A linear mixed-effects model was used for the photosynthetic rate data taken in June. The same model with an added log transformation on the response was used for the photosynthetic rate data taken in July and October, as well as for transpiration rate and stomatal conductance data for all months. Treatment was included as a fixed effect and a random intercept was included for the replicates for all analyses. Models used for data taken in July and October included the month and the interaction between month and treatment as fixed effects. If the interaction between month and treatment was not significant, the interaction term was removed from the model. All pairwise comparisons were interpreted using Tukey's HSD *p*-value adjustment to control the family wise error rate.

All statistical analyses were performed with R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria) [40]. The 'glmmTMB' package was used for the production of all models [41] and the 'DHARMa' package was used to evaluate the overall fit of the generalized linear mixed-effects models using randomized quantile residuals [42].

3. Results

3.1. Site Environmental Conditions

Mean temperatures in June, July, and October, when sampling occurred, were 18.7, 19.9, and 13.3 °C, respectively (Table 2). Mean temperatures in June–August remained above 18 °C until September (Table 2). Min and max temperatures in June were 3.6 and 30.2 °C, respectively. Min and max temperatures in July and October were 9.3 and 30.0 °C, and 3.7 and 25.0 °C, respectively. Compared to monthly normal temperatures recorded from 1981 to 2010 from the NOAA database [43], the mean temperatures in June, July, and October, of the year 2021, were 0.5 °C lower, 1.4 °C lower, and 2.2 °C higher, respectively. In 2021, mean precipitation in June, July, and October, when sampling occurred, was 0.17 mm, 3.97 mm, and 3.79 mm, respectively (Table 2). After June, monthly precipitation remained above 3.0 mm until December. The mean monthly normal precipitation recorded across 1981–2010 from the NOAA database [43] remained above 50.0 mm from January to December, with the highest mean precipitation occurring in May at 104.4 mm. The overall precipitation in 2021 was 35.8 mm, ranging monthly between 0.17 and 4.09 mm, indicating that 2021 was a dry year compared to the data collected from 1981 to 2010.

3.2. Temperature Inside vs. Outside Mesh Cages

While cages were applied, the location of the data logger (inside vs. outside of the mesh cage) and month interaction was statistically significant (X^2 = 45.98, df = 5, $p < 0.0001$). Except for the month of May, temperature was higher in cages; however, only for the month of May was the difference statistically significant (X^2 = 5.38, df = 1, $p < 0.02$; Table 3). Average monthly temperatures were also significantly different by month (X^2 = 5986.42, df = 5, $p < 0.0001$; Table 3).

3.3. Experiment 1—*L. nigrinus* Impacts on HWA Sistens Densities

HWA ovisac disturbance was significantly greater on treatment branches with *L. nigrinus* compared to treatment branches without predators (X^2 = 41.45, df = 2, $p < 0.0001$; Figure 2A). HWA winter mortality was similar across treatments (X^2 = 3.71, df = 2, $p = 0.16$; Figure 2B). *Laricobius nigrinus* larvae were only collected from L4 and L8 treatment branches. Even though L8 branches initially had a higher density of *L. nigrinus* adults, the average number of *L. nigrinus* larvae collected from both treatments were not significantly different (X^2 = 1.34, df = 1, $p = 0.25$; Figure 2C). *Laricobius nigrinus* larval recovery was positively affected by the initial HWA density on treatment branches (X^2 = 5.79, df = 1, $p = 0.02$).

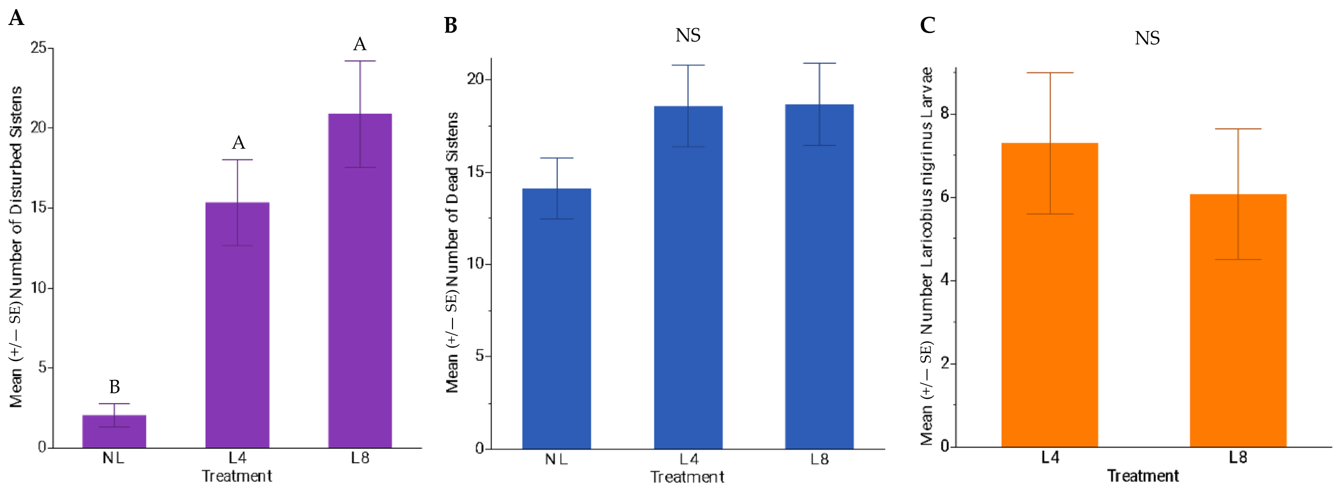

Figure 2. Experiment 1. (**A**) Mean (+/− SE) number of disturbed sistens across treatments. Different letters above standard error bars indicate significant ($p < 0.05$) differences, Tukey post hoc adjustment. (**B**) Mean (+/− SE) number of sistens dead due to winter conditions across treatments. Letters NS above standard error bars indicates no significant differences ($p > 0.05$). (**C**) Mean (+/− SE) number of Laricobius nigrinus larvae collected from treatment branches. Letters NS above standard error bars indicates no significant differences ($p > 0.05$).

3.4. Experiment 2—*L. nigrinus* Impacts on Tree Growth and Next Generation HWA Sistens Population Density

The number of new shoots produced was significantly different among treatments ($X^2 = 12.19$, df = 3, $p = 0.007$). Branches in the negative control and the L8 treatment produced significantly more new shoots than the NL treatment, whereas the L4 treatment resulted in an intermediate number of new shoots (Figure 3A). The initial number of new shoots did not have a significant effect on the number of new shoots that were produced at the end of the study ($X^2 = 0.55$, df = 1, $p = 0.46$). There were no treatment effects on the probability of new shoot growth nor on the length of new shoot growth ($p = 0.524$; Table 4). The final HWA density, settled HWA sistens, was significantly reduced for L4 and L8 treatments compared to the NL treatment ($X^2 = 13.32$, df = 3, $p = 0.004$; Figure 3B).

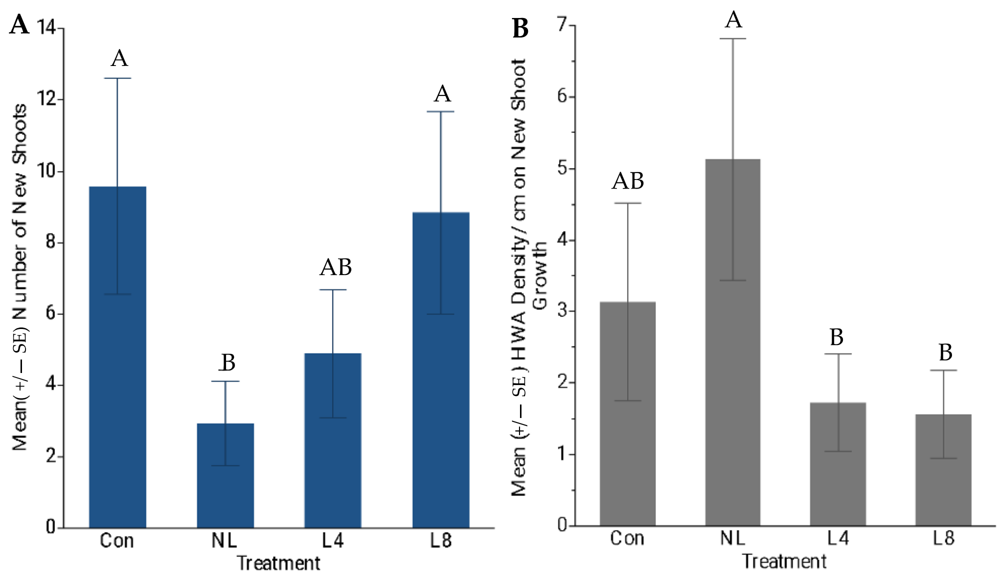

Figure 3. Experiment 2—-External Tree Measurements. (**A**) Mean (+/− SE) number of new shoots across treatments. Different letters above standard error bars indicate significant ($p < 0.05$) differences,

Tukey post hoc adjustment. (**B**) Mean (+/− SE) HWA density (HWA/cm) on new shoot growth across treatments. Different letters above standard error bars indicate significant ($p < 0.05$) differences, Tukey post hoc adjustment. All measurements were taken on eastern hemlock. Con = no predators at low HWA densities (<1/cm), NL = no predators at moderate to high HWA densities (<1 HWA/cm), L4 = 4 and L8 = 8 *L. nigrinus* at moderate to high HWA densities.

Table 4. Zero-inflated linear mixed model analysis on the effect of treatment on new shoot growth production and new shoot growth length.

Treatment	Zero Model		Shoot Length Model	
	Mean Probability (%) [a]	**Standard Error (+/–)**	**Mean New Shoot Length (cm)** [b]	**Standard Error (+/–)**
Control	11.52	10.82	28.3	8.94
NL	49.13	21.15	13.9	11.15
L4	18.67	14.65	21.4	9.33
L8	6.15	6.96	32.2	8.57

[a] Mean probability represents the probability that that treatment branch will not produce new shoots. [b] Means in the shoot length model are means only for hemlocks that produced new growth. Means calculated by dividing the total new shoot length by the total number of shoots.

The average photosynthetic rate taken in June, on 1-year-old shoot growth, was significantly different among treatments ($X^2 = 11.40$, df = 3, $p = 0.01$; Figure 3A). The photosynthetic rate in the negative control was significantly greater than the NL treatment, whereas treatments L4 and L8 did not differ significantly from the negative control or the NL treatment (Figure 4A). The average photosynthetic rates measured in July and in October on new shoot growth was not significantly different among treatments although patterns were similar to June ($X^2 = 6.29$, df = 3, $p = 0.10$; Figure 4A). Average photosynthetic rate was significantly greater in October than it was in July ($X^2 = 31.82$, df = 1, $p < 0.0001$). The average transpiration rate in June was significantly different among treatments ($X^2 = 13.83$, df = 3, $p = 0.003$). The negative control had a higher average transpiration rate than the L4 and NL treatments, whereas the L8 treatment did not significantly differ from the other treatments (Figure 4B). The average transpiration rate in July and October did not significantly differ by treatment ($X^2 = 6.82$, df = 3, $p = 0.08$; Figure 4B), nor by month ($X^2 = 2.59$, df = 1, $p = 0.11$). In June, the average stomatal conductance was significantly higher in the negative control than in the L4 and NL treatments ($X^2 = 15.92$, df = 3, $p = 0.001$; Figure 3C.). The L8 treatment had an intermediate stomatal conductance (Figure 4C). In July and October, the average stomatal conductance did not significantly differ among treatments ($X^2 = 6.70$, df = 3, $p = 0.08$; Figure 4C). However, the average stomatal conductance was significantly higher in October ($X^2 = 16.34$, df = 1, $p < 0.001$).

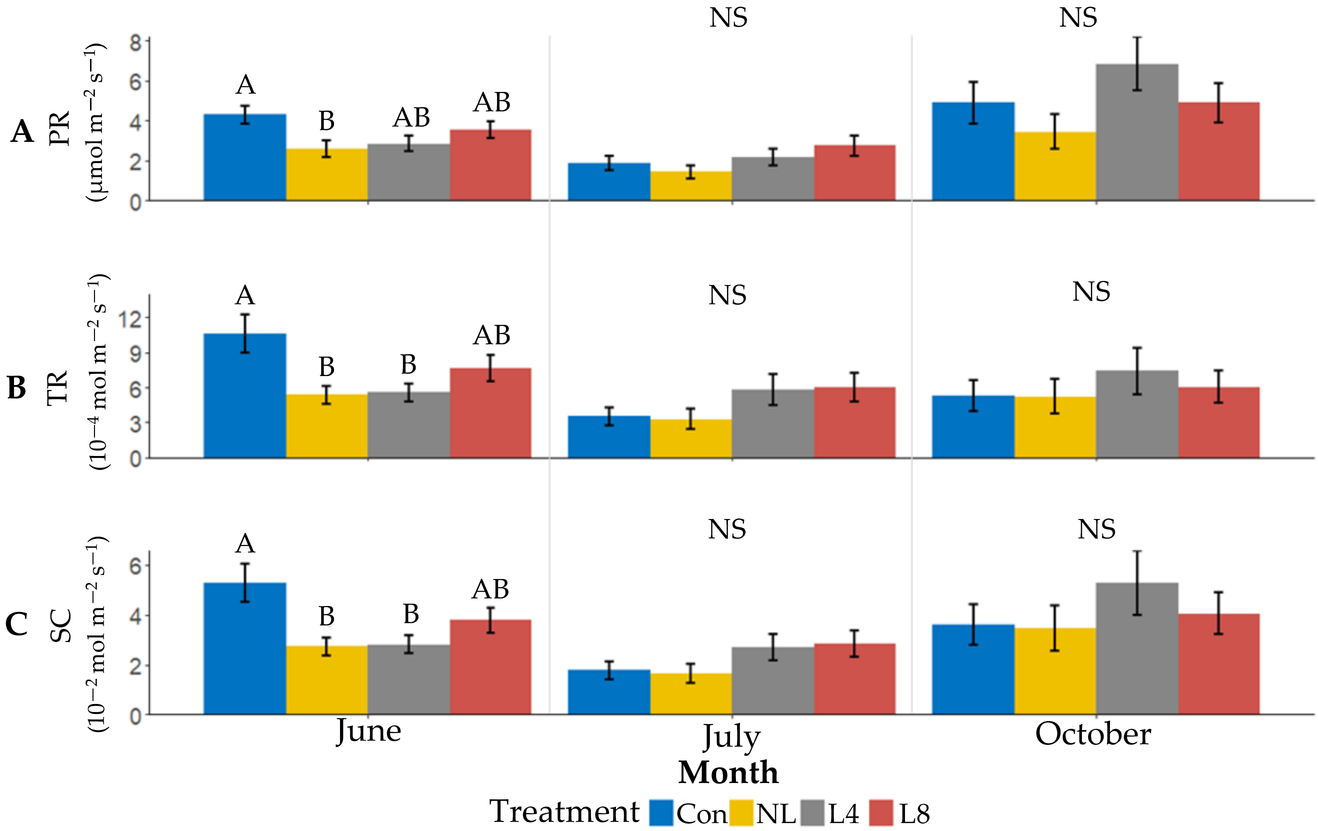

Figure 4. Experiment 2—Tree Physiological Measurements. (**A**) Model adjusted mean (+/− SE) photosynthetic rate (PR) across treatments for June, July, and October. Different letters above standard error bars indicate significant ($p < 0.05$) differences, Tukey post hoc adjustment. Statistical analyses are separated by month. (**B**) Model adjusted mean (+/− SE) transpiration rate (TR) across treatments and separated by month. Letters NS above standard error bars indicate no significant ($p < 0.05$) differences. Statistical analyses are separated by month. Units were converted to 10^{-4} for easier viewing. (**C**) Model adjusted mean (+/− SE) stomatal conductance (SC) across treatments for June, July, and October. Units for stomatal conductance were converted to 10^{-2} for easier viewing. Letters NS above standard error bars indicate no significant ($p < 0.05$) differences. Statistical analyses are separated by month. All measurements were taken on eastern hemlock. Con = no predators at low HWA densities (<1/cm), NL = no predators at moderate to high HWA densities (<1 HWA/cm), L4 = 4 and L8 = 8 *L. nigrinus* at moderate to high HWA densities.

4. Discussion

This is the first study to investigate if *L. nigrinus* predation can affect hemlock tree health. Use of inclusion cages on branches allowed us to evaluate whether different densities of *L. nigrinus* adults reduced the number of HWA sistens, and whether this translated into improved host health assessed at the branch level. Though the effects were temporary, this study demonstrated that, by reducing the HWA sistens population, *L. nigrinus* predation has a positive effect on hemlock tree health. The results support the continuation of classical biological control efforts through releasing and redistributing *L. nigrinus*.

4.1. Potential Cage Effects

In order to observe the impact of *L. nigrinus* predation at different densities, mesh inclusion cages were applied to all treatment branches including negative controls. This prevented *L. nigrinus* already present in the field from gaining access to the branches and ensured that *L. nigrinus* adults released onto the branches remained. A main concern with using mesh cages for this study was the potential of creating a different microclimate within the cage that would have an effect on HWA populations and hemlock tree health. One previous study indicated that the utilization of large 60 × 100 cm nylon cages showed no differences in temperature or humidity from outside of the cages, but they could potentially reduce the length of new shoot growth [44]. Considering this, large L100 cm × W 66 cm nylon cages were selected. In our study, we were unable to test for differences in humidity, but we did find a temperature difference in May, where it was 14.65 °C outside of the cage and 13.47 °C inside the cage (Table 3). Cages were removed at the end of May before new growth was produced. Therefore, we were able to limit the effect the cages would have had on new shoot growth production. In addition, winter mortality of HWA sistens was similar across treatments, indicating that all treatment branches were exposed to the same environmental conditions (Figure 2B). Therefore, we could also assume that HWA populations present on all treatment branches were also exposed to the same environmental conditions with minimal cage effects.

4.2. Experiment 1—*L. nigrinus* Impacts on Winter HWA Sistens Densities

Based on our results for experiment 1, HWA sistens ovisac disturbance was significantly greater on treatment branches where *L. nigrinus* adults were released compared to treatment branches without *L. nigrinus*.

Several studies have utilized HWA sistens ovisac disturbance to assess the impact of *L. nigrinus* on HWA populations in the eastern US [11,30–32,39]. In our study, *L. nigrinus* predation produced a significantly greater number of disturbed ovisacs compared to the NL treatment, which did not contain *L. nigrinus* adults (Figure 2A). This supports the findings of Jubb et al., 2020 [31] and Mayfield et al., 2015 [32], where exclusion cages were used at sites where *L. nigrinus* was established.

In this study, two different densities of *L. nigrinus* adults were used. The L4 treatment consisted of two females and two males, representing a low density of *L. nigrinus* adults and the L8 treatment consisted of four females and four males representing a high density of *L. nigrinus* adults. As of 2018, *L. nigrinus* establishment has been confirmed at 18 release sites across Virginia, with a range of 0–80 adults collected from a site, and a predator prey ratio ranging from 0.000 to 0.144 larvae per adelgid [29]. This indicates that naturally in the field, the number of *L. nigrinus* present on HWA infested hemlock branches is variable and it would be possible to have the same densities of *L. nigrinus* adults used in this study to be present on the same branch, though the higher density would likely represent a "best case scenario". In addition, a 1:1 sex ratio was used for this study to ensure reproduction and to address the difference in prey handling time and prey preferences seen in *L. nigrinus*. Male *L. nigrinus* adults are known to significantly kill more HWA sistens adults than females and have a longer handing time [45]. Considering these differences between the sexes, it was more realistic to include an equal number of males and females for each treatment.

Laricobius nigrinus larvae were collected from both L4 and L8 treatments, confirming that the adults released into the inclusion cages were able to reproduce. Interestingly, the number of *L. nigrinus* larvae collected from L4 and L8 treatment branches were not significantly different, even though the starting densities of adults was different (Figure 2C). During the initial field site set up, treatment branches, except the positive control, were selected with similar HWA population densities. Prey abundance could have been the limiting factor. In lab bioassays, one *L. nigrinus* female could produce approximately 100 eggs over 13.2 weeks and one larva was capable of consuming 225 HWA progrediens eggs and 252 HWA progrediens eggs at temperatures 12 and 18 °C, respectively [26]. If there were two females in the L4 treatment cages and four in the L8 treatment cages, then it would be possible that up to 200 *L. nigrinus* eggs and 400 *L. nigrinus* eggs were produced for each treatment, respectively. *Laricobius nigrinus* adults and larvae are known to exhibit a type II functional response [45]. Meaning that as the HWA population increases, predation increases up to a certain point. For this study, NL treatment branches had an average HWA density of 2.83 adelgid/cm, L4 treatment branches had an average HWA density of 3.08 adelgid/cm, and L8 treatment branches had an average density of 3.20 adelgid/cm. If we include the initial length of shoot growth, we can estimate the average number of HWA sistens that were present on each branch. This would equate to 156 HWA, 154 HWA, and 154 HWA in total on NL treatment branches, L4 and L8 treatment branches, respectively, not including the HWA progrediens eggs that they would have produced. HWA sistens are known to produce an average of 49 eggs per ovisac [10]. With that in mind, there potentially were 7546–7644 progrediens eggs available. If there were 200 *L. nigrinus* larvae present on the branches, then the larvae could consume up to 45,000 progrediens eggs in the L4 treatment cages and 90,000 progrediens eggs in the L8 treatment cages, suggesting that there may have not been enough prey available if the *L. nigrinus* adult females reproduced to their full potential. Therefore, some larvae may have starved and were unable to reach the fourth instar. However, the mesh cages were large and additional branch material was included in the cages, so additional HWA sistens could have been present on that branch material and could have provided more prey. By looking at only 20–30 cm terminal branch sections, we have limited our observations as to how many *L. nigrinus* larvae were produced within the mesh cages. There could have potentially been more *L. nigrinus* larvae present on the remaining branch material within the cages.

4.3. Experiment 2—L. nigrinus Impacts on External Tree Growth and Next Generation HWA Sistens Population Density

One of the main effects of HWA feeding on eastern hemlock is the reduction of shoot growth [46–48]. In this study, branches in the NL treatment produced the fewest number of shoots. The negative control and the L8 treatment produced the greatest number of shoots, suggesting that a greater density of *L. nigrinus* beetles may reduce HWA populations sufficiently enough on branches to reduce some of the negative effects caused by HWA feeding. However, there were no significant differences among treatments in the probability of new shoot growth being produced and mean new shoot growth length (Table 4). Looking closer at the data, the mean probability that NL treatment branches would not produce new shoot growth was 49.13%, meaning that almost half of the time, NL treatment branches, which did not have *L. nigrinus* predation, would not produce new shoots (Table 4). In contrast, L4 treatment branches had a 18.67% mean probability of not producing new shoots and L8 treatment branches had a 6.15% mean probability of not producing new shoots (Table 4). Numerically, there are differences between treatments and there seems to be a clear treatment effect, where *L. nigrinus* predation may result in a higher probability of new shoot growth and longer new shoot growth compared to branches without *L. nigrinus* predation, but the variability in shoot growth measurements is large, suggesting high variability between selected trees. We suggest that future work should increase tree sample size and include multiple replicates per tree to reduce variability in shoot production variables.

By the end of this study, HWA density was reduced on the L4 and L8 treatment branches and greatest on the negative control and NL treatment branches. According to McClure [23], hemlock new growth production did not occur if the HWA density was greater than four adelgids/20 mm^2 of branch, which would be approximately two adelgids/cm. For this study, branches with HWA densities lower than one adelgid/cm were selected for the negative control, which was below the damaging threshold that was observed by McClure [23]. Without *L. nigrinus*, the negative control branches, by the end of the study, ended up with HWA densities above two adelgid/cm similar to the final HWA densities observed for the NL treatment. Additionally, final HWA densities for the L4 and L8 treatments were close to two adelgid/cm, which would still be above the damaging threshold. Indicating that even with *L. nigrinus* feeding on the sistens generation, new growth on HWA infested hemlocks would still be in decline. This supports ongoing efforts to establish additional predators, such as *Leucotaraxis argenticollis* (Zetterstedt) (Diptera: Chamaemyiidae) and *Leucotaraxis piniperda* (Malloch) (Diptera: Chamaemyiidae), which are known to feed on both generations of HWA [49,50]. With additional predators, there could be potential to reduce HWA densities below the damaging threshold and to prevent HWA populations from rebounding after predation [51].

4.4. Experiment 2—*L. nigrinus* Impacts on Tree Physiology

To examine the effect *L. nigrinus* predation has on hemlock tree health, we also measured the photosynthetic rate, transpiration rate, and stomatal conductance on 1–2-year-old growth and current year growth. For this study, photosynthetic rates ranged between 2 and 5 µmol m^{-2} s^{-1}, which were consistent with other studies [16–19,48]. In June, on 1–2-year-old growth, negative control branches had a greater photosynthetic rate than NL branches, which initially had a higher HWA population and did not have *L. nigrinus* predation (Figure 4A). The same pattern found for the photosynthetic rate was also observed for transpiration rate and in stomatal conductance (Figure 4B,C). The slight increase in the photosynthetic rate for L4 and L8 treatment branches, in June, suggest that *L. nigrinus* predation on HWA populations in the context of this study, can have an impact on hemlock tree health. This would mean that by decreasing the pest population, the stress caused by HWA feeding was reduced, which led to an improvement in tree physiology. However, this pattern was not consistent, so the effect of *L. nigrinus* predation may be temporary. A similar pattern was observed for the transpiration rate and stomatal conductance. A larger sample size may have aided in determining statistical differences.

The status of the current year growth and field site conditions could help explain why the treatment effect was lost in October and why the overall photosynthetic rate was significantly greater in October versus July. In July, at the time of sampling, current year growth was not fully elongated, and the stems were still a dark green. At the time of sampling in October, the current year growth was fully elongated, and the stems were no longer green and beginning to form woody tissue. It is possible that by October, the foliage efficiency of current year growth increased and instead of splitting energy resources between growth and function, current year growth could now focus solely on function and reach photosynthetic rates at its full potential. Field site conditions before and during sampling could also play a role. For this study, we were able to record temperature at the site and utilize weather stations nearby to record precipitation. Temperatures in June and July were relatively similar to each other with average monthly temperatures being 18.7 and 19.9 °C, respectively, while the average temperature in October was lower at 13.3 °C (Table 2). A previous study suggested that temperature may have little effect on photosynthesis from late spring to early fall [52]. In addition, another study stated that optimum temperatures for photosynthesis for hemlock ranged between 14 and 19 °C, and higher temperatures resulted in a decrease in net photosynthesis [53]. Therefore, temperature may not have had much of an influence on the differences in the photosynthetic rate, transpiration rate, or stomatal conductance of treatment branches in this study. Precipitation may be more of a factor in affecting the photosynthetic rate. The eastern hemlock is known to grow best on moist fertile soils [54], and is intolerant of drought [54,55]. Average precipitation was 0.17, 3.97, and 3.79 mm in June, July, and October, respectively (Table 2). From July to October, precipitation was consistently above 3.00 mm (Table 2). This could have led to a reduction in stress due to dry conditions, resulting in higher photosynthetic rates in October compared to July.

5. Conclusions

This study demonstrated that *Laricobius nigrinus* predation on HWA populations has a positive effect to hemlock tree health at the branch level. Although the effect was temporary, *L. nigrinus* predation resulted in an increase in the photosynthetic rate, transpiration rate, and stomatal conductance in June. *L. nigrinus* predation of HWA populations on treatment branches lead to an increase in the number of new shoots produced at the end of the growing season, which was equivalent to the number of new shoots produced by the negative control that started with HWA densities below the damaging threshold. However, the final HWA density for all treatments was above the damaging threshold, which would potentially cause a reduction in the number of new shoots produced the following year. This strongly suggests that *L. nigrinus* is an important biological control agent of HWA and is capable of having a positive impact on hemlock tree health. However, additional management strategies are needed to further reduce the negative effects to hemlock tree health caused by HWA.

Author Contributions: Conceptualization, C.E.P. and S.M.S.; methodology, C.E.P., A.A., J.R.S. and S.M.S.; software, C.E.P., A.A. and J.R.S.; validation, C.E.P., J.R.S. and S.M.S.; formal analysis, C.E.P. and A.A.; investigation, C.E.P.; resources, J.R.S. and S.M.S.; data curation, C.E.P.; writing—original draft preparation, C.E.P.; writing—review and editing, C.E.P., A.A., J.R.S. and S.M.S.; visualization, C.E.P. and A.A.; supervision, S.M.S.; project administration, C.E.P. and S.M.S.; funding acquisition, S.M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the USDA Forest Service, grant number 19DG11083150009.

Data Availability Statement: Data for this study is not publicly available due to it being included as a part of a graduate dissertation.

Acknowledgments: We would like to thank John Peterson for his help with field data collection and equipment maintenance. We also thank members of the Forest Entomology Lab at Virginia Tech for their help with field site set up and for their help in the lab. We would like to thank the USDA Forest Service for providing funding for this work. We would also like to thank Virginia Tech Libraries and the Open Access Subvention Fund for providing financial support to publish this article in an open access journal.

Conflicts of Interest: The authors declare no conflict of interest.

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