Establishment of *Laricobius nigrinus* (Coleoptera: Derodontidae) in Virginia and assessment of its impact on hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), throughout the eastern U.S.

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Abstract

Management of hemlock woolly adelgid (HWA), *Adelges tsugae* (Hemiptera: Adelgidae), is currently being implemented through several different methods including the release of host-specific predators such as *Laricobius nigrinus* Fender (Coleoptera: Derodontidae). Releases of this predator began in 2003. In 2014, an assessment of the efficacy of *L. nigrinus* at release sites from New Jersey to Georgia. Field sites were chosen based on the presence of moderate to high densities of HWA, that *L. nigrinus* was released at least four years prior to the start of the study, and that the predator was determined to be established at that site. Three treatments were set up at each of the sites: no cage, closed exclusion cage, and open cage. Three assessments were taken during key points throughout the season in order to monitor both HWA and *L. nigrinus* populations. Larval predator were recovered from most of the sites in year one and in higher numbers in year two. Many sites at which *L. nigrinus* were recovered showed high predation rates of HWA in uncaged samples ranging from 1.5 to 47.3% in year one and 0 to 66 % in year 2. A survey for *L. nigrinus* establishment at previous release sites in VA was conducted. These sites date back as far as 2003 and as recently as 2015. Beat sheeting and branch clippings were conducted to recover *Laricobius* spp. adults and larvae, respectively. The recovered insects were then identified to species through genetic analysis. A mix of the introduced *L. nigrinus* and the native *L. rubidus* LeConte (Coleoptera: Derodontidae) were recovered at some sites, and only *L. rubidus* were recovered from others. Higher numbers of *Laricobius* were recovered in spring of year two. Overall, tree health in Virginia decreased from spring 2015 to 2016.

Abstract for General Audiences

Laricobius nigrinus is a beetle predator of hemlock woolly adelgid (HWA), an aphid like insect covered by wool. HWA is a serious pest of both eastern and Carolina hemlock, two important tree species found in Appalachia which provide species diversity and habitat to numerous animal and plant species. Damage to hemlock trees include death, dieback, and disease. We began releasing *L. nigrinus* in 2003 in the eastern United States to manage HWA. There are now over 900 documented releases of *L. nigrinus* from Maine to Georgia. Nine field sites were set up to assess the impact *L. nigrinus* is having on HWA populations throughout the geographic range of its releases. At many of these sites we found that *L. nigrinus* adults and larvae are significantly reducing HWA populations. A large proportion of the predators of HWA recovered at these sites are *L. nigrinus*, which shows that this species is primarily responsible for the observed predation. The second study conducted assessed for establishment of *L. nigrinus* at release sites around Virginia. *Laricobius nigrinus* was found at four of the fourteen sites sampled. Continued sampling is necessary to get a more accurate assessment of establishment since many of the sites were recovering from low HWA populations resulting from extreme cold temperatures in 2014 and 2015.

Atributions

Several colleagues aided in the data collection for chapter 2. A brief description of their contribution is included here.

Chapter 2: Impact assessment of *Laricobius nigrinus*, a predator of hemlock woolly adelgid in the eastern United States

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Chapter 1: Introduction

1.1 Biology and Damage of the Hemlock Woolly Adelgid

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), was first discovered in the eastern United States near Richmond, VA in the early 1950s (Souto et al. 1995). HWA is native to the western United States and Asia and feeds on *Tsuga* spp. in those areas (Havill et al. 2011a). The strain of HWA introduced to eastern North America is from Japan (Havill et al. 2006). HWA is not considered a pest in its native range where populations are regulated by host resistance, native predators, and climatic conditions (McClure 1989, McClure and Cheah 1999, Skinner et al. 2003). In the eastern United States, HWA feeds on two native *Tsuga* species, eastern hemlock, *Tsuga canadensis* (L. Carriere) and Carolina hemlock, *Tsuga caroliniana* (Engelman) (Havill et al. 2011a). Eastern hemlock's range extends from New England southward to Georgia and westward to Minnesota (Godman and Lancaster 1990). Cold temperature and climatic conditions may impact the spread of HWA (Evans and Gregorie 2007). Both hemlock species in the eastern U.S. are susceptible to HWA, due in part to a lack of host resistance and lack of specialized native predators present in eastern North America (Wallace and Hain 2000).

Adelgids are closely related to both aphids (Hemiptera: Aphidae) and phylloxerans (Hemiptera: Phylloxeridae) and share many characteristics with them including a complex life cycle with asexual and sexual generations (McClure 1987). Adelgids differ

from both aphids and phylloxerans in having shorter antennal segments and no cornicles. Adelgid hosts are limited to gymnosperms, whereas their relatives feed on both angiosperms and gymnosperms (Heie 1987). In adelgids, both sexual and asexual females lay eggs only on conifers (Drooz 1985). In the eastern U.S., HWA is made up of two parthenogenic generations, consisting of only wingless females (McClure 1987, Havill and Foottit 2007). In Asia, HWA has two hosts. Their primary host is tigertail spruce, *Picea torano* Koehne, and secondary hosts are *Tsuga* spp. The holocyclic life cycle is not found in North America, due to the lack of its primary host (McClure 1987, Havill and Foottit 2007). The sistens generation spans between 9-10 months, from early summer to the following spring. This generation aestivates during the summer months, and becomes active in the fall, and continues to develop through the winter (McClure 1989). The progrediens generation is the offspring of the sistens generation, occurring from spring to early summer (McClure 1989). All life stages, except for eggs and mobile crawlers, produce a waxy “wool” outer covering to protect themselves from predators and the elements (Jones et al. 2014), and remain sedentary on the twigs for the duration of their life. HWA’s life cycle in southern U.S. is accelerated due to warmer temperatures (Mausel et al. 2008).

Hemlock woolly adelgid feeds on *Tsuga* species of all ages, feeding predominantly on the most recent shoot growth, by inserting its stylet bundle into the xylem tissue at the base of a needle and feeding on parenchyma cells (Young et al. 1995). High densities of HWA cause significant damage to the tree including chlorosis and bud mortality, due to the sessile nature of HWA’s lifecycle leading to prolonged feeding at the same site.

HWA feeding depletes the hemlock's nutrients, thus leaving the tree vulnerable to dieback, death, and disease (McClure 1987, Fidgen et al. 2006, Preisser et al. 2014). Feeding by HWA is known to cause a hypersensitive response in hemlock trees due to the HWA infestation (Radville et al. 2011). Most of the feeding occurs in winter to later spring or early summer, when there is a lack of predators to prey on HWA (McClure 1989). The sessile lifestyle, physical deterrents against predation, and number of progeny produced contribute to the success of HWA (McClure 1989, Jones et al. 2014).

1.2 Importance of Eastern and Carolina Hemlock in the Eastern United States

While eastern hemlock is more abundant in the Northeast and along the Appalachian Mountains and is rarely found in the S.E. Piedmont (Morin et al. 2011). It is a long-lived tree species that serves an important role in the forest ecosystems, providing habitat for many native animal species including birds, small mammals, amphibians, and fish species (Evans et al. 1996). Eastern hemlock is often found associated with well drained moist soils in riparian areas (Peattie 1950). The loss of eastern hemlock changes the forest ecosystems in which it occurs with respect to long-term species composition and microclimate (Orwig and Foster 1998, Lovett et al. 2006). Loss of hemlock results in an increased soil temperature, due to canopy openings. When eastern hemlocks die, they are replaced by *Acer, Quercus, Betula* and *Rhododendron* species (Orwig and Foster 1998, Brown 2004); none of which provide the same ecosystem functions that hemlocks do. Eastern hemlocks are commonly planted in ornamental urban landscapes.

Carolina hemlock is a rare tree, native to the Appalachian Mountains and Upper Piedmont in the southeastern United States (Jetton et al. 2008). It is typically located in dry locations including mountain bluffs and ridges, though they are occasionally found in cool moist valleys and ravines (Rentch et al. 2000). Carolina hemlock, like eastern hemlock, is a host for HWA and faces loss from their native range (McClure et al. 2003). Carolina hemlock typically dominates stands later successionally than most species due to its slow growth and ability to tolerate water, nutrient, and shade (Jetton et al. 2008). Eastern and Carolina hemlocks occasionally grow together in riparian zones and rich soils along mountain streams (Farjon 1990).

1.3 Management of Hemlock Woolly Adelgid

There are several ways that HWA is managed, including application of chemical insecticides and releases of biological control agents. An effective, yet limited option is the application of insecticides in urban and suburban settings, where hemlock is valued as an ornamental plant, and in forest settings. Application methods include foliar sprays, bark sprays, soil injections, and horticultural oils (Fidgen et al. 2002). Application of systemic insecticides have proven to be the most successful way to suppress populations of HWA on individual trees (Cowles et al. 2006) and treatments can remain efficacious for up to five years (Fidgen et al. 2002, Mayfield et al. 2015, Benton et al. 2016). However, there are concerns associated with runoff of insecticides to local streams and aquatic environments (Cowles et al. 2006). Chemical treatments can be costly to implement, at the stand level, however combining both chemical and biological control has potential (Mayfield et al. 2015).

1.4 Biological Control of Hemlock Woolly Adelgid

Successful establishment of natural enemies is a critical step in suppressing HWA (Mausel et al. 2010, Havill et al. 2011, Onken and Reardon 2011). Success using biological control is characterized by a decrease in the population of the species being preyed upon and an increase in the population of the released natural enemy, both species end up stabilizing overtime (Beddington et al. 1978). With regards to HWA, a successful biological control effort is marked by an initial increase in the predator population which stabilizes over time, an initial decrease in the HWA population which stabilizes over time, and the improved health of the infested trees to levels which are acceptable (Lenger and Bellows 1999). Biological control programs usually incorporate predators or parasites that are specific to the pest species (Dahlsten and Mills 1999). Finding a complex of predators to introduce into the eastern U.S. has potential to help reduce HWA population below injurious levels (Havill et al. 2011a). HWA is an excellent candidate for a biological control program due to it sessile lifestyle. Explorations to locations where HWA are native, Pacific Northwest and Asia, found natural enemies of HWA in these locations. To date there are no known parasitoids of HWA, however several predators have been found in both Asia and the Pacific Northwest and are the focus of biological control programs against HWA (Onken and Reardon 2011).

1.4.1 Overview of Predators Considered for Biological Control

In the southeastern United States, specifically in North Carolina and Virginia, where HWA is invasive, it was found that HWA was preyed upon by generalist insect predators in the families Coccinellidae, Chrysopidae, Hemerobiidae, and Cecidomyiidae (Wallace and Hain 2000). In the Pacific Northwest, 55 species in 14 families have been found to associate with HWA, with Derodontidae being the most abundant predatory family found (Kohler et al. 2008). *Leucopis* spp. were found in the Pacific Northwest and are present for a longer period of time than *L. nigrinus*. *Leucopis* spp. larvae were present during HWA's progrediens and sistens egg stages compared to *L. nigrinus* being present during only the progrediens egg stage (Kolher et al. 2016). Scymnus *(Pullus) coniferarum* Crotch, are found to feed upon adelgids on hemlocks and pines (Montgomery and Keena 2011). In Japan, the search for predators of HWA began in 1992 (McClure 1995). *Sasajiscymnus tsugae* (Sasaji and McClure) (Coleoptera: Coccinellidae) was one species recovered and approved for release in 1995 (Sasaji and McClure 1997). In the mid-1990s, three coccinellid species, *Scymnus (Neopullus) camptodromus* Yu & Lui, *Scymnus (Neopullus) sinuanodulus* Yu & Yao, and *Scymnus (Neopullus) ningshanesis* Yu & Yao were discovered in China. All three species were imported to the U.S., however there have been limited releases of two species, and one species has not yet been approved for release (Montgomery and Keena 2011). *Scymnus (Neopullus)* lady beetles are predominantly opportunists that feed on a wide variety of prey (Montgomery and Keena 2011). A renewed effort to survey in Japan helped to identify two new predatory species, *Laricobius osakensis* Montgomery and Skiyake (Coleoptera: Derodontidae) Montgomery and Skiyake (Montgomery et al.

2011), and *L. naganoensis* Leschen, which has contaminated colonies of *L. osakensis*. *Laricobius naganoensis* delayed initial release of *L. osakensis* and has subsequently complicated *L. osakensis* rearing (Fischer et al. 2014).

1.4.2 *Laricobius* spp. as Biological Control Agents

Laricobius spp. belong to the family Derodontidae, the tooth-necked fungus beetles. The name refers to indentations on the pronotum, and that three of the four genera in this family are known to feed predominantly on fungi; the one exception being *Laricobius* spp. (Bright 1991, Hava 2006). *Laricobius* spp. are adelgid specialists both as adults and larvae (Bright 1991). Several species of *Laricobius* have been used as biological control agents to date, including *L. nigrinus* from the Pacific Northwest. There have been several species of *Laricobius* found to associate with adelgid species throughout the world. *Laricobius rubidus* (Le Conte), native to the eastern United States and a predator of the pine bark adelgid (PBA), *Pineus strobi* Hartig, feeds on HWA (Zilahi-Balogh et al. 2005). *Laricobius rubidus* completes its life cycle on HWA and is often found feeding in the same location as *L. nigrinus* (discussed later) (Mausel et al. 2008). These two species hybridize with each other (Havill et al. 2012), yet impacts on both HWA and PBA systems appears limited in stands containing both white pine and hemlock (Fischer et al. 2015).

Several species of *Laricobius* occur throughout Asia in association with *A. tsugae*. Two *Laricobius* species are found associated with HWA on *Tsuga chinensis* in the Sichuan Province, China: *Laricobius baoxingensis* Zilahi-Balogh and Jelinek, and *Laricobius*

kangdingensis Zilahi-Balogh and Jelinek (Zilahi-Balogh et al. 2007). *Laricobius osakensis* was first discovered in Japan in 2005 and approved for release in 2010 (Lamb et al. 2011). *Laricobius osakensis* shows promise controlling HWA due to its voracious feeding behavior, and feeds on more on ovisacs than *L. nigrinus* (Story et al. 2012). *L. osakensis* females were found to lay more eggs than *L. nigrinus* at increasing prey densities (Vieira et al. 2012). When *L. osakensis*, *L. nigrinus*, and *L. rubidus* are together in laboratory assays, they do not negatively impact the rate of predation with regards to the amount of prey consumed (Story et al. 2012).

1.5 *Laricobius nigrinus* as a Biological Control Agent

Laricobius nigrinus is native to western North America (Lawrence 1989), has been closely associated with *A. tsugae* on western hemlock, *Tsuga heterophylla* Rafinesque Sargent, and is considered to be a potential biological control agent (Lawrence 1989, Zilahi-Balogh et al. 2003, Kohler et al. 2008). *L. nigrinus* was imported to the eastern U.S. from western North America, where it was evaluated and determined to be host-specific (Zilahi-Balogh et al. 2002). Releases in the eastern United States began in 2003 and F_2 adults were later recovered at the release sites in 2005 (Lamb et al. 2006, Salom et al. 2008, Mausel et al. 2010). Releases continue to this day throughout much of the HWA range in the eastern U.S. (Roberts et al. 2011). Two strains of *L. nigrinus,* coastal and interior, have been identified with the latter being more cold hardy than the coastal strain (Mausel et al. 2011a). To date, *L. nigrinus* has established in sites in plant hardiness zones 6a - 6b (Mausel et al. 2010) and 7a (Mayfield et al. 2015). *Laricobius nigrinus* has limitations as a predator of HWA with regards to it becoming

dormant during the later portion of HWA's progrediens generation and leaving that generation largely unchecked by *L. nigrinus*. *Laricobius nigrinus* is an ideal species as a biological control agent of HWA, since it can develop only on HWA, *L. nigrinus* follows closely with HWA's lifecycle (Zilahi-Balogh et al. 2002), and *L. nigrinus* is shown to predate on HWA in the lab (Story et al. 2012).

1.5.1 Morphology of *Laricobius nigrinus*

Zilahi-Balogh et al. (2006) provided a detailed morphological description of *L. nigrinus*. Male and female adults cannot be distinguished externally, but can be determined by microscopic examination of the distended genitalia (Shepherd et al. 2014). *Laricobius nigrinus* adults are characterized by a uniformly shiny black body covered in fine hairs. Adults are approximately 2.3 - 2.9 mm in length and are dorsally convex and ventrally flattened. The head is partially concealed from above by the pronotum and antennae and tarsi are a dull red color. The pronotum is 1.2 to 1.5 times wider than it is long. *Laricobius nigrinus* and *L. rubidus* adults can be distinguished based on the male aedeagus with *L. nigrinus* being more tapered compared to *L. rubidus* which is more obliquely truncate (Leschen 2011). *Laricobius nigrinus* eggs, found in HWA ovisacs, are shiny bright yellow oval in shape, approximately 0.37-0.50 mm in length and 0.24-0.33 mm in width. There are four larval instars. The larvae are oligopod and slightly fusiform with abundant prominent setae. Young *L. nigrinus* larvae are yellow and older larvae are a yellow-green to yellow-brown. The pupae are exarate and yellow in color. Males and females can be distinguished, female genitalia have two valvifers and the male has a 3-lobed aedeagus.

1.5.2 Rearing of *L. nigrinus*

Current rearing methods for *L. nigrinus* are labor intensive but have been successful. *Laricobius nigrinus* is reared using protocols presented by Lamb et al. (2005), based on lab experiments described in Salom et al. (2012). HWA-infested hemlock branches are collected from the field and then placed into containers containing feeding *L. nigrinus*. The containers' design consists of a 30 cm diam ca. 45 cm high Dura-Lar® acetate cylinder with the top vented with 0.14 mm nylon mesh and the bottom open placed on a galvanized steel funnel with the top diameter of 30 cm with hardware cloth 5 x 5 mm^2 placed inside of the funnel. A 0.24 L mason jar is attached to the funnel and filled with 10 mL pupation medium that consists of sphagnum moss, peat moss, and play sand. New foliage is provided every 12 days, which allows for constant supply of food for *L. nigrinus* to complete its life cycle on HWA and allow for mature larvae to drop into the soil to pupate. The pupae are then placed into 0.95 L and 1.9 L clear plastic containers containing at least 5 cm of pupating medium and stored in growth chambers at 15°C and moistened with distilled water once a week to keep moisture levels between 30-40% (Lamb et al. 2006) until July. The containers containing pupating and aestivating *L. nigrinus* are moved to 19°C until mid-September and are watered weekly. The containers are checked every other day beginning in late August for adult emergence. Ideally, the beetles emerge from October to December, and are maintained and fed until they are sent to cooperators for field releases, using the procedures documented in the HWA Predator Release and Monitoring Database (Roberts et al. 2011).

1.5.4 Releases of *Laricobius nigrinus*

Releases of the coastal strain *L. nigrinus* began in 2003, and by 2005 it had been released in 22 locations from Massachusetts to Georgia (Mausel et al. 2010). As of September 2016, over 900 documented releases of *L. nigrinus* have been made from Georgia to Maine (Roberts et al. 2011). The HWA Predator Release and Monitoring Database was created to record the release of HWA predators, monitor their recovery, and make the record public to indicate where predators have been released. However, there have been limited recovery efforts to see if predators have established at the release sites. Monitoring releases for several years helps improve our understanding of how the predator population acts at a specific field site. Mausel et al. (2010) assessed 22 sites from Georgia to Massachusetts across plant hardiness zones 5a to 7a to assess if there was establishment of *L. nigrinus*. Numbers of adult *L. nigrinus* released at the sites ranged from 75 to 1,200. Sampling consisted of beat sheeting for adult *L. nigrinus* and branch clippings for larval *L. nigrinus*. They found establishment at 13 of the 22 sites sampled, where the only variable correlated with larval recovery at the sites was minimum winter temperature. No variables were correlated with adult recovery. They found that releases during fall to early spring and across all densities of HWA were successful. From this study they concluded that establishment probability for the coastal strain was high in plant hardiness zones 6a to 7a and low in plant zones 5a and 5b.

1.5.3 *Laricobius nigrinus* and *L. rubidus* Hybridization

Laricobius nigrinus and *L. rubidus* will mate with each other (Mausel et al. 2008) and collection of beetles with mixed coloring of both species led to the determination that hybrids were being produced (Davis et al. 2011, Havill et al. 2012). *Laricobius rubidus* is often found associated with and can complete its development on HWA (Zilahi-Balogh et al 2005). However, it has not been observed to impact HWA populations (Havill et al. 2010). Although adult *L. nigrinus* and *L. rubidus* adults can be distinguished using male genitalia, the process is labor intensive and damage to the specimens is possible (Zilahi-Balogh et al. 2006, Havill et al. 2010). Through genetic testing, it was found that there is hybridization occurring at *L. nigrinus* release sites (Havill et al. 2012). Currently, sampling for presence of *L. nigrinus* at release sites requires genetic testing to quantify the level of hybridization occurring. Mayfield et al. (2015) found that in sites where hemlock trees were treated with low dosages of imidacloprid, most of the predators (77%) recovered were *L. nigrinus*, followed by *L. rubidus* at 12%, and hybrids at 11%. They found that the hybrid rate remained stable over time. Understanding hybridization levels at a site may provide important information regarding success of establishment and if hybrids are contributing or inhibiting impacts of *L. nigrinus* at release sites.

1.6 Research Rationale

It is important to assess the impact that biological control agents have in a forest setting to see if they are succeeding in controlling the target pest. Sampling efforts may be conducted to check for establishment of natural enemies. When the natural enemies are

found at generally increasing numbers in relation to their prey, it is considered a successful establishment (Mausel et al. 2010). However, in this study, if *L.nigrinus* is recovered at a site year after year, it was determined to be established at the site. Once the natural enemies become established at a release site, further sampling should be conducted to assess the degree of increased mortality to the pest and decreasing density of the pest (Van Driesche et al. 2008). Assessment of the natural enemy can be conducted using plots that contain the natural enemy and ones without the natural enemy. Assessment of the natural enemy can be conducted using cages to exclude the predator. Analyzing sites or cages with and without the natural enemy can be used to show the impact of the natural enemy on the prey species (Calderon et al. 2012). It is important to determine if the biological control agent is effectively controlling the prey species. If not, more agents (additional species) or releases of the agent may be needed (Van Driesche 2014). Since establishment of *L. nigrinus* has had success in plant hardiness zones 6a - 7a, an early cage exclusion study was conducted at a hemlock plantation located in McCoy, VA two years after beetles were released. Greater predation took place outside the cages compared to inside the cages and was attributed to *L. nigrinus* (Mausel et al. 2008). More effort is needed to see what is happening across the establishment range *of L. nigrinus* to determine the efficacy of *L.nigrinus* as a predator in the field.

1.7 Objectives:

The first objective of this study is to assess the degree of impact *L. nigrinus* and winter mortality is having on the HWA at release sites where the predator is established. The

second objective is to assess success of establishment of *L. nigrinus* at previous release sites in Virginia. Both studies are critical in assessing the importance of *L. nigrinus* as a biological control agent out in a field setting. It is the first step toward a long-term effort to assess whether the predator contributes to mortality of the pest and improved health of the trees.

Chapter 2: Impact Assessment of *Laricobius nigrinus*, a predator of hemlock woolly adelgid in the eastern United States

Abstract

Hemlock woolly adelgid (HWA) is a pest of eastern and Carolina hemlock and causes hemlock tree death and dieback. It is native to both Asia and the Pacific Northwest. Also native to the Pacific Northwest is *Laricobius nigrinus,* a host-specific predator whose lifecycle is synchronous with HWA. Its release as a biological control agent throughout the eastern U.S. has been ongoing since 2003. Establishment of *L. nigrinus* at release sites is well documented; however, investigations of its impact on HWA populations have been limited. Therefore, we report on a study attempting to assess the impact *L. nigrinus* is having on populations of HWA sistens throughout the eastern U.S. The nine field sites used in the study had moderate to high densities of HWA, had releases made at least four years prior to the study, and had recovery of beetles for at least two years since their release. Three treatments were set up at each of the sites: no cage, closed exclusion cage, and open cage, all on accessible branches. Three assessments were taken during key points throughout the season in order to monitor what was occurring for both HWA and *L. nigrinus* populations at the release sites. Of the nine sites sampled where *L.nigrinus* had been released at least four years prior, it was recovered at six of them (67%). Predation rates of HWA increased later in the season when both larvae and adult *L. nigrinus* were present. Despite changes in HWA populations from year to year, significantly more HWA were disturbed on open and uncaged branches than on caged branches at most of the sites in plant hardiness zones 6b to 7a with disturbance in assessment three ranging from 1.5 to 47.3% in year one and 0 to 66% in year two.

This shows promise for *L. nigrinus* as a biological control agent at many of the sites sampled.

Keywords: Biological control, *Laricobius nigrinus*, predator, *Adelges tsugae*, exclusion cages, *Tsuga canadensis*

2.1 Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is a non-native invasive pest of both eastern (*Tsuga canadensis* (L.) Carriere) and Carolina hemlock (*Tsuga caroliniana* Engelmann) (McClure 1996). The strain of HWA that is invasive in the eastern United States originates from Japan and was found in the early 1950s near Richmond, Virginia on imported nursey stock (Souto et al. 1996, Havill et al. 2006). In its native range of Asia and western North America, HWA is not considered a pest on the native *Tsuga* spp. due to climatic conditions, native predators, and host resistance (McClure 1989, McClure and Cheah 1999, Skinner et al. 2003). In the eastern United States, eastern hemlock and Carolina hemlock are highly susceptible to HWA attack (McClure 1991).

Methods for controlling HWA include the application of chemical and biological control. Chemical control has proven to be effective at controlling HWA populations. The most common method for applications of chemical insecticides (mostly neonicotinoids) is

through soil or tree injections (Cowles 2006). Such applications are appropriate on a small scale, but not sustainable for large scale area-wide efforts (Fidgen et al. 2002).

A more sustainable long-term approach for keeping eastern hemlocks healthy in the forest setting is the use of classical biological control (Mausel et al 2010, Onken and Reardon 2011). Explorations to Asia and the western United States, where HWA is native, was conducted but success was limited (Wallace and Hain 2000, Onken and Reardon 2011). Several species were recovered from these locations notably *Symnus sinuanodulus* Yu & Yao (Coleoptera: Coccinellidae), *Sasajiscymnus tsugae* Sasaji (Coleoptera: Coccinellidae), *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae), and *L. nigrinus* Fender (Coleoptera: Derodontidae) (Mausel and Salom 2013). Other notable species outside of the order Coleoptera are the *Leucopis* spp. (Diptera: Chamaemyiidae) found in the Pacific Northwest. Namely *Leucopis argenticollis* Zetterstedt (Diptera: Chamaemyiidae) and *Leucopis atrifacies* (Aldrich) (Diptera: Chamaemyiidae) (Kolher et al. 2008).

Laricobius spp. are known to feed on adelgids (Lawrence 1989). After adequate risk assessment studies (Zilahi-Balogh et al. 2002, Vieira et al. 2011), *L. nigrinus* and *L. osakensis* were permitted for release and introduced into the eastern United States to help control HWA. Meanwhile, *L. rubidus*, a native species that preys primarily on pine bark adelgid, *Pineus strobi* (Hartig) (PBA) (Clark and Brown 1960), was found to colonize HWA when hemlocks and eastern white pine trees, *Pinus strobus,* were both part of the forest ecosystem (Zilahi-Balogh et al. 2005). Hybridization is known to occur

between *L. nigrinus* and *L. rubidus* (Havill et al. 2012), but is thought to be inconsequential (Fischer et al. 2015).

Open releases of the coastal strain of *L. nigrinus* began in 2003 in the eastern United States. By 2005, the beetles had been released in 22 locations from Massachusetts to Georgia and determined to be established at 13 of these sites (Mausel et al. 2010). The coastal strain was the first strain to be found in the Pacific Northwest and became the main strain to be researched and released. The key factors for establishment were plant hardiness zones (a proxy for minimum temperature) and numbers of beetles released per site. It was concluded that plant hardiness zones 6a to 7a were suitable for release of the coastal strain.

As of September 2016, over 900 documented releases of *L. nigrinus* have been made from Georgia to Maine (Roberts et al. 2011). The HWA Predator Release and Monitoring Database was created to record the release of HWA predators, monitor their recovery, and make the record public to indicate where predators have been released. Revisiting release sites and recording establishment has occurred for only a small fraction of the sites. But from these efforts, several sites have been identified to consistently have *L. nigrinus* present when HWA was present. Given the maturity of the release effort for *L. nigrinus*, we decided to assess the predator's impact on HWA populations at sites where *L. nigrinus* establishment was well documented. Additionally, it was decided that the effort should encompass the geographic range of the coastal strain's establishment.

In this study, we assessed the impact that *L. nigrinus* had on HWA populations at sites located from New Jersey to Georgia. This is the first step toward determining if *L. nigrinus* is contributing to HWA mortality in a significant way. Results from this work could be used to provide justification for continued investment or disinvestment in the release of this and other biological control agents in hemlock stands.

Study Objective: Impact of *Laricobius nigrinus* on HWA Populations

Impact assessment was conducted at nine separate release sites with samples taken at three distinct times in HWA and *L. nigrinus* lifecycles. The first sample was taken in October or November after HWA broke dormancy. The second sample was taken in February or March, during peak oviposition of HWA, to measure HWA mortality and predation, as well as HWA disturbance. The third sample was taken in April, during peak Ln larval activity. At the end of sampling, the degree of predation was assessed.

Hypothesis

1. If *L. nigrinus* is established at a site, then we should see a difference in the number of disturbed HWA on branches where predators were and were not excluded.

2.2 Materials and Methods

2.2.1 Field site and tree selection

Nine field sites were chosen (Table 2.1 and Fig 2.1) in six states: New Jersey, Maryland, Virginia, North Carolina, Tennessee, and Georgia. The sites encompass plant hardiness zones 6a to 7a (planthardiness.ars.usda.gov). Each field site was chosen based on a list of criteria including: 1) release of *L. nigrinus* at least four years prior to the set-up of the test (before 2010), 2) moderate-high densities of HWA at the start of the study, and 3) and previous recoveries of *L. nigrinus,* suggesting the predator has established. The same sites were used for both years of the study.

2.2.2 Assessment 1: Field site set up and determining initial HWA densities

Exclusion cages were set up on branches of hemlock trees within each of the sites to assess the level of predation by *L. nigrinus*. The many sites 15 pairs of uncaged and closed fine nylon mesh (41 x 37 mesh/square cm, mesh aperture 30 µm) 1 m long cages (MegaView Science, Taichung, Taiwan) were placed on hemlock branches. An additional five open caged treatments were set up at most of the sites to account for any possible cage effect. The mesh used for the cages was fine enough to exclude predators and allow the HWA population to grow in the absence of predators. Branches were chosen and tests were set up in October and November of 2014 and 2015, soon after the HWA sistens generation breaks diapause and began to develop. All test branches chosen had moderate-high density of adelgids, with a goal of 2-3 HWA/cm. Such a density level was not always possible, so the branches with the greatest amount of HWA were chosen for the study. After branches were selected and marked, branchlets were labeled, and the number of adelgids present on new growth was

counted from the base to distal tip of the branch, and branchlet lengths were measured. At the site in New Jersey (NJ 1), adelgids on both the old and new growth were counted due to lack of adelgids on new growth. After counting the number of adelgids and cm of new growth, one of three treatments, no cage, open cage, or closed cage, was assigned to the branch. At NJ 1 in both year one and year two and at NC 1 in year two, only closed and no cage treatments were set up.

2.2.3 Assessment 2: Adult *Laricobius* predation of sistens during peak oviposition of HWA

Assessment two was taken during the peak oviposition time for HWA sistens, when approximately 75% or greater of the live HWA sistens were producing eggs (in either February or March depending on site). Samples were taken from non-study branchlets and taken back to the lab to determine the percentage of adults laying eggs. Once 75% of the HWA ovisacs contained eggs, the second branchlet samples were clipped and taken back to the lab for examination under a microscope, where the number of 1) intact HWA that contained live HWA, 2) HWA disturbed or preyed upon, and 3) winter killed HWA (HWA that were not disturbed but contained dead sistens) were counted. HWA ovisacs were classified as disturbed due to the ovisac appearing to be shredded. Ovisacs that contained *Laricobius* larvae or eggs were recorded. Winter mortality and ovisac disturbance data were collected separately at NJ 1, NC 1, TN 1, TN 2, TN 3, and GA 1; overall mortality including disturbed HWA data were taken at MD 1, VA 1, and VA 2 in year one and winter mortality and ovisac disturbance were assessed separately at these sites in year 2 .

2.2.4 Assessment 3: Cumulative predation of HWA by adult and larval stage *Laricobius*

Assessment 3 took place 2 - 4 weeks after Assessment 2, to coincide with the blooming of eastern redbud (*Cercis canadensis*) L., a known phenological indicator of when *L. nigrinus* larvae are present as late instars (third or fourth instars) (Mausel et al. 2010). For this assessment, the remainder of the branches were removed from the tree and taken back to the lab and set up in 19 L buckets, small cups, or in larval rearing funnels (Salom et al. 2012). The collection funnels were checked every 1 - 2 d for the next 6 - 8 weeks. Any larvae that had dropped were placed into 95% ethanol. The branchlets were then examined after the larvae finished dropping. The number of intact and disturbed sistens ovisacs was recorded.

2.2.5 Genetic analysis of *Laricobius* spp. recovered at field sites

Laricobius larvae and adults recovered from each site underwent genetic testing to determine species and hybridization between the native *L. rubidus* and the introduced *L. nigrinus* in order to determine the species composition at the sites. Genetic testing between *L. nigrinus* and *L. rubidus* was conducted using the procedure published by Davis et al. (2011). The genetic testing was conducted using Qiagan blood and tissue extraction kit and the methods set forth by Qiagan (Qiagan 2006). The mitochondrial cytochrome oxidase I (CO1) gene was then amplified using PCR. Once PCR was completed a gel was run to confirm the amplification of the CO1 gene. Once the amplification of the CO1 gene was confirmed it was sent to Yale's DNA Analysis Facility

on Science Hill for further processing. DNA was then analyzed using DNAstar Seqman Pro to determine species.

2.2.5 Statistical analysis

Analysis of variance (ANOVA) was used to test the effect of treatment (no cage, closed cage, open cage) on HWA densities, percent overwintering mortality, and percent ovisacs disturbed. Means were separated using Tukey's HSD. A log transformation was performed on HWA densities from Assessment 1 to normalize the data. An arcsine square root transformation was performed to normalize the data for both winter mortality and percent HWA disturbed in both Assessments 2 and 3 and an ANOVA was performed with Tukey HSD.

2.3 Results

2.3.1 Assessment 1: Field site set up and determining initial HWA densities

There were no significant differences in HWA densities among treatments pooled for all of the sites in year 1 ($F = 0.338$, $DF = 2, 135.9$, $P > 0.7136$) and year 2 ($F = 1.07$, $DF = 2, 84.5$, $P > 0.3493$). There were also no significant differences observed among treatments within each of the sites (Table 2.2). Initial densities of HWA increased from year one to year two at MD 1, TN 1, TN 2, and TN 3. Visual observation of the data showed densities of HWA stayed relatively the same at NJ 1, VA 1, and VA 2. There was a noticeable decrease in HWA densities at NC 1 and GA 1. The densities of HWA varied considerably among sites ranging in year one from fewer than 1 to nearly 6 HWA/cm

(Fig. 2.2A) and in year two from under 0.5 to ca. 5 HWA/cm on average at the sites (Fig 2.2B).

2.3.2 Assessment 2: Adult *Laricobius* predation of sistens during peak ovipositioning of HWA.

Winter mortality and ovisac disturbance was assessed in both years at NJ 1, NC 1, TN 1, TN 2, TN 3, and GA 1; overall mortality (including HWA disturbed) was assessed at MD 1, VA 1, and VA 2 in year 1 and winter mortality and ovisac disturbance in year 2. This distinction in data collected lead to analyzing each group separately. In year 1, mean winter mortality at each of the sites ranged from 27 – 74.3%, and showed a north-south pattern with sites further north typically experiencing higher rates of mortality (Fig 2.3 A). Winter mortality in year 2 ranged from 15 – 95% based on site (Fig 2.3 B). There were no significant differences in mortality rates among treatments for either year 1 ($F= 1.823$, $df= 2, 81.8$ $p > 0.1681$) or year 2 ($F= 1.518$ $df= 2, 97.9$, $p > 0.2243$) with regards to data pooled across all sites. However, when data were analyzed for differences within sites, it was found that differences among treatments were observed in year 1 at NC 1, with greater mortality of HWA occurring in caged compared with HWA on uncaged branches (Fig 2.3 A). Generally, cages did not have an effect on winter mortality.

There were no significant differences in overall mortality of HWA among treatments in the pooled data for year 1 ($F= 0.558$, $df= 2, 21.2$, $p<0.5803$) at sites MD 1, VA 1, and VA 2. There were no significant differences among the treatments within each site (Fig. 2.3A).

Year 1: The numbers of HWA ovisacs disturbed during Assessment 2 was used to estimate levels of predation at each of the sites except for MD 1, VA 1, or VA 2 in year one. No data were collected from open cage samples at TN 1, TN 2, or TN 3. No cage samples had high rates of disturbance, followed by open cage; while, caged samples had the lowest rates of disturbance (Fig. 2.4 A). When analyzed by site there were significant differences observed between caged and no caged samples at NC 1 and TN 2 (Fig 2.4 A and Table 2.2). At two of the three sites that did not show significant differences among treatments, there was a trend of greater disturbance on no cage branches compared to caged branches. This is likely due to *Laricobius* spp. since few other predators were recovered throughout the duration of this study, however other predators could account for a proportion of the disturbance seen in the HWA populations.

Year 2: Ovisac disturbance was typically greater on both the open and no cage branches compared with closed cage branches (Fig 2.4B), and was likely due to cages excluding predators. There were significant differences among treatments at VA 1 and VA 2 (Fig 2.4B and Table 2.2). Ovisac disturbance was greatest on the open cage branches and least on the caged branches. At many of the sites, either the open cage samples or the no caged samples showed trends toward greater rates of disturbance than on caged branches, similar to what was observed in year one.

Laricobius larvae were recovered during Assessment 2 at seven of the nine sites in year one and four of the nine sites in year two (Table 2.3). Overall, greater amounts of *L. nigrinus* were found in both years compared to *L. rubidus*. *L. rubidus* was found at some of the sites, though they were found more in year two than year one (Table 2.4). There were large numbers of *Laricobius* specimens that could not be identified through genetic tests. Many of the sites that showed high rates of disturbance in Assessment 2 also had *Laricobius* larvae recoveries.

2.3.3 Assessment 3: Cumulative predation of HWA by adult and larval stage *Laricobius*

In Assessment 3, the number of HWA disturbed was recorded after larvae dropped from the infested hemlock foliage after 6-8 weeks in the lab. In year one, rates of disturbance in assessment 3 were typically greater in the no cage samples than the caged samples, and significantly different in five of the nine sites (Fig. 2.5a). In year two, a similar trend was observed with significant differences in six of the nine sites (Fig. 2.6b). Significant differences were seen among treatments in both year one ($F=17.04$ $df= 2, 97.9$, $p<0.0001$) and year two with the data pooled across sites ($F= 16.63$, $df= 2,130.2$, $p<0.0001$) (Fig. 2.6). Disturbance averaged around 20% in the no bag treatment in year one and over 40% in no and open bag treatments in year 2.

Laricobius larvae were recovered at five of the nine sites in year one and eight of the nine sites in year two (Table 2.3). *L. nigrinus* was the primary species that was

recovered, though *L. rubidus* where recovered from some of the sites. A large proportion of the beetles recovered could not be identified genetically (Table 2.4).

2.4 Discussion:

This was the first multi-state effort to assess the impact of *L. nigrinus* on HWA populations. Previous work has determined efficacy of *L. nigrinus* in laboratory settings (Story et al. 2012) or at an individual site (Mausel et al. 2008, Mayfield et al. 2015). Mayfield et al. (2015) found in a field study that the density of undisturbed ovisacs of HWA was twice as high on branches that were protected from predators than those exposed to them. Work has also been conducted on how *L. nigrinus* compares with both *L. rubidus* and *L. osakensis. Laricobius rubidus* oviposited fewer eggs than either *L. nigrinus* or *L. osakensis* and that overall *L. osakensis* preyed upon the greatest amount of HWA ovisacs (Story et al. 2012). However, this does not discount that *L. nigrinus* is not a sufficient predator but rather that *L. osakensis* predation rates are higher.

Post-release impacts of biological control agents can be economic or environmental. Economic impact includes an increase in crop yield or decrease in costs to control a pest species. Environmental impacts include off-target effects of the biological control agent, but also the post-release impacts of the biological control agent on the pest population. Several studies have conducted similar tests with exclusion cages, similar to what we used to assess the impact that *L. nigrinus* was having on HWA populations.

One such study used biological check methods and paired-cage techniques that excluded the predator, *Pheidole megacephala* (Fabr.) (Hymenoptera: Formicidae), from feeding on the pink pineapple mealybug, *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae) (Gonzales-Hernandez et al. 2002). Another study monitored predation of *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) without cages to exclude predators (Rosenheim et al. 1999). They chose not to use a cage so that they could determine that the cage was not impacting mortality observed. We chose to use cages to exclude predators because this would allow us to monitor the same sample out in the field over a long period of time without frequent trips to monitor what is occurring in the population. We also found that there was little to no cage effect with regards to winter mortality of HWA, so we determined that this method worked well to assess impact of *L. nigrinus* on HWA populations.

Our Assessment 1 pre-treatment densities of HWA did not differ among treatments, which was important for making comparisons among the treatments during Assessments 2 and 3. Additionally the differences in pre-treatment numbers from year one to year 2 show that the populations can change dramatically from year to year. The tremendous increases observed at some sites in year two were most likely the result of populations recovering from very high mortality documented throughout the eastern U.S. after the extreme low temperature events that took place in both January of 2014 and February 2015.

In Assessment 2, non-predator winter mortality did not differ among treatments at any of the sites with the exception of NC 1 in year one. This demonstrated that the cages did

not seem to influence the results of this measure. Overall mortality (winter mortality plus disturbed ovisacs), which was calculated for MD 1, VA 1, and VA 2 in only year one due to HWA disturbed not being recorded. Mortality at these sites did not differ among treatments, suggesting that adult *Laricobius* spp. feeding during the winter are not having a measureable impact on the HWA population up to that point in the season at these three sites. Instead it appears that winter mortality caused by cold temperatures might have contributed to most of the HWA mortality.

Laricobius spp. were recovered at many of the sites in both years, especially where high rates of ovisac disturbance were recorded. Overall, greater rates of disturbance were observed in Assessment 3 compared to Assessment 2. There were some levels of disturbance observed in the caged samples. This could be due to numerous factors including the cage disturbing the HWA throughout the winter either through snow weighting the cage down or wind, which would cause the cage to rub up against the HWA. HWA could have also been disturbed when the samples were taken off the trees and placed into plastic bags to be transported to the lab, which could have resulted in some of those samples showing high rates of disturbance. The results are still valid since HWA disturbance was greatest in either the open cage or uncaged seen at many of the sites.

Sites such as NJ 1 showed low levels of HWA ovisac disturbance in year two, in Assessment 2 and no disturbance in Assessment 3. In year two at NJ 1, no *Laricobius* spp. were recovered and little to no disturbance was observed. Few *Laricobius* spp. were recovered in year one and none were recovered in year two. Previous research

by Mausel et al. (2010) found that *L. nigrinus* established in plant hardiness zones 6a-6b and Mayfield et al. (2015) determined that it establishes in plant hardiness zone 7a. NJ 1 is located in plant hardiness zone 6a and compared to the other sites, experiences colder average temperatures. The polar vortex experienced in winter 2014 and 2015, which brought colder than average temperatures, likely led to few if any *Laricobius* spp. recovered. However, the polar vortex experienced in winter 2014 and 2015 is a rare event, once HWA populations recover sufficiently; it is likely that *L. nigrinus* will be recovered again. Continued sampling is necessary to verify this.

It appears that most of the ovisac disturbance is a result of *Laricobius* feeding and oviposition. Virtually no other predators were recovered from the sites with an exception of a ladybird beetle larvae at one of the TN sites and a syrphid larvae at the MD site. Through genetic analysis, it was observed that most of the *Laricobius* specimens collected were *L. nigrinus*. The species composition at these sites fits closely to what Fisher et al. (2015) found in the field setting; *L. nigrinus* numbers increase after a release, *L. rubidus* numbers decrease at a site, and the hybridization rate remains stable over time. The majority of species recovered at these release sites were *L. nigrinus*. *Laricobius rubidus* was recovered but less frequently than *L. nigrinus*. Hybrid testing has not yet occurred to determine the percent of hybrids at each field site. The results from the genetic analysis strengthen the likelihood that *Laricobius* spp. are responsible for much of the disturbance seen at these sites.

Several samples were lost throughout the season in both year one and year two at many of the sites. Some of the sample branches were broken while others died and remained completely intact. Samples that were broken off the branch were due to weather conditions, either snow or heavy winds; wildlife pulling or removing the cages and destroying the samples; and vandalism, which resulted in samples to go completely missing. Samples that went missing over the field season to present an issue with regards to sample size. However, the data are robust enough and encompasses nine field sites, most of which show similarities with regards to overwintering mortality and ovisac disturbance.

There were a considerable number of specimens that could not be identified through genetic analysis. This was likely a result of the DNA becoming degraded due to exposure to high temperatures or theoretically due to the equipment malfunctioning in the lab. In spite of this, there was still a sizeable proportion of the *Laricobius* population that were successfully sampled at each of these sites.

This study shows that by Assessment 3, *L. nigrinus* is significantly reducing HWA sistens populations at many of the release sites. Most of the sistens ovisac disturbance occurs as a result of the combined impact of adult feeding and oviposition and larval feeding. Additionally, over the course of two years, impacts occurred at sites ranging from plant hardiness zones 6a to 7a. There are some implications regarding the biological control of HWA. *Laricobius* spp. are some of the most commonly used biological control agents to manage HWA. However, *Laricobius* spp. predation of the

sistens generation and only minimally predate the progrediens generation by feeding on their eggs. This leaves a hole in managing HWA populations, with this generation of HWA left unchecked by predators and HWA prolific nature HWA populations can rebound at a site. Less is known regarding how *L. nigrinus* is impacting progrediens population densities, predator-prey relationships in the eastern United States compared to the western U.S., or the long-term effects on tree health at release sites. Future work will be conducted on these topics to better assess what is occurring at these field sites.

Table 2.1: Summary of *Laricobius nigrinus* impact assessment sites in the eastern United States: including location, year of previous releases, number of trees used, treatments set up at each site, and plant hardiness zone.

State	Site	Lat, Long	Ln releases	Total no. Trees		Total no. Caged and Uncaged Branches		Total no. Open Cage Branches		Plant Hardiness Zone*
				2014	2015	2014	2015	2014	2015	
NJ	1	41.12 N, -74.91W	2007, 2008	20	10	20	10	0	0	6a
MD	1	39.70 N, -78.67 W	2004	12	7	15	15	5	5	6b
VA	1	37.64 N, -78.80 W	2005	20	5	15	15	5	5	7a
VA	2	37.21 N, -80.59 W	2003	13	12	15	15	5	5	6b
NC	1	35.81 N, -82.19 W	2005	9	7	12	14	4	0	6b
TN	1	35.76 N, -83.30 W	2007	5	5	15	15	5	5	7a
TN	2	35.69 N, -83.87 W	2008	5	5	15	15	5	5	7a
TN	3	35.66 N, -83.59 W	2006	5	5	15	15	5	5	7a
GA	1	34.79 N, -83.76 W	2008, 2010	4	4	15	15	5	4	7a

*****Plant hardiness zones taken from planthardiness.ars.usda.gov

Table 2.2. Statistical differences among treatments: in year one (A) and year two (B) by site for HWA density (Assessment 1), overwintering mortality, and ovisacs disturbed (Assessments 2 and 3).

A. Year 1

	Assessment 1 HWA/cm			Assessment 2 Winter Mortality			Assessment 2 Ovisacs Disturbed			Assessment 3 Ovisacs Disturbed		
Site	F	DF	p	F	DF	p	F	DF	p	F	DF	p
NJ 1	0.53	1, 36	0.47	0.54	1, 31	0.47	0.004	1, 31	0.95	2.8	1, 26	0.11
MD 1	1.19	2, 32	0.32	0.34*	2, 18*	0.72*	N/A	N/A	N/A	0.0008	2, 21	0.99
VA 1	0.34	2, 32	0.71	0.34*	2, 30*	0.72*	N/A	N/A	N/A	3.66	2, 25	0.04
VA 2	0.61	2, 33	0.55	0.23*	2, 32*	0.79*	N/A	N/A	N/A	3.34	2, 29	0.049
NC 1	0.24	2, 25	0.79	9.6	2, 26	0.0001	13.69	2, 24	0.0001	28.6	2, 25	0.0001
TN 1	2.77	2, 32	0.078	0.17	1, 27	0.68	2.19	1, 27	0.15	3.74	2, 27	0.037
TN 2	0.45	2, 32	0.65	1.48	1, 27	0.23	9.65	1, 27	0.004	5.14	2, 31	0.012
TN 3	2.93	2, 32	0.068	1.32	1, 25	0.26	3.52	1, 25	0.073	2.7	2, 30	0.0819
GA 1	0.014	2, 32	0.99	0.45	2, 32	0.64	0.12	2, 32	0.89	4.91	2 32	0.014

B. Year 2

	Assessment 1 HWA/cm			Assessment 2 Winter Mortality			Assessment 2 Ovisac Disturbance			Assessment 3 Ovisac Disturbance		
Site	F	DF	p	F	DF	P	F	DF	P	F	DF	P
NJ 1	2.28	1, 18	0.15	0.00	1, 18	0.95	1.00	1, 18	0.33			
MD 1	1.30	2, 33	0.29	1.86	2, 21	0.18	0.50	2, 21	0.62	58.80	2, 16	0.00010
VA 1	0.46	2, 31	0.64	0.62	2, 30	0.55	4.39	2, 30	0.02	11.74	2, 31	0.00020
VA 2	0.31	2, 32	0.74	0.66	2, 31	0.52	6.12	2, 31	0.01	0.46	2, 29	0.64000
NC 1	0.01	1, 26	0.93	0.51	1, 26	0.48	3.64	1, 26	0.07	16.20	1, 24	0.00050
TN 1	0.08	2, 12	0.93	1.01	2, 12	0.39	1.70	2, 12	0.22	2.48	2, 12	0.13000
TN 2	0.56	2, 12	0.59	0.68	2, 12	0.52	1.03	2, 12	0.39	11.33	2, 12	0.00170
TN 3	0.11	2, 12	0.90	0.25	2, 14	0.78	2.28	2,12	0.15	2.00	2, 12	0.18000
GA 1	0.79	2, 31	0.46	1.94	2, 31	0.16	1.62	2, 31	0.21	7.29	2, 30	0.00260

*Overall mortality was taken in assessment 2 in year 1

Table 2.3. Number of *Laricobius* beetles recovered from field sites during Assessments 2 and 3: by treatment for both years.

Site	NJ 1		MD 1		VA 1		VA 2		NC 1		TN 1		TN 2		TN 3		GA 1	
	AD	LRV	AD	LRV	AD	LRV	AD	LRV	AD	LRV	AD	LRV	AD	LRV	AD	LRV	AD	LRV
Assessment 2, Year 1																		
Open Cage	N/A		0	0	0	2	0	7	0	3	N/A		N/A		N/A		0	1
No Cage	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	2
Cage	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
Assessment 3, Year 1																		
Open Cage	N/A		0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
No Cage	0	0	0	0	0	0	0	6	0	32	0	2	0	2	0	0	0	12
Cage	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Assessment 2, Year 2																		
Open Cage	N/A		0	0	0	0	0	1	N/A		0	0	0	3	0	0	0	0
No Cage	0	0	0	7	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Cage	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Assessment3, Year 2																		
Open Cage	N/A		0	13	0	0	0	17	N/A		0	0	0	0	0	0	0	6
No Cage	0	0	0	19	0	4	0	5	0	42	0	3	0	1	1	0	0	68
Cage	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 2.4. Total *Laricobius* spp. adult and larvae proportions of species collected in year one and year two from the impact assessment field sites. Bold-faced rows indicate sites and year when significant ovisac disturbance was recorded from open or no cage branches

		Species Recovered		
Year	Site	Total *Laricobius* spp. recovered	% *L. nigrinus*	% *L. rubidus*
Year 1	NJ 1	1	0	0
	MD 1	0	0	0
	VA 1	**2**	**0**	**0**
	VA 2	**13**	**100%**	**0%**
	NC 1	**38**	**100%**	**0%**
	TN 1	3	0	0
	TN 2	**2**	**0**	**0**
	TN 3	1	0	0
	GA 1	**16**	**50%**	**50%**
Year 2	NJ 1	0	0	0
	MD 1	**43**	**87%**	**13%**
	VA 1	**5**	**100%**	**0%**
	VA 2	23	89%	11%
	NC 1	42	77%	33%
	TN 1	3	0	0
	TN 2	**1**	**0**	**0**
	TN 3	2	100%	0%
	GA 2	**74**	**97%**	**3%**

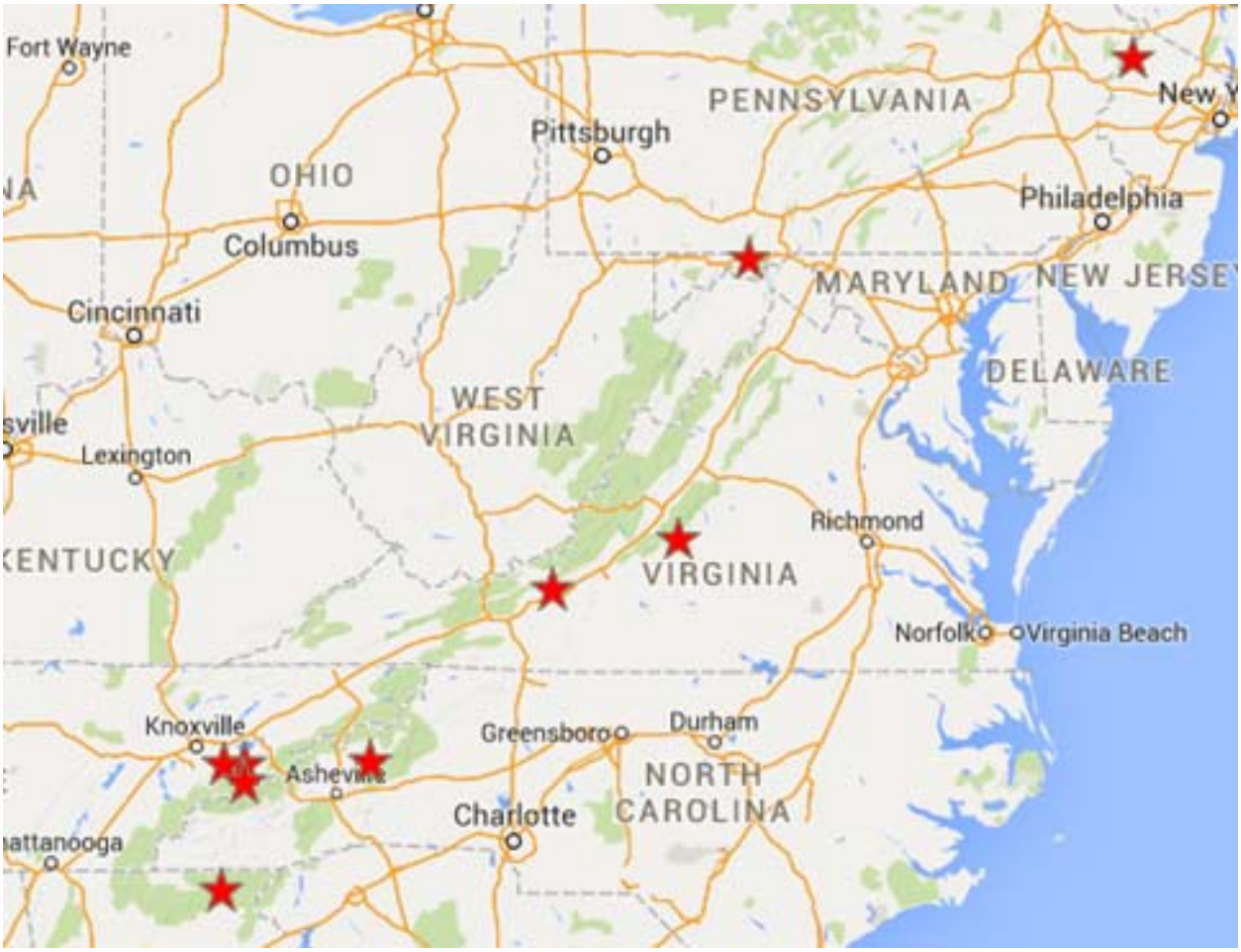

Figure 2.1. Map of *L. nigrinus* assessment field sites. The red stars mark site locations of field sites used in the study.

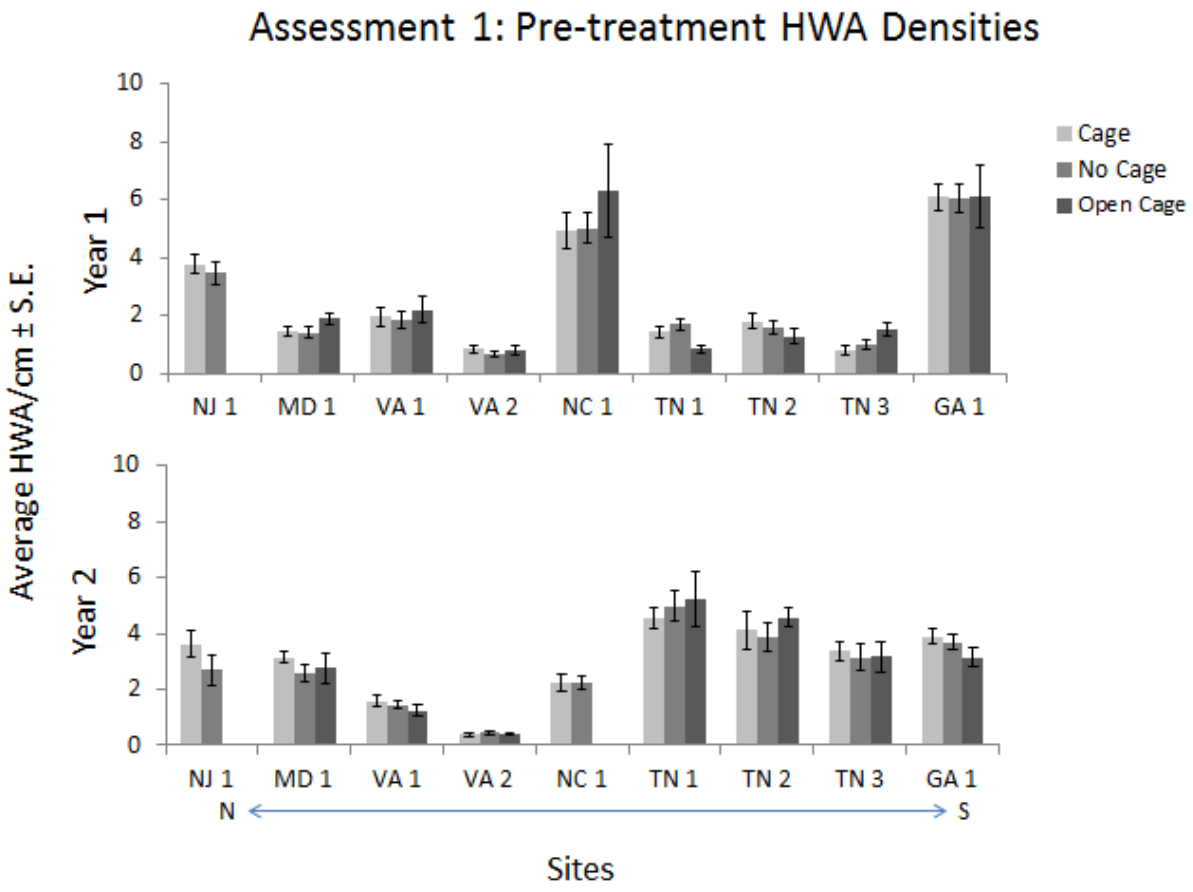

Figure 2.2. Mean ± S.E. HWA density from Assessment 1 in years one (A) and two (B) for each treatment. Open caged samples were not set up at NJ 1 in both years and at NC 1 in year two. Sites range from north to south (left to right) on the x-axis.

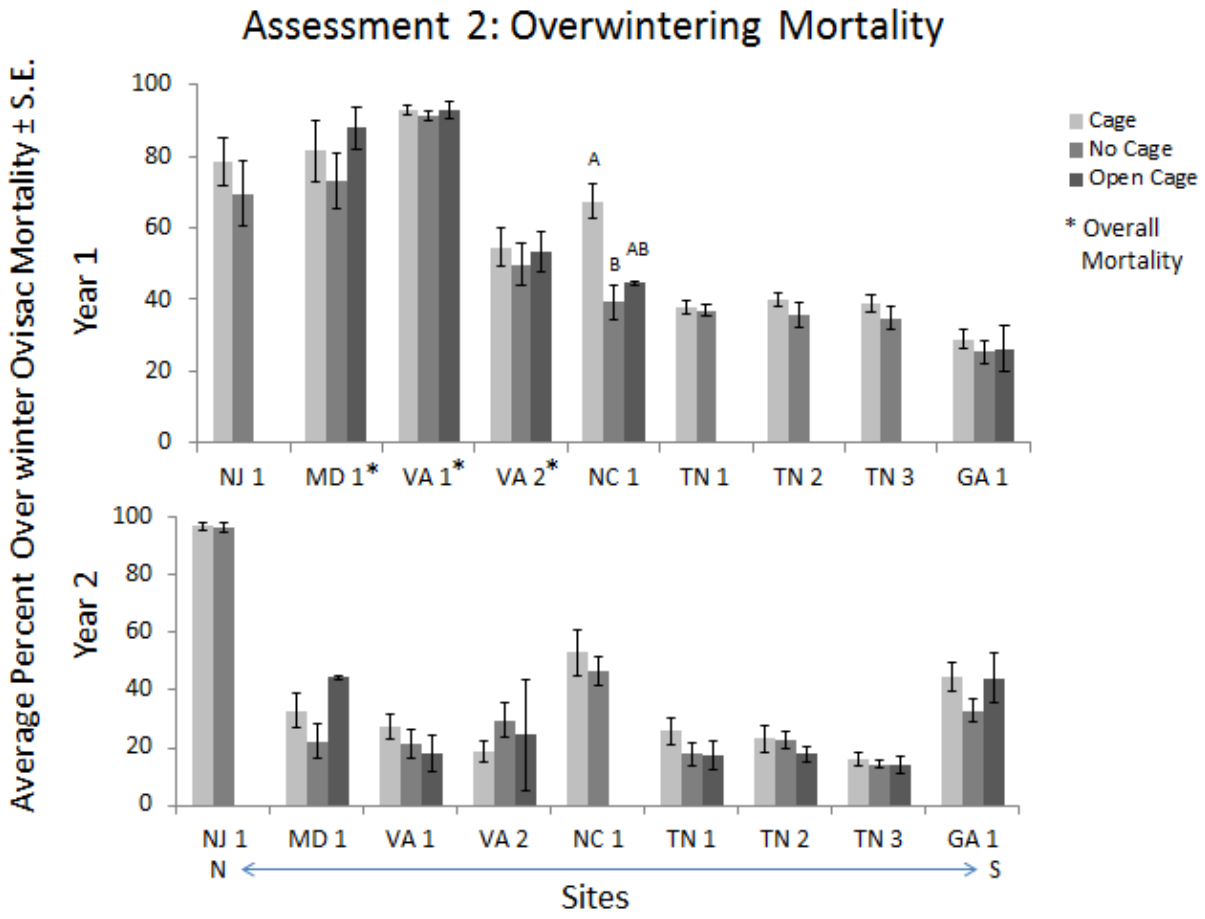

Figure 2.3. Winter mortality Assessment 2 of HWA ± S.E. in year one (A), where NJ 1 does not have open cage samples and in year two (B), where NJ 1 and NC 1 do not have open caged samples. Overall mortality was taken in MD 1, VA 1, and VA 2 in year one. An ANOVA was performed to assess for differences between the treatment for each of the sites and either a Tukey's HSD. Sites range from north to south (left to right) on the x-axis.

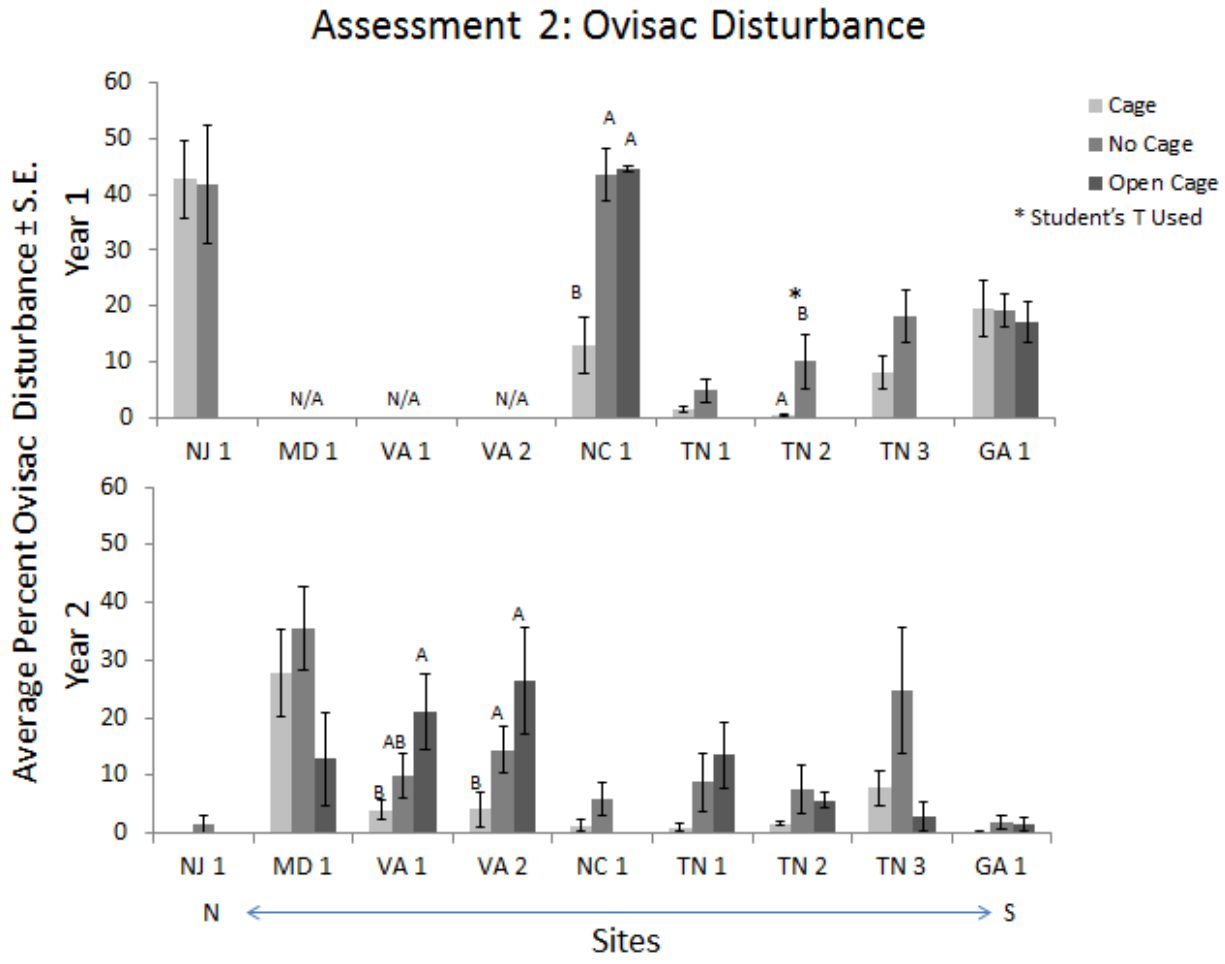

Figure 2.4. Mean percent ± S.E. HWA ovisacs disturbed in Assessment 2 in years one (A) and two (B). Open cage samples were not set up in NJ 1 in either year. Open cage samples were not collected from TN 1, TN 2, or TN 3 in year one. HWA disturbed in Assessment 2 was not recorded from MD 1, VA 1, or VA 2. An ANOVA was performed to assess for differences between the treatments for each of the sites and either a Tukey's HSD or a student's T-test was used. Sites range from north to south (left to right) on the x-axis.

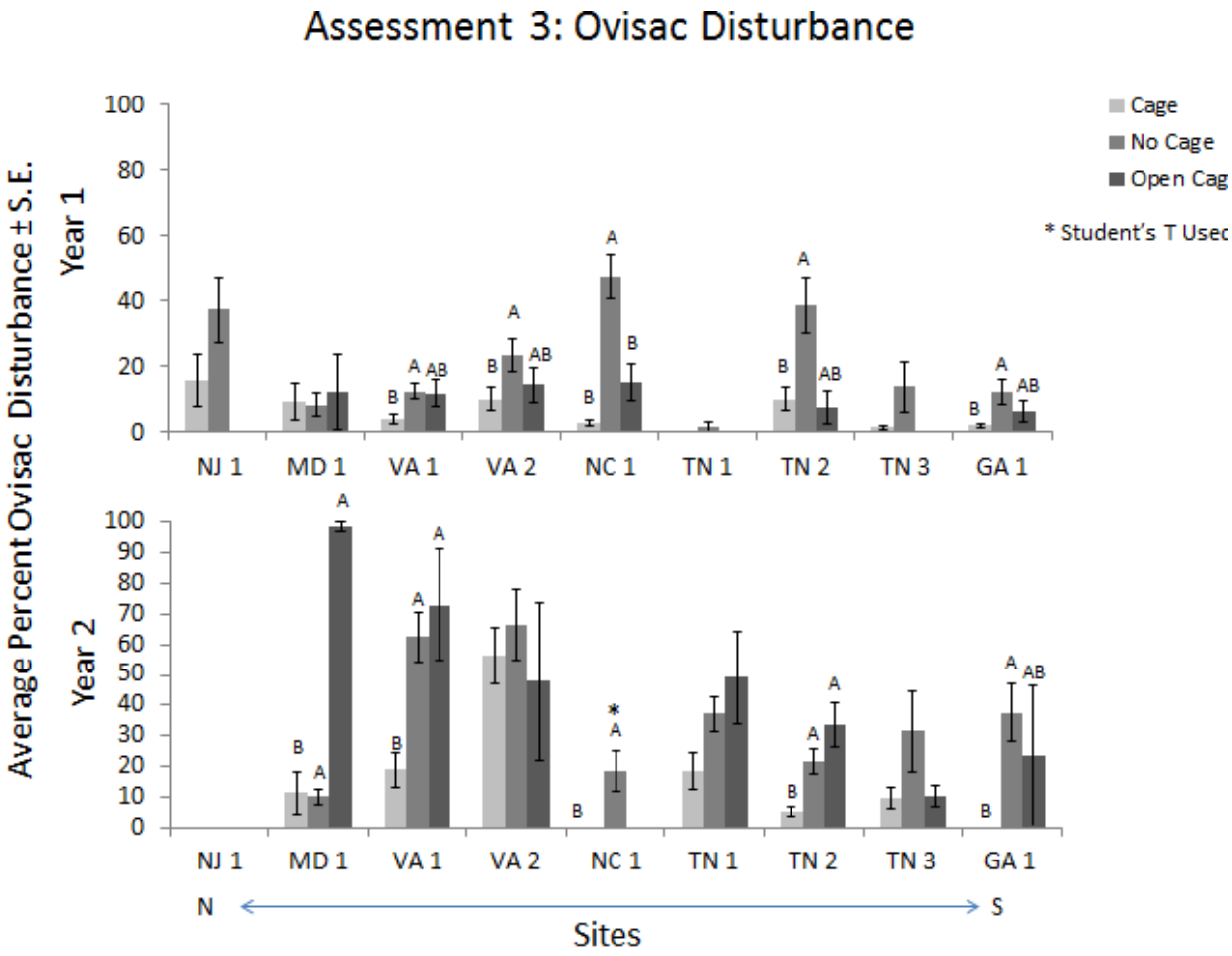

Figure 2.5. Mean ± S.E. percent HWA disturbed in Assessment 3 in years one (A) and two (B). Open cage samples were not set up in NJ 1 in either year. Open cage samples were not set up at NC 1 in year two. An ANOVA was performed to assess for differences between the treatments for each of the sites and either a Tukey's HSD or a student's T-test was used. Sites range from north to south (left to right) on the x-axis.

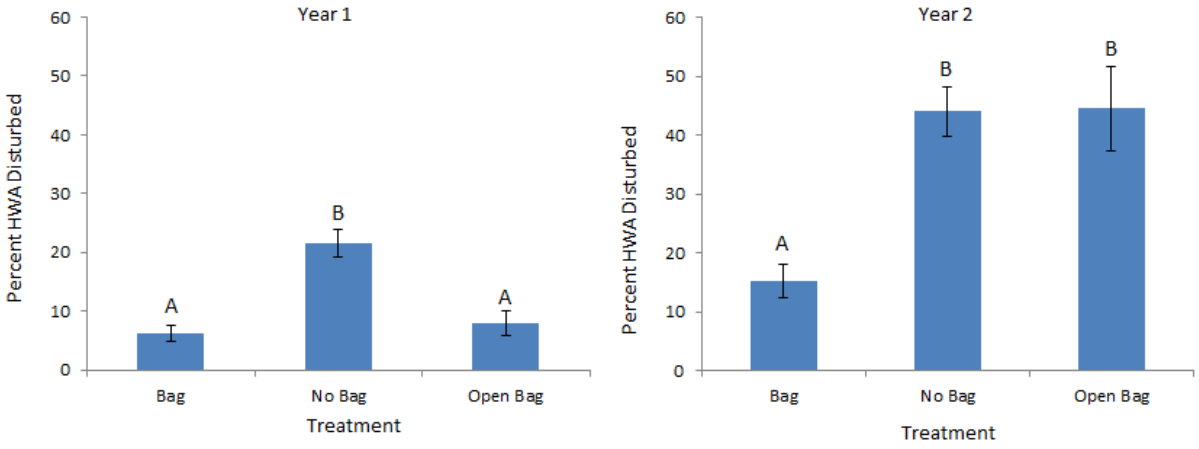

Figure 2.6. Overall mean ± S.E. of HWA ovisacs disturbed in Assessment 3 for years one and two.

Chapter 3: Recovery of *Laricobius nigrinus* at Previous Release Sites in Virginia

Abstract

Hemlock woolly adelgid is a serious pest of both eastern and Carolina hemlock. One of the most actively researched and a released predator of this pest is the adelgid specialist, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae). Inoculative releases of *L. nigrinus* began in 2003 and now number over 900 releases throughout the eastern U.S. There is strong evidence of its establishment at numerous sites, yet systematic efforts to assess establishment have not been carried out. In Virginia, this insect has been released at 21 documented sites, yet only sporadic efforts to assess the insect's establishment have been carried out. *L. nigrinus* was sampled at 14 of those sites mainly due to their accessibility. Samples for *L. nigrinus* using two standard techniques: beat sheet sampling to target primarily adults (spring and fall 2015, and spring 2016) and branch clippings to target primarily larvae (spring 2015 and spring 2016). *Laricobius nigrinus* was recovered from four of the sites. Two polar vortex events in January 2014 and February 2015 likely influenced the recovery of *Laricobius* spp. due to the near disappearance HWA, their food supply. Overall, tree health at the sampled sites declined from spring 2015 to spring 2016, however, these data are largely preliminary and more sampling should occur.

Keywords: *Laricobius nigrinus*, *Adelges tsugae*, *Tsugae canadensis*, biological control agents, field recovery, establishment

3.1 Introduction

Hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is a serious pest of both eastern hemlock (*Tsuga canadensis* L. Carriere) and Carolina hemlock (*Tsuga caroliniana* Engelmann). The adelgid feeds by inserting its stylet bundle into the hemlock at the base of the branch where the needles are attached (McClure 1987, Young et al. 1995). Damage from HWA to these trees includes needle loss, chlorosis, dieback, and tree death (McClure 1987). Since its introduction near Richmond, VA (Souto et al. 1996), HWA has spread throughout a large portion of eastern and Carolina hemlock's range (Havill et al. 2014). Infested forest trees are difficult to treat chemically at an area-wide level (Fidgen et al. 2002). For over 20 years, biological control has been considered a key part of any long-term IPM strategy for helping save the hemlocks in the east (Onken and Reardon 2011, Mausel and Salom 2013)

Explorations of western North America and Asia resulted in several species that prey on HWA. One species of note was *Laricobius nigrinus* Fender (Coleoptera: Derodontidae). *Laricobius nigrinus* was shipped into VA beginning in 1997 and studied in quarantine (Mausel and Salom 2013). This insect was determined to be host-specific to HWA (Zilahi-Balogh et al. 2002). Open releases began in the eastern U.S. in 2003 (Mausel et al. 2010). Following releases of *L. nigrinus*, mating was observed in the field on HWA-infested hemlock branches between *L. nigrinus* and *L. rubidus* LeConte (Coleoptera: Derodontidae) (Mausel et al. 2008), a native predator of pine bark adelgid, *Pineus strobi* (Hartig) (Hemiptera: Adelgidae) (Zilahi-Balogh et al. 2005). Mixed coloration of some

adult beetles led to the observation that hybrids were being produced (Davis et al. 2011, Havill et al. 2012). *L. rubidus* was also found to be able to complete its development on HWA (Zilahi-Balogh et al 2005). However, it is not known whether hybridization between *L nigrinus* and *L. rubidus* will impact the efficacy of biological control of HWA (Fischer et al. 2015). Adult *L. nigrinus* and *L. rubidus* adults can be distinguished using male genitalia, but the process is labor intensive and damage to the specimens is possible (Zilahi-Balogh et al. 2006, Havill et al. 2010). Ultimately, through the use of genetic techniques *L. nigrinus* and *L. rubidus* can be distinguished from one another.

Successful establishment of predators is a critical step in suppressing HWA (Mausel et al. 2010, Havill et al. 2011, Onken and Reardon 2011). Success using biological control is characterized by a decrease in the population of the species being preyed upon and an increase in the population of the released natural enemy (Beddington et al. 1978). With regards to HWA, a successful biological control effort would be marked by a decrease in the HWA population, an increase in the predator population, and improved health of the infested trees. In a successful biological control program, natural enemies would be present year after year, with the ultimate goal of suppressing the pest population enough to save the threatened host (Lenger and Bellows 1999).

Several studies have been conducted on the efficacy and recovery of *L. nigrinus*. Lamb et al. (2006) showed high predation rates of *L. nigrinus* in caged branch studies in the field. In small field plot studies, Mausel et al. (2008) and Mayfield et al. (2015) found that

predators were having an impact on HWA populations through exclusion cage experiments. There have been limited systematic efforts to sample for *L. nigrinus* establishment (Mausel et al. 2010, Hakeem et al. 2011). Mausel et al. (2010) sampled 22 sites from Massachusetts to Georgia in plant hardiness zones 5a to 7b. *Laricobius nigrinus* was recovered from 13 of the 22 sites and releases during fall to early spring and across all densities of HWA were successful. It was concluded that establishment probability was high in plant hardiness zones 6a to 7a and probability of establishment increased with greater numbers of beetles released.

The HWA Predator Release and Monitoring Database was created to record the release of HWA predators, monitor their recovery, and make the record public to indicate where predators have been released (Roberts et al. 2011). In Virginia 6,185 individual *L. nigrinus* have been released at 21 locations since 2003 (Roberts et al. 2011). Frequent sampling for the predator has taken place at only a small portion of these sites. As a result, there is limited knowledge on how well *L. nigrinus* is establishing in Virginia.

Objective: Survey Previous Release Sites of *Laricobius nigrinus* to Assess Degree of Establishment

Fourteen *L. nigrinus* release sites in Virginia were sampled to assess for presence of *L. nigrinus*. Tree health assessment and a general site assessment were performed. Due to lack of morphological differences between adult and larval *L. nigrinus* and the native *L. rubidus*, genetic testing was conducted to identify species. Assessments involved:

a. Assessing the presence or absence of *L. nigrinus* at each release site,

b. Determine if there is a correlation between tree health and number of *Laricobius* recovered, and

c. Determine the relative proportion of *Laricobius* spp. present at each site.

3.2 Materials and Methods

3.2.1 Site Selection

The coastal strain of *L. nigrinus* has been released at 21 documented locations around Virginia (Table 3.1) (Roberts et al. 2011). Many of these sites have been identified through the HWA predator release and recovery database maintained by Virginia Tech (Roberts et al. 2011) or through papers that documented the release. Both Nature Camp and Cherokee Flats had releases of *L. nigrinus*, however both sites were not listed in the HWA predator release and monitoring database, but they were documented on paper (McAvoy, unpublished data). The predator database records the GPS coordinates, species released, date, and site conditions at the time of release (Roberts et al. 2011). Recovery collections were added to the predator database.

3.2.2 Sampling for HWA density

HWA density was categorized for each site by randomly sampling 20 to 30 branches on the outer edges of the trees at mid to low canopy height in a manner similar to Jones et al. (2015) to assess for HWA densities. An approximation of cm of new growth was taken on those branches and HWA on new growth were approximated. Categories used for HWA densities did vary from Jones et al. (2015). Approximation of HWA/ cm of new

growth were used to determine estimates of HWA densities due to ease of classification into these four categories. The categories used were:

a. No HWA - HWA were not found.
b. Low HWA- HWA were present less than ca. 1/100 cm of new growth.
c. Moderate HWA - HWA were present between 1/100 to 10/100 of new growth
d. High HWA - HWA were present at > 10/100 cm of new growth.

At the time of sampling, climatic conditions, including temperature and cloud cover were recorded. Beat sheeting was conducted in fall 2015, and both spring and fall 2016. Beat sheet sampling consisted of going to the field site and finding branches that had HWA present on them and they were beat for a few seconds to assess if any *Laricobius* spp. fell on to the sheets. Branch clippings were collected in spring 2015 and 2016. Branches were sampled by collecting branches populated with HWA and removing branches between 0.3 – 2.5 m above the ground from the tree and then taken back to the lab. In the lab, they were placed in floral foam saturated with water and placed within 19 L buckets, and monitored for larval drop for the next 6-8 weeks. If *Laricobius* spp., either adults or larvae, were found through beat sheeting or through branch clippings, they were placed in a 1.5 ml vial of 95% ethanol with the site and date recorded on the vial for genetic analysis later.

3.2.3 Tree health

Tree health was measured at each sample site using the Forest Inventory and Analysis (FIA) method. The FIA method measures 1) crown density, 2) transparency, 3) dieback, and 4) live crown ratio (USDA Forest Service 2011). Crown density was determined by the amount of plant material that block light from penetrating through the crown. Transparency is measured by the amount of light visible through the tree foliage. Dieback was measured by the amount of mortality of branches and twigs on the tree. Live crown ratio is the portion of the tree that is alive compared to the portion that is dead though the lower dead branches are not counted in this measure. Tree health was averaged for each of the sites for spring 2015 and spring 2016. An average was compiled using all sites to get an overall picture of tree health at all *L. nigrinus* release sites across Virginia. All tree health data underwent an arcsine square root transformation to normalize the data. A paired t-test was used to assess if there were significant differences in tree health within that site from spring 2015 to spring 2016.

3.2.3 Genetic Analysis of *Laricobius* spp. recovered

The *Laricobius* larvae and adults recovered from each site underwent genetic testing to determine species and hybridization between the native *L. rubidus* and the introduced *L. nigrinus*. Genetic testing between *L. nigrinus* and *L. rubidus* was conducted using the procedure published by Davis et al. (2011). The genetic testing was conducted using Qiagen blood and tissue extraction kit and the methods set forth by Qiagen (Qiagen 2006). The mitochondrial cytochrome oxidase I (CO1) gene was then amplified using

PCR. Once PCR was completed a gel was run to confirm the amplification of the CO1 gene. Once the amplification of the CO1 gene was confirmed it was sent to Yale's DNA Analysis Facility on Science Hill for further processing. DNA was then analyzed using DNAstar Seqman Pro to determine species. Hybrids of *L. nigrinus* and *L. rubidus* were analyzed using procedures described by Havill et al. (2010).

3.4 Results

3.4.1: Spring 2015

In April 2015, 12 release sites were visited and samples were taken to assess for presence of *Laricobius* spp. Temperatures were found using a smart phone application (Weather Underground), which gave weather conditions from the closest weather station to the site. Weather data were then compared to other nearby weather stations to ensure the app's accuracy. Temperatures ranged from 6 to 18°C. Weather conditions were mostly cloudy or partially sunny during the days that were sampled at the site (Table 3.2). At most of the sites, HWA densities were low. Moderate densities were recorded from two sites. One site did not have HWA present. There were no *Laricobius* recovered from the beat sheets at any of the sites during April 2015. *Laricobius* were recovered from branch clippings from both sites where HWA populations were rated moderate. A total of 14 larvae were recovered, six larvae were recovered from Kentland Farm, Whitethorne, VA on eastern hemlock; and eight larvae from McCoy, Virginia on Carolina hemlock. The larvae underwent genetic analysis to

determine species. All six larvae from Kentland were determined to be *L. nigrinus*. The eight larvae from McCoy were a mix of 50% *L. nigrinus* and 50% *L. rubidus*.

3.4.2: Fall 2015 – Winter 2016

Thirteen sites were sampled for *Laricobius* presence during fall 2015 and winter 2016 (Table 3.3). Only beat sheeting was carried out. The time spent sampling varied from 15 minutes to 110 minutes at the larger sites. The average time spent sampling was 29 minutes. Temperature ranged from -1 to 21°C at the time of sampling. HWA levels at seven of the sites were low, moderate at two sites, and not present at four sites. Adult *Laricobius* were only recovered at one of the sites, McCoy, VA on Carolina hemlock, a moderate site. Genetic analysis was conducted on the beetles and found that one of the beetles was *L. rubidus* and two of the beetles recovered could not be identified genetically. Through initial visual identification the latter two beetles are likely *L. rubidus*.

3.4.3: Spring 2016

In spring 2016, 14 sites were sampled for *Laricobius* (Table 3.4). The weather conditions were variable, with temperatures ranging from 1 to 21 °C and time sampling ranging from 10 to 40 minutes. *Laricobius* was recovered at 6 of the 11 moderate sites and 2 of the 7 low sites, primarily through branch clippings, with the exception of Nature Camp where two adult beetles were recovered through beat sheeting and were visually identified as *L. rubidus*. Unfortunately, both beetles escaped and could not have genetic

analysis performed on them. Genetic analysis was conducted on all the larvae recovered. The *Laricobius* species recovered varied by site ranged from a mix of *L. nigrinus* and *L. rubidus* to only one species being recovered at a site (Table 3.5) and higher numbers of *Laricobius* spp. were recovered in year two compared to year one. Two sites, Kentland and James River had predominantly *L. nigrinus* recovered.

Laricobius spp. recovered from three sites, Nature Camp, Cherokee Flats, and Poverty Creek were predominantly or entirely *L. rubidus.* These three sites only had recoveries in spring 2016, making the genetic composition somewhat preliminary in nature. McCoy was a unique site in that it had both eastern and Carolina hemlock present at the site in high numbers. In year one and in year two, *L. nigrinus* was found on Carolina hemlock, but higher numbers of *L. rubidus* were found on Carolina hemlock. *L. rubidus* was not found on eastern hemlock at McCoy during the sample period. *Laricobius* spp. were recovered from several sites in both year one and year two. They were recovered from six of the 15 sites over the two-year period. Genetic make-up appeared to vary based on what site was sampled with some sites being a mix of both *L. nigrinus* and *L. rubidus*, while other sites were only *L. rubidus* (Fig. 3.2 and Table 3.5).

3.3.4: Tree health changes at release sites

Tree health was measured at each of the sites in spring 2015, fall 2015, and spring 2016. Since transparency data were not collected in spring 2016 due to human error, it was left out of the analysis, but the other measures of tree health provide an idea of what tree health was like at each of the sites. Since seasonality may have an impact on

tree health, it was only analyzed in spring of both years to establish what has occurred from year one to year two. There were observable differences in some of the measures of tree health for the FIA method between spring 2015 to spring 2016, namely crown density ($F= 5.3$, $DF= 1, 0224$, $p< 0.0111$), and percent foliage density ($F= 4.513$, $df=1, 224$, $p<0.0174$) (Fig. 3.1). It was found that both measures showed a considerable decrease over the course of a year. Crown density was around 33% in year one and dropped to 27.3% by year two. This was similar to percent foliage density which was around 34.8% and dropped to 28.8%. There were no statistical differences observed in dieback of hemlock trees ($F= 0.119$, $df= 1, 224$, $p < 0.365$) and live crown ratio ($F=0.58$, $df= 1, 224$, $p < 0.223$). Dieback seen in the trees stayed relatively the same from year one to year two from 38 to 37%. Respectively, live crown decreased from year one to year two from 48.2 percent to 45.7%. Overall, at *L. nigrinus* release sites around Virginia, eastern hemlock tree health appears to slight decline in a one-yr period. More sampling is needed to see if this trend continues

3.4 Discussion

It is likely that the first year's results were impacted by the polar vortex events that occurred in January 2014 and February 2015. The polar vortex brought unusually cold temperatures to the region that impacted both *L. nigrinus* and HWA populations at many of these field sites. A study conducted by Elkington et al. (2016) found that adelgids in colder locations have lower supercooling points than those in warmer locations. Elkington et al. (2016) experienced a sudden cold event that brought colder

temperatures to some of their locations; at these locations they observed higher mortality. Since the *L. nigrinus* released in VA are from the coastal strain, they are less cold hardy than the interior strain (Mausel et al. 2011b). The impact of the polar vortex and the coastal strain of *L. nigrinus* could explain the low amounts of *Laricobius* spp. recovered in year one compared to year two and why only *L. rubidus* were recovered at some of the sites.

Even through considerable HWA mortality has been observed throughout the region (McAvoy et al. 2016), densities of HWA at many of these sites remained the same from spring 2015 to spring 2016. HWA densities did increase from low to moderate at Poverty Creek. Two sites, Big Stoney and Pinnacle HWA densities decreased from low to no HWA present. Big Stoney is a relatively small and isolated site compared to other sampled due to it being located next to a road on one side and a creek on the other side. Big Stoney is at plant hardiness zone 5b (Table 3.1) and with colder than average temperatures being experienced in winters 2014 and 2015 and the small size of the site could explain the decrease in HWA densities at this site.

A mix of *L. nigrinus* and *L. rubidus* was found at both at Poverty Creek, Kentland, and McCoy. James River appeared to have only *L. nigrinus.* Other sites such as Nature Camp and Cherokee Flats had only *L. rubidus*. Many of the sites that show a mixture of *L. nigrinus* and *L. rubidus*; however, they were predominantly populated with *L. nigrinus*, with the exception of McCoy, which is likely due to the high amounts of Carolina hemlock present at that site. The relative proportions of *L. nigrinus* and *L. rubidus* at the Virginia sites is similar to what Mayfield et al. (2015) found with regards to species composition at the sites that were surveyed by Mayfield et al. (2015). Though more

surveys of the sites should be conducted to better understand the relative proportion of each species.

Based on timing of data collection on spring 2015 and 2016 data were able to be compared to one another. These data are largely preliminary regarding tree health, due to measurements only being taken over a year. However, it does appear that tree health decreased at many of these sites from 2015 to 2016. This does not mean that biological control is insufficient but rather not enough time has passed to see what is occurring with regards to tree health. Some measures showed a significant difference including crown density and percent foliage density.

One such explanation as to why tree health is declining is the HWA populations before the polar vortex experienced in winters 2014 and 2015. It is unknown if the densities of HWA were high before the polar vortex. If it is assumed that they were, this could explain why hemlock tree health is still declining. This may take several years to see if the polar vortex, which knocked back HWA populations, will lead to improved health of the trees. There are no data at these sites before the polar vortex hit regarding tree health, so I can only speculate as to what is occurring at these sites. It should also be noted that we did not see significant differences in crown dieback and live crown ratio. This shows that the trees have close to the same amount of the live crown and dieback as the year before.

This is only showing what is occurring at release sites in Virginia over the course of one year. This study should be conducted several years to better understand what is occurring with tree health and adelgid populations at these sites. The polar vortex,

which brought unprecedented cold temperatures to the region, offered a unique opportunity to see how cold temperatures impact HWA and *L. nigrinus* as well as assessing how tree health may be impacted due to this event.

Though the polar vortex had a huge impact on both HWA and likely *L. nigrinus* populations throughout the eastern United States *L. nigrinus* was still recovered at four of the sites sampled. Since *L. nigrinus* was recovered at these four sites (Kentland, Poverty Creek, James River, and McCoy), it is established at these sites because *L. nigrinus* was still successfully recovered after at least one field season of the release. Even though *L. nigrinus* was not recovered at many of the sites it should not be concluded that it is not present at these sites. *L. nigrinus* populations at these sites could be in low numbers making it difficult to recover. Continued sampling after the polar vortex will help determine if *L. nigrinus* is present at these sites. This information coupled with monitoring HWA densities and tree health will help in identifying sites that need augmented releases.

There were many issues experienced while performing the genetic analyses on the *Laricobius* species recovered at field sites. This ultimately led to some of the specimens recovered not being identified to species. Possible reasons as to why this could have occurred include the warm temperature experienced in the lab space (which may have resulted in degradation of the DNA) or possibly an equipment malfunction. Though not all specimens were able to be identified to species, we had enough data to begin to

understand the genetic makeup of *Laricobius* spp. at these sites. Continued monitoring should be conducted at these sites to assess if *L. nigrinus* is present, and if they are, assess whether species composition varies from year to year.

Table 3.1. *Laricobius nigrinus* release sites in Virginia: (HWA Predator Release and Monitoring Database accessed March 2015 and January 2017). Sites that were sampled are in bold.

Site	GPS	Initial Release Date	# Beetles Released	Plant Hardiness Zone
James River SP	**37.641 N, -78.800 W**	**17-Nov-05**	**300**	**6b**
Kentland Farm	**37.209 N, -80.590 W**	**18-Nov-03**	**258**	**6a**
North Fork	**37.443 N, -80.515 W**	**8-Dec-03**	**600**	**6a**
Big Stony	**37.416 N, -80.507 W**	**8-Dec-03**	**300**	**5b**
Mountain Lake	37.367N, -80.537 W	4-Jun-09	500	6a
		23-Dec-09	42	
Mtn. Lake Drainage Pond and Blueberry Ridge	37.369 N, -80.537 W	18-Nov-10	1800	6a
Poverty Creek	**37.256 N, -80.534 W**	**7-Mar-10**	**150**	**6a**
		22-Nov-10	**1000**	
		15-Oct-14	**239**	
		4-Nov-14	**300**	
McCoy	**37.215 N, -80.602 W**	**3-Mar-13**	**150**	**6a**
		6-Nov-14	**267**	
Gullion Fork	**36.995 N, -81.273 W**	**27-Mar-13**	**225**	**6a**
Lick	37.011 N, -81.427 W	4-Nov-04	150	6b
Dickey Creek	**36.737 N, -81.432 W**	**8-Feb-05**	**75**	**6a**
Hurricane	**36.722 N, -81.488 W**	**30-Mar-03**	**300**	**6a**
Channels State Forest	**36.829 N, -81.963 W**	**1-Nov-10**	**1000**	**6b**
Highland	**36.692 N, -81.517 W**	**4-Nov-04**	**1200**	**6a**
Pinnacle Natural Area	**36.962 N, -82.053 W**	**28-Nov-06**	**310**	**6b**
Burns Creek	36.925 N, -82.537 W	19-Feb-08	300	6b
Devils Fork	36.820 N, -82.630 W	1-Jan-08	300	6b
Big Cherry Reservoir	36.828 N, -82.692 W	25-Nov-08	500	6b
Big Cherry Reservoir	36.832 N, -82.700 W	9-Dec-08	500	6b
Nature Camp	**37.876 N, -79.214 W**	**06-Jan-12**	**400**	**7a**
Cherokee Flats	**37.414 N. -80.583 W**	**20-Nov-14**	**400**	**5b**

Table 3.2. Number of *Laricobius* larvae recovered: from branch clippings and relative hemlock woolly adelgid density at *L. nigrinus* release sites from sampling in spring 2015.

Site	Date	*L. nigrinus* Recovered	*L. rubidus* Recovered	HWA density level	Time collecting	Temp. (°C)	Weather conditions
Kentland	4/17/2015	6	0	Moderate	20 minutes	10	partially cloudy
McCoy*	4/17/2015	4	4	Moderate	30 minutes	10	partially cloudy
Poverty Creek	4/17/2015	0	0	Low	45 minutes	13	partially cloudy
Big Stony	4/17/2015	0	0	Low	15 minutes	13	partially cloudy
Cherokee Flats	4/17/2015	0	0	Low	45 minutes	16	partially cloudy
North Fork	4/17/2015	0	0	Low	30 minutes	18	partially cloudy
Highland	4/21/2015	0	0	Low	45 minutes	6	partially sunny
Hurricane	4/21/2015	0	0	No HWA	20 minutes	10	partially sunny
Dickey Creek	4/21/2015	0	0	Low	20 minutes	10	partially sunny
Channels State Forest	4/21/2015	0	0	Low	30 minutes	13	partially sunny
Pinnacle Natural Preserve	4/21/2015	0	0	Low	45 minutes	13	partially sunny

*Both eastern and Carolina hemlock sampled. *Laricobius* larval recovery only on Carolina hemlock

Table 3.3. Number of *Laricobius* adults recovered and relative hemlock woolly adelgid density at *L. nigrinus* release sites from sampling conducted in fall 2015/winter 2016.

Site	Date	*Laricobius* Recovered	HWA density level	Time collecting	Temp. (°C)	Weather conditions
Kentland	11/21/2015	0	Low	30 minutes	13	clear
McCoy	1/2/2016	3	Moderate	30 minutes	-1	clear
Poverty Creek	1/2/2016	0	Low	20 minutes	-1	clear
Big Stony	1/2/2016	0	Low	20 minutes	-1	clear
Cherokee Flats	1/2/2016	0	Low	30 minutes	4	clear
North Fork	1/2/2016	0	None	20 minutes	4	clear
Highland	12/16/2015	0	Low	1 hour 50 minutes	10	clear
Hurricane	12/16/2015	0	None	20 minutes	7	clear
Dickey Creek	12/16/2015	0	None	15 minutes	10	clear
Channels State Forest	12/16/2015	0	None	15 minutes	13	clear
Pinnacle Natural Preserve	12/16/2015	0	Low	25 minutes	21	clear
Gullion Fork	12/16/2015	0	Moderate	25 minutes	2	clear

Table 3.4. Number of *Laricobius* adults and larvae recovered from branch clippings and relative hemlock woolly adelgid density at *L. nigrinus* release sites from sampling conducted in spring 2016.

Site	Date	No. of *Laricobius* Recovered	HWA density level	Time collecting	Temperature (°C) approximation	Weather conditions
Kentland	2/26	0	Moderate	branch collected	1°c	Snow
Kentland	3/4	2	Moderate	branch collected	5°c	Snow
Kentland- Sample 3	4/5	22	Moderate	branch collected	N/A	N/A
McCoy eastern	3/4	0	Moderate	branch collected	5°c	Snow
McCoy Carolina	3/4	13	Moderate	branch collected	5°c	Snow
McCoy eastern	3/26	5	Moderate	20 minutes	7°c	Cloudy
McCoy Carolina	3/26	0	Moderate	20 minutes	7°c	Cloudy
Poverty Creek	3/4	0	Moderate	branch collected	5°c	Cloudy
Poverty Creek	3/26	3	Moderate	20 minutes	7°c	Cloudy
Big Stony	3/26	0	No HWA	15 minutes	7°c	Cloudy
Cherokee Flats	3/26	4	Low HWA	25 minutes	7°c	Cloudy
North Fork	3/26	0	Low HWA	15 minutes	7°c	Cloudy
Highland	4/23	0	Low HWA	40 minutes	10°c	Cloudy
Hurricane	4/23	0	no HWA	20 minutes	10°c	Cloudy
Dickey Creek	4/23	0	Low HWA	10 minutes	10°c	Cloudy
Channels SF	4/23	0	Low HWA	25 minutes	13°c	Cloudy
Pinnacle NP	4/23	0	No HWA	20 minutes	13°c	Cloudy
Gullion Fork	4/23	0	Low HWA	20 minutes	13°c	Cloudy
Nature Camp	3/8	3	Low HWA	40 minutes	21°c	Clear
James River	3/8	0	Moderate	branch collected	21°c	Clear
James River-Sample 3	4/8	4	Moderate	branch collected	N/A	N/A

Table 3.5. Genetic make-up of *Laricobius* spp. recovered from field sites in spring 2015, fall 2015, and spring 2016.

		Species Recovered					
		L. nigrinus		*L. rubidus*		No ID	
Site	Date	Adult	Larvae	Adult	Larvae	Adult	Larvae
Kentland	Spring 2015	0	6	0	0	0	0
Kentland	Spring 2016	0	1	0	2	0	3
James River	Spring 2016	0	2	0	0	0	2
McCoy (Carolina)	Spring 2015	0	4	0	4	0	0
McCoy (Carolina)	Fall 2015	0	0	1	0	2	0
McCoy (Carolina)	Spring 2016	0	1	0	10	0	2
McCoy (eastern)	Spring 2016	0	3	0	0	0	2
Nature Camp	Spring 2016	0	0	2	1	0	0
Cherokee Flats	Spring 2016	0	0	0	4	0	0
Poverty Creek	Spring 2016	0	0	0	1	0	2

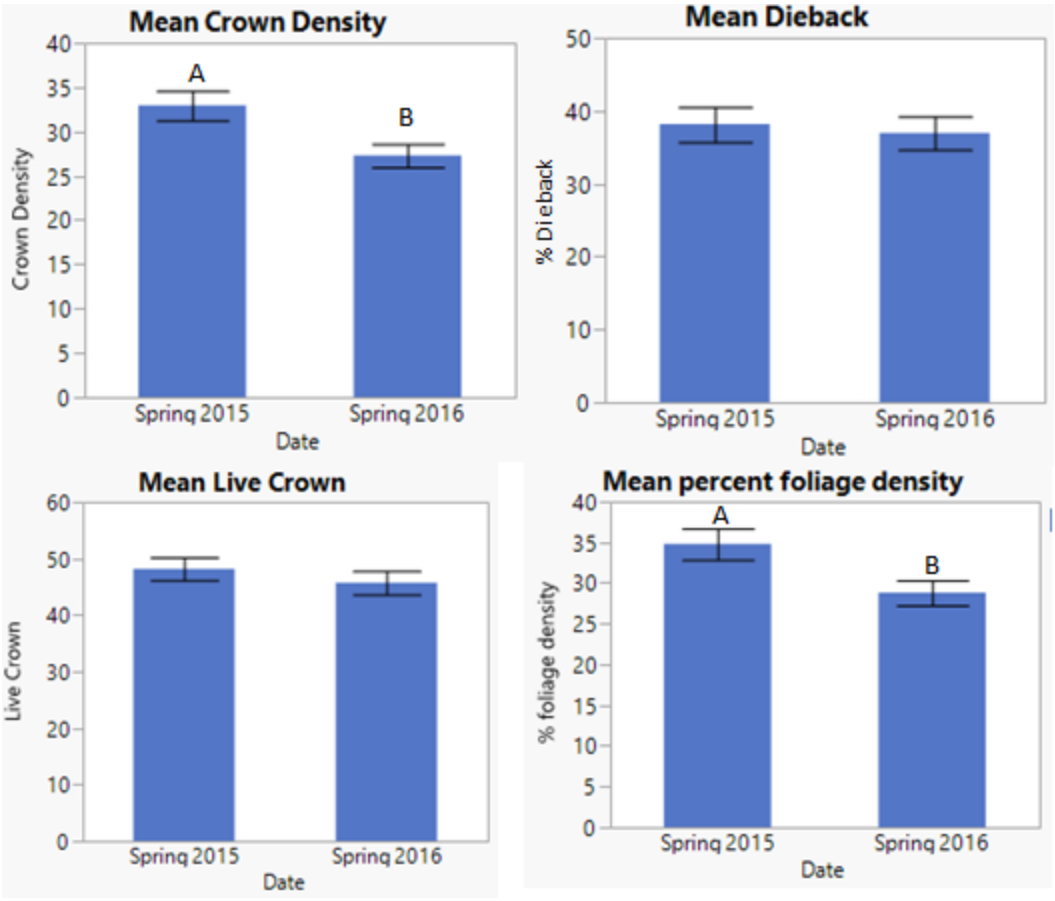

Figure 3.1. Changes in tree health between spring 2015 and spring 2016 at *L. nigrinus* release sites in Virginia. Using four measures including crown density, dieback, percent live crown, and percent foliage density.

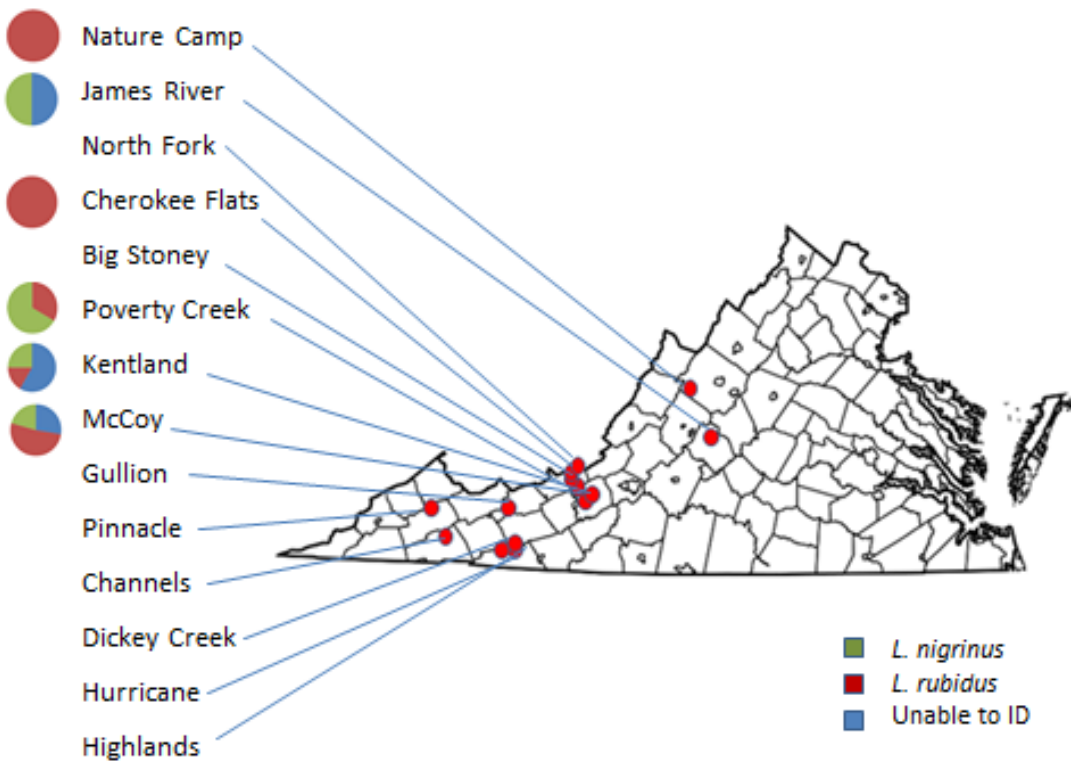

Figure 3.2: Virginia *L. nigrinus* release sites visited between spring 2015 and spring 2016 and genetic make-up of *Laricobius* spp. at each of the sites. Sites without circles had no collections.

Chapter 4

Summary

Eastern and Carolina hemlock are important tree species in the eastern United States that provide services that no other tree species can. Hemlock woolly adelgid, *A. tsugae*, has impacted hemlocks through a substantial portion of their range in eastern United States. Biological control of HWA is our best hope for long term sustainable management of HWA in forests. *Laricobius nigrinus,* from the Pacific Northwest, was released in 2003 in the eastern United States. It has proven to be an effective predator in laboratory settings though less was known about how it predates in the field. Through sampling at release sites, we began to assess if *L. nigrinus* are still present at release sites and their impacts on HWA populations.

Chapter 2: *L. nigrinus* Impact Assessment

Hemlock woolly adelgid is a serious pest of both eastern and Carolina hemlock. *L. nigrinus*, a predator of hemlock woolly adelgid, was released beginning in 2003 and continues to be released today. Work conducted on the efficacy of *L. nigrinus* in the laboratory setting and Mausel et al. (2010) and Mayfield et al. (2014) and found *L. nigrinus* in plant hardiness zones 6a-7a. However, less was known about the efficacy of *L. nigrinus* in the field until now. We have observed and recovered *L. nigrinus* from many of the field sites assessed in both year one and year two, as well as observed differences in percent HWA disturbed in both year one and year two in assessment three and in year two in assessment two. High rates of disturbance were found in assessment two and three. This coupled with the lack of other HWA predators

recovered strengthens that *Laricobius* spp. are primarily responsible for preying on HWA and are reducing HWA populations in assessment three with disturbance in uncaged samples of HWA ranging from 1.5 to 47.3% in year one and 0 to 66 % in year 2. This shows promise for *L. nigrinus* as a biological control agent at many of the sites sampled. There are some limitations for the biological control program regarding *L. nigrinus* to control HWA populations namely a lack of predation by HWA on the progrediens generation of HWA which may lead to a repopulation of HWA at these sites when *L. nigrinus* is absent. Even with this limitation, *L. nigrinus* appears to contribute to the overall mortality of the sistens generation where the predator is established.

Chapter 3: Recovery of *L. nigrinus* at Release Sites in Virginia

Weather can have an enormous impact on insect populations whether it is in the summer or the winter. The polar vortex experienced in January 2014 and 2015 brought extreme cold temperatures to the region and impacted HWA and likely *L. nigrinus* populations. The number of *Laricobius* spp. specimens collected in year two was much higher than year one and *Laricobius* spp. were collected at more sites in year two than year one. This is likely due to the polar vortex experienced in year one and with more seasonal temperatures seen in year two. *Laricobius* spp. were collected at many of the sites. Most of the sites had a mix of *L. nigrinus* and *L. rubidus*; however, at two sites there were only *L. rubidus* recovered. This could be due to *L. nigrinus* being impacted by extreme cold temperatures; whereas, *L. rubidus* may not have been as affected. Tree health overall appears to be decreasing from 2015 to 2016 at *L. nigrinus* release sites in crown density and percent foliage density. Data collected for tree health was

only analyzed for spring 2015 and 2016. However, to better understand what is occurring at these sites more sampling should occur to assess tree health and *Laricobius* spp. make-up at the sites.

Future work

Progress has been made in assessing the impact that *L. nigrinus* is having on HWA populations though there still is much to understand about what is occurring at each of the sites. Future directions for the impact assessment include assessing progrediens population density, quantifying the predator-prey relationship and comparing that in the eastern United States to the Pacific Northwest, where HWA and *L. nigrinus* are native, and assessing long-term effects on tree health at release sites. Continued efforts to sample at release sites of *L. nigrinus* around Virginia may provide more information on changes in tree health, presence of *L.nigrinus*, and changes in HWA densities. Information regarding establishment of *L. nigrinus* in Virginia may provide information on whether or not more releases of *L. nigrinus* in Virginia are necessary for controlling HWA populations at these sites.

Laricobius larvae collected in the field still need to undergo genetic testing to assess if hybrids are present at the site. This will allow us to better understand the genetic make-up of *Laricobius* species present at the site.

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