Evaluation of the establishment of predatory beetle, *Laricobius nigrinus* (Coleoptera: Derodontidae) in Virginia, and assessment of its impact on hemlock woolly adelgid (Hemiptera: Adelgidae) at release sites in the eastern U.S.

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Evaluation of the establishment of predatory beetle, *Laricobius nigrinus* (Coleoptera: Derodontidae) in Virginia, and assessment of its impact on hemlock woolly adelgid (Hemiptera: Adelgidae) at release sites in the eastern U.S.

Carrie Sue Jubb

Abstract (Academic)

The predatory beetle, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), has been released in the eastern U.S. since 2003 for the management of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). The establishment of *L. nigrinus* at release sites in Virginia was assessed in 2017 and 2018. Sampling was performed in both years to determine presence or absence *L. nigrinus*. Stand level HWA densities, tree health, predator-prey ratios, and *Laricobius* spp. identifications were also assessed at each site. *Laricobius nigrinus* established at 82% of sites and was the primary species recovered. HWA densities appeared to decline, and tree health appeared to improve in year two. Predator-prey ratios were lower than those indicated in the native range, however much is left to be understood about the dynamics of this system in its introduced range. A separate two-year study to assess the impact of *L. nigrinus* on HWA was initiated in 2014 (Phase One) at nine sites in the eastern U.S. Significant predation of HWA sistens ovisacs was demonstrated during this period, therefore, it was continued from 2016-2018 (Phase Two) to provide longer-term evaluations. Predator exclusion cages were used to monitor predator and prey populations. In Phase Two of the study, mean ovisac disturbance rates on no-cage branches were significantly greater than caged branches and were as high as 80%,

suggesting that *L. nigrinus* can have a significant impact on the sistens generation. Microsatellite analysis of *Laricobius* larvae indicated that *L. nigrinus* was the primary species recovered at study sites.

Abstract (General Public)

Hemlock woolly adelgid (HWA), an invasive insect native to Asia and western North America, is a significant threat to two native hemlock species in the eastern U.S. Since 2003, a predatory beetle, *Laricobius nigrinus*, has been released for management of HWA. In biological control programs such as this one, it is important to evaluate the ability of predators to establish and impact prey in areas where they are introduced, as this helps guide future management decisions. As such, a study was conducted to examine the ability of *L. nigrinus* to establish after being released at 26 locations in Virginia. In those investigations we found that *L. nigrinus* established at 82% of the sites. Although promising, longer-term studies are needed to understand if the predation of HWA by *L. nigrinus* helps improve the health of our native hemlocks. A separate two-phase study was carried out from 2014-2016 and 2016-2018 evaluating the impact of *L. nigrinus* on HWA at nine release sites in the eastern U.S. In Phase Two reported here, cages were used to exclude *L. nigrinus* on some HWA infested branches while on others, no cage was applied to allow free access to HWA. Comparisons between branches were made to determine the level of predation by *L. nigrinus*. These studies showed a significant impact by *L. nigrinus* on the winter generation of HWA with as many as 80% of those insects being attacked on study branches which indicates that this species has potential as an effective predator.

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I would like to thank a multitude of people for supporting this effort – it truly did take a village. I am grateful to the USDA Forest Service for their support of this research through cooperative agreement 14-CA-11420004-028. I thank S.M. Salom, my major advisor, who has offered significant guidance and input during this process and in the development of my skills as a researcher. I also thank my committee members, A.B. Mayfield, T. Kuhar, and D. Pfeiffer for sharing their expertise and for offering insightful review of this project. Several collaborators; J. Elkinton (University of Massachusetts), J. Grant, and G. Wiggins (University of Tennessee) and A. Mayfield (USDA Forest Service), as well as additional personnel including B. Mudder, R. Crandall, M. Mayer, K. Britt (and an army of others) helped expand the geographical scope, and thus the robustness, of these investigations and provided valuable data. Significant assistance with molecular protocols was contributed by N. Havill and K. Stanley. I am grateful to L. Chamberlin, K. Mooneyham, and D. Gaston (Virginia Department of Forestry), S. Passwaters (James River State Park), and B. Thompson (Maryland DNR Forest Service) for site assistance. C. Brewster and A. Mayfield offered helpful guidance with statistical analyses. A. Heminger, my project predecessor, shared data and critical guidance. Excellent assistance with field and laboratory components of this project were received from T. McAvoy, A. Dechaine, R. Mays, A. Toland, and J. Wahls. So much of this work could not have been completed without the support I received from the insect mass rearing laboratory staff, "Team Lari"; N. Morris, K. Jeffries, and K. Stanley. I thank the graduate students of the VT Department of Entomology and also the Forest Entomology lab; R. Brooks, M. Ragozzino, A. Toland, J. Foley, and H. Wantuch, for their camaraderie and advice – you are all inspiring. Thank you also to K. Shelor for the critical support with student administrative details. I'm indebted to H. Scoggins who first encouraged me to work towards this goal as well as other friends that bolstered me along the way. I thank my family, especially my parents who always encouraged my curiosity of the natural world. Finally, I have eternal gratitude for my husband, Christopher Elledge, who kept the home fires burning during this process. His unending patience, support, motivation, and Microsoft® Office Excel and Word wizardry saw me to the end of this project (T.S. Unite!).

Dedication

I dedicate this thesis to my entomologist father, “Dr. Bug”. After all these years, who would have thought that I would follow in your footsteps?

Attributions

Several colleagues aided in the design, implementation, and data collection in Chapter 3, and genetic analysis in Chapters 2 and 3. These same colleagues were also involved in study implementation, data collection, and genetic analysis for Appendix 1.

Albert E. Mayfield, PhD (Environmental and Forest Biology, SUNY College of Environmental Science and Forestry) is currently a Research Entomologist with the USDA Forest Service Southern Research Station. He assisted with the study design for Chapter 3 and was involved with setup and data collection at the North Carolina and Georgia sites for Chapter 3 and Appendix 1.

Bryan Mudder, MS (Forest Resources, Clemson University) is a Biological Science Technician at the USDA Forest Service Southern Research Station. He was involved with study setup in Chapter 3 and Appendix 1 and collected data at the North Carolina and Georgia sites.

Jerome Grant, PhD (Department of Entomology, Clemson University) is a Professor of Entomology and Plant Pathology at the University of Tennessee. He assisted with the study design as well as setup and data collection at the three Tennessee sites in Chapter 3 and Appendix 1.

Gregory Wiggins, PhD (Department of Entomology and Plant Pathology, University of Tennessee) is the NIMBioS Education and Outreach Coordinator at the University of Tennessee. He was involved with study setup in Chapter 3 and Appendix 1 and collected data at the three Tennessee sites.

Joseph Elkinton, PhD (Department of Entomology, University of California, Berkley) is a Professor in the Department of Environmental Conservation at the University of Massachusetts, Amherst. He assisted with Chapter 3 study design and directed research related activities at the New Jersey site.

Ryan Crandall, BA (Department of Environmental and Forest Biology, State University of New York) is a currently a M.S. graduate research assistant in the Department of Environmental Conservation at the University of Massachusetts, Amherst. He was involved with study setup in Chapter 3 and Appendix 1 and collected data at the New Jersey site.

Kari Stanley, AS (School of Science, Technology, Engineering & Mathematics, Virginia Western Community College,) is currently an undergraduate student in the Department of Biology and full-time staff in the Department of Entomology at Virginia Tech. She performed the microsatellite molecular protocols on *Laricobius* spp. samples recovered in Chapters 2 and 3.

Nathan Havill, PhD (Department of Ecology & Evolutionary Biology, Yale University) is currently a Research Entomologist at the USDA Forest Service Northeast Center for Forest Health Research. He provided significant guidance with microsatellite protocols and performed the final analysis for determining species assignment of *Laricobius* spp. recoveries using Structure and New Hybrids software program in Chapters 2 and 3.

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Chapter 1: Literature Review

1.1 Hemlock woolly adelgid biology and damage to native hemlock

Adelges tsugae Annand (Hemiptera: Adelgidae), (Hemlock woolly adelgid, HWA), is an exotic, invasive pest that is a significant threat to the health and longevity of both eastern hemlock (*Tsuga canadensis* (L.) Carriere), and Carolina hemlock (*Tsuga caroliniana* Englelm) in forests in the eastern United States. The presence of HWA was first recorded in Richmond, VA in 1951 (Havill et al. 2006), and may have originally been introduced via ornamental plantings (Souto et al. 1995). The current spread of HWA covers more than half the range of *T. canadensis* from Canada south to Georgia with recent detections noted in Michigan and in Nova Scotia, Canada (Fig. 1.1).

HWA is native to eastern Asia and western North America and is found in association with all nine-known species of *Tsuga* spp. (Havill et al. 2016), though it is only a serious threat to hemlocks in the eastern U.S. (Havill et al. 2006). In its native range in western North America and Asia, HWA is not considered a pest due to population regulation by natural predators and host resistance (McClure 1989, Cheah and McClure 1995, McClure and Cheah 1999). In eastern forests however, there are no natural enemies that can effectively manage HWA populations (Wallace and Hain 2000). Havill et al. (2006) used mitochondrial DNA was used to determine the source of the infestation of HWA in the eastern U.S. HWA in these locations have a single haplotype, which is also shared with adelgids in Japan found in association with southern Japanese hemlock (*Tsuga sieboldii* Carr.) at low elevations.

The distribution of *T. canadensis* stretches from the Canadian Maritime Provinces over to the Great Lakes and south along the Appalachian Mountain and Plateau regions to Alabama and Georgia (Fig 1.1). The range of *T. caroliniana* is greatly reduced and found primarily in the Blue Ridge Province of the southern Appalachian Mountains from northern Georgia to southwest Virginia, however, some pocket populations are found in upper Piedmont areas in these regions (Jetton et al. 2008). Hemlocks are a shade-tolerant, evergreen, foundation species that add diversity to predominantly deciduous forests in the eastern U.S. (Evans et al. 1995). They thrive in moist environments such as north and east facing slopes or gorges with high humidity and low temperatures (Peattie 1950). As foundation species, hemlocks play a large role in defining forest structure and ecosystem dynamics. Their tolerance for shade allows them to form dense canopies which provide habitat and food for wildlife, particularly avian species including several warblers. These species are sensitive to the loss of these canopies and are often displaced as a result of hemlock mortality (Tingley et al. 2002). Hemlocks also help regulate stream temperatures critical for aquatic species such brook trout (Evans et al. 1995) and macroinvertebrates (Snyder et al. 2002). Loss of these trees in eastern forests alters species composition and microclimates (Lovett et al. 2006). When hemlock mortality occurs, trees are replaced by other species, such as *Acer*, *Betula, Liriodendron, or Rhododendron* which do not serve the same ecosystem functions (Brantley et al. 2013). This can cause an increase in soil temperatures, a change in soil chemistry, and a decrease in seed bank density in these riparian areas due to the loss of the regulating effects of hemlock foliage (Orwig and Foster 1998, Jenkins et al. 1999, Brantley et al. 2013, Dharmadi et al. 2019).

HWA is a small insect (0.4-1.4 mm long), which produces a distinctive cotton-like flocculence. Although mostly sessile, it is readily dispersed by wind, birds, mammals or human activity (McClure 1990). In the eastern U.S., HWA has a complex life cycle which consists of two parthenogenic generations per year (McClure 1989). The first instar nymphs (crawlers) of the sistens generation hatch in late-spring to early-summer and immediately settle at the base of needle fascicles on new growth and begin to feed. Shortly thereafter, they enter a period of summer aestivation. They resume development in the fall and progress through four nymphal instars until reaching maturity in January of the following year. They begin to produce small amber colored eggs (0.36 mm long by 0.23 mm wide), which hatch in late-March to April. Most of these eggs become the sessile progrediens generation which settle on previous year's growth, while a small portion may occasionally develop into winged sexuparae. These sexuparae fly from hemlock in search of spruce, their alternate host species, however, the preferred spruce is not found in the eastern U.S. (McClure 1991). In Japan, this morphological form of HWA is found on tigertail spruce, *Picea torano* (K. Koch) Koehne, where they form galls and give rise to an oviparous sexual generation (Havill et al. 2011). With a lack of an appropriate spruce, the sexuparae generation in the eastern

U.S. does not reproduce and causes a reduction in the HWA population. Even without this generation however, their asexual reproductive biology allows them to quickly build their populations (McClure 1989). HWA feed on hemlocks by inserting their stylet bundles into xylem ray parenchyma cells where they then uptake the plants' nutrients (Young et al. 1995). This nutrient uptake by HWA combined with their high-density colonization of twigs can eventually cause shoot growth reduction and branch dieback. Trees can then become susceptible to secondary insect attacks or disease. Tree mortality is highly variable within stands and among sites in the eastern U.S. but has been reported to occur in as little as 4-10 years after initial infestation (McClure et al. 2001).

1.2 Management of hemlock woolly adelgid:

Current management of HWA is a multi-faceted approach involving chemical and biological control, resistance breeding and genetic conservation (Potter et al. 2012, Vose et al. 2013). Chemical controls such as neonicotinoids are effective and have been used in forests at the stand level, however there are concerns regarding potential negative effects to surrounding ecosystems (Cowles 2009) and wide-spread application can be costly (Vose et al. 2013). As such, classical biological control, with its long-term tactics of management through the use natural enemies, has been a significant focus of HWA management since the late-1990's. Studies were conducted in the eastern U.S. to determine if natural enemies of HWA existed and if they were able to adequately control HWA populations. Surveys in New England found *Scymnus suturalis* Thunberg, a coccinellid native to Europe, and a native derodontid, *Laricobius rubidus* LeConte, feeding on HWA (Montgomery and Lyon 1995). Later, Wallace and Hain (2000) reported the presence of the coccinellid, *Harmonia axyridis* Pallas, predatory flies (Diptera: Cecidomyiidae), and lacewings (Neuroptera: Chrysopidae) on HWA in survey sites in Virginia and North Carolina. Cage exclusion experiments in this study determined that predators were not abundant enough to effectively control populations of HWA. Because of the inability of native natural enemies to reduce populations of HWA below damaging levels, foreign exploration for potential biological control agents began in 1992 (Cheah 2011). These surveys focused on the collection of natural enemies of HWA in areas where the adelgid was native, such as; Japan, China, and the western North America. Several predators including, *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) from Japan, *Scymnus* spp. from China and western North America, *Laricobius nigrinus* Fender from western North America, *Laricobius osakensis* Montgomery and Shiyake from Japan and chamaemyiid flies from western North America have been collected, evaluated in quarantine and released. The status and details of those efforts have been summarized by Onken and Reardon (2011).

1.3 *Laricobius nigrinus* biology

Laricobius are one of four genera included in the family Derodontidae. These species are distributed in cooler, northern continents and are known predators of adelgids (Lawrence 1989). There are three species of *Laricobius* that are of primary interest to the biological control of HWA in the eastern United States; the native *Laricobius rubidus* and two species imported for classical biological control; *Laricobius osakensis* from

Japan and *Laricobius nigrinus* from western North America. During a survey of hemlock in the western U.S., Kohler et al. (2008) found that the most abundant predator associated with HWA was a small beetle, *Laricobius nigrinus*. As a result of its abundance *L. nigrinus* was imported to the Virginia Tech Beneficial Insects Quarantine Laboratory beginning in 1997 for evaluation as a potential biological control agent (Zilahi-Balogh et al. 2003b).

Laricobius nigrinus is native to British Columbia, Washington, Oregon and Northern Idaho (Fender 1945, Hatch 1962, Lawrence 1989). Adults are small (2.31-2.94 mm long) and black in color with glossy bodies covered in fine hairs. Eggs measure up to 0.50 mm long and 0.33 mm wide and are oval and bright yellow in color. Larvae are oligopod and fusiform with fine setae and develop through four instars. Pupae are yellow and exarate with setae present and develop in pupation chambers created within the soil. It is only at this stage that the sex of the individual can be determined without dissection, as genital structures retract into the body when eclosion to the adult stage occurs (Zilahi-Balogh et al. 2006, Shepherd et al. 2014). Zilahi-Balogh et al. (2003c) determined that it was univoltine and active primarily from fall through winter, with a period of subterranean aestivation below the soil surface from May to mid-September. Zilahi-Balogh et al. (2003a) reported that approximate development time from egg to adult was 64.8 ± 2.58 days at 15°C. *Laricobius nigrinus* is highly host-specific to HWA and requires HWA to complete its life cycle. They are also phenologically synchronous with the sistens generation of HWA and both species have a coinciding summer aestivation. *Laricobius nigrinus* also shows an ovipositional preference for HWA (Zilahi-Balogh et al. 2002), and both adult and larval *L. nigrinus* feed on HWA. Larvae feed primarily on eggs of HWA and adults feed primarily on nymphs and adults (Zilahi-Balogh et al. 2003b). In field studies, where *L. nigrinus* was released within cages on branches, they significantly decreased HWA sistens densities when compared to densities on branches that were not caged (Lamb et al. 2005a). In this study, *Laricobius nigrinus* significantly lowered sistens populations during oviposition but also during the pre-oviposition period from November to January when no other predators were present. These results suggested that *L. nigrinus* had potential to be an effective biological control agent (Zilahi-Balogh et al. 2002, Lamb et al. 2005a).

1.4 Release of *L. nigrinus* and hybridization with native congener

After significant host range and suitability testing, mass rearing protocols were developed (Lamb et al. 2005b, Salom et al. 2012), and the first operational controlled releases of *L. nigrinus* in the eastern U.S. began in 2003. Since that time, over 400,000 beetles have been released from field and lab-reared sources at over 700 sites (Virginia Tech 2019). Releases have been made by a variety of public agencies in most states where HWA has been detected. To organize these data, the HWA Predator Database was created in 2007. These records serve to assist in the overall management of HWA biological control program by providing a reference for releases and monitoring, mapping, and reporting. These tools help managers make informed strategic decisions about future biological control efforts. The establishment of *L. nigrinus* populations at several release sites in the eastern U.S. was determined by Mausel et al. (2010), and evaluation of its dispersal habits was later assessed in Davis et al. (2012).

Laricobius nigrinus and its eastern North America native congener, *L. rubidus* are closely related sister species that were found to have recently diverged (Montgomery et al. 2011). *Laricobius rubidus* is similar in size to *L. nigrinus* and can only be differentiated in the adult stage by its reddish elytra and unique male genitalia (Leschen 2011, Montgomery et al. 2011). It is an important predator of *Pineus strobi* Hartig, (pine bark adelgid - PBA) on *Pinus strobus* (eastern white pine) (Clark and Brown 1960, Wantuch et al. 2019). Since the introduction of HWA, however, *L. rubidus* has capitalized on this abundant food source in locations where hemlock and white pine occur together (Mausel et al. 2008). It is known to be able to complete its development on HWA (Zilahi-Balogh et al. 2005). Both *L. nigrinus* and *L. rubidus*, have been found co-mingling, mating, and successfully producing hybrid offspring at many existing *L. nigrinus* release sites (Havill et al. 2012). These hybrid offspring are impossible to identify through morphological means, because their resulting coloration can vary greatly and overlap with coloration exhibited by either parent species (Havill et al. 2012). As a result, individuals must undergo genetic testing for accurate identification. In a study by Fischer et al. (2015), populations of *L. nigrinus* were shown to increase at some sites from 2007-2012 on hemlock; whereas, populations of *L. rubidus* decreased, and the incidence of hybridization remained steady proportionally. In another study conducted at a separate site from 2011-2013, Mayfield et al. (2015) found that *L.*

nigrinus were the primary species recovered and although the prevalence of *L. rubidus* did not decrease significantly over time, there was a very low incidence of the beetle on hemlock at those sites in the last year of the study (<1%). Hybrid levels in this study remained relatively consistent. It is important to continue to monitor hybridization levels at release sites over time in order to understand what effects it will have on HWA, PBA, and populations of *L. nigrinus* and *L. rubidus*.

1.5 Assessing efficacy of biological control of forest pests

Successful biological control is indicated by a reduction of pest populations and a maintenance of those populations below economic and ecological damage thresholds over time by natural enemies (Bellows and Hassell 1999). The outcomes of many biological control programs are, however, not well understood. Further complicating matters are the difficulties involved with quantifying impact in biological control programs using predators. When studying parasitoids, mathematical models can be used to understand parasitoid-prey interactions. Predator-prey interactions involve more parameters and are more difficult to assign values to (Beddington et al. 1978). Confirmation of establishment of natural enemies is the first critical post-release evaluation conducted in classical biological control programs. Establishment of *L. nigrinus* was initially confirmed at numerous sites in the eastern U.S. by Mausel et al. (2010). Once establishment occurs, it is important to determine whether the predator is impacting the prey population, and if so to what extent. Impact studies are for critical for assessing the success of biological control programs. Typically, there are two main tactics employed to assess the effects of natural enemies on their hosts. First, life tables can be constructed to quantify mortality caused by natural enemies. Duan et al. (2015), used six study sites to evaluate the effects of three introduced parasitoids and avian predators of emerald ash borer (EAB) by felling trees, removing bark and observing larval galleries to determine life stages affected, and cause of mortality. Through the creation of life tables, they were able to attribute a decline in emerald ash borer spread to parasitism by one of the three introduced species. As an alternative to life tables, experimental techniques can be used in the field to estimate impact (Luck et al. 1999). For example, a bi-monthly sampling scheme was employed by Culliney et al. (1988), to assess the effectiveness of *Leucopis obscura* Haliday on *Pineus pini* Macquart on exposed branches of cluster pine, *Pinus pinaster* Aiton. Counts of all life stages of predator and prey were conducted at each sample period. Correlation analysis was used to analyze the density-dependent relationships between predator and prey. Results suggested that the predator was effectively controlling population densities of *P. pini* below an economic tree loss threshold.

Predator exclusion cages can be a useful technique for assessing the effect of predation on a pest population (Luck et al. 1988). With this technique, prey counts are made in samples where predators have access to prey, and also where they are excluded from the cages. The counts are then compared after the study period to determine the amount of mortality caused by the predator (Castellanos et al. 2015). Predator exclusion cages were used in two studies to determine impact of *L. nigrinus* on HWA at a release site in Virginia (Mausel et al. 2008) and in Georgia (Mayfield et al.

2015). These studies utilized HWA ovisac disturbance as a proxy for *L. nigrinus* predation. Branches which were exposed to *L. nigrinus* showed significantly higher ovisac disturbance when compared to caged branches. Although predatory exclusion methods can be effective in assessing the cause of mortality in certain insects, these experiments can be subject to bias. Castellanos et al. (2015) found that *Orgyia leucostigma* (Lepidoptera: Erebidae) caterpillars would drop from plants in response to the presence of predators. On plants where predators had access to the caterpillars, it was therefore difficult to determine population changes due to mortality caused by predator or drop-response by prey. Additionally, predator exclusion cages can create microclimate variation, affecting temperature, humidity and light penetration and effects are often variable based on cage materials used. This can affect both predator and prey behavior in addition to plant responses (Nelson and Rieske 2014).

Often it is helpful to study natural enemies and their effects on host species in their native environments. These studies can help define which species might be most successful in managing pest populations in invasion areas. Mausel et al. (2017) reported that *L. nigrinus* had a significantly positive functional and numerical response to HWA densities at two study sites in a portion of its native range in Seattle, WA. Adults increased their feeding, aggregation, and reproductive responses as HWA densities increased. Similar studies could also be performed at *L. nigrinus* release sites in the eastern U.S. to determine how this species responds to varying HWA densities. These types of studies could help researchers elucidate differences that may exist between introduced and native ranges. In Fidgen et al. (2013), a target density of 0.4 HWA sistens per cm of branch was indicated as a threshold whereby higher densities might negatively affect tree health. This density could be used as a measure for determining impact assessment of predators of HWA (Mausel et al. 2017). It is critical that biological control agents be able to respond to increased densities of prey in terms of aggregation and reproduction (Beddington et al. 1976). If the results of impact studies indicate a lack of appropriate mortality on the prey population, a return to native regions may be necessary to search for additional natural enemies (Van Driesche 2014).

1.6 Research rationale

Both eastern and Carolina hemlock are ecologically important species in eastern forests, and their health and longevity is severely threatened by HWA. Management of HWA has included the controlled release of a beetle predator, *L. nigrinus* since 2003. Over 400,000 beetles have been released in the eastern U.S. including over 14,000 in Virginia. Although previous studies have determined establishment at several locations within Virginia (Mausel et al. 2010, Heminger 2017), no comprehensive evaluation of all release locations has been completed. Evaluation of establishment is a pivotal task as it can aid in decision making for future management efforts within Virginia and provides insight into the ability of this predator to persist in the introduced range. If establishment can be confirmed, it suggests that *L. nigrinus* is adaptable to climates outside of its native range, an important attribute of introduced natural enemies. It is also imperative to determine the impact which the introduced predator has upon the invasive pest. To date, significant funding from a variety of agencies has been funneled into *L. nigrinus* rearing and release activities. If this species is not able to effectively impact HWA, management funding may need to be redirected to other efforts including evaluation of other potential predators. Although previous studies have investigated impact on a smaller scale (Mausel et al. 2008, Mayfield et al. 2015), a more regional effort is necessary to understand how *L. nigrinus* performs within a wider geographical range in the eastern U.S.

1.7 Research objectives

1. To assess the establishment of *L. nigrinus* at all release sites located in Virginia.
2. To measure the impact that *L. nigrinus* has on HWA populations at established release sites in the eastern U.S.

Both objectives reported here were first initiated by Ariel Heminger in the fall of 2014, in cooperation with the University of Massachusetts, University of Tennessee, and the USDA Forest Service (Heminger 2017). This impact assessment study indicated that *L. nigrinus* was significantly reducing HWA sistens populations on study branches at many sites. Ovisac disturbance rates were as high as 66% on no-cage study branches where predators had free access to the prey. During the period of the study in January 2014 and February 2015, the eastern U.S. experienced two polar vortex events, which caused extreme low winter temperatures (Tobin et al. 2017, Waugh et al. 2017). As a result, populations of both HWA and *L. nigrinus* declined at all field sites. This was confirmed in both the *L. nigrinus* impact assessment study and the survey of Virginia *L.*

nigrinus release sites during that period. Low numbers of *L. nigrinus* were recovered in the spring of 2015 when compared to the same sample period in 2016 (Heminger 2017). By 2016, populations of both predator and prey had begun to rebound due to a return of more typical winter temperatures. In an effort to gain a better understanding of the impacts of *L. nigrinus* on HWA over time and in a variety of seasonal temperatures, it was proposed that the study be extended from 2016-2018.

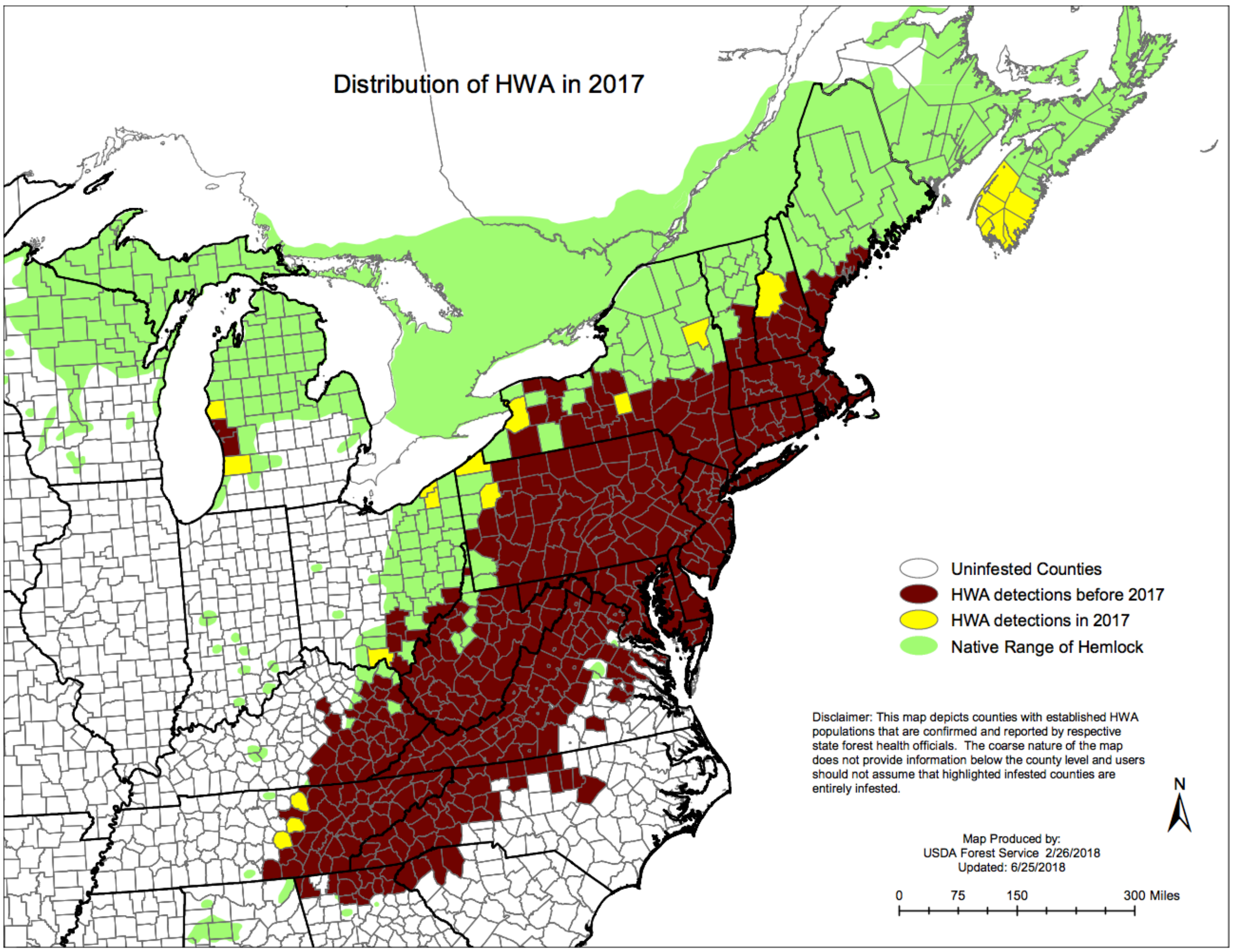

Figure 1.1. HWA Infestation by state and county in the range of *Tsuga canadensis* in the eastern U.S. Source: USDA Forest Service

1.7 References

Beddington, J. R., M. P. Hassell, and J. H. Lawton. 1976. Components of arthropod predation: II. The predator rate of increase. J. Anim. Ecol. 45: 165-185.

Beddington, J. R., C. A. Free, and J. H. Lawton. 1978. Characteristics of successful natural enemies in models of biological-control of insect pests. Nature 273: 513-519.

Bellows, T. S., and M. P. Hassell. 1999. Theories and mechanisms of natural population regulation, pp. 17-44. In T. W. Fisher, T. S. Bellows, L. E. Caltagirone, D. L. Dahlsten, C. B. Huffaker and G. Gordh (eds.), Handbook of Biological Control. Academic Press.

Brantley, S., C. R. Ford, and J. M. Vose. 2013. Future species composition will affect forest water use after loss of eastern hemlock from southern Appalachian forests. Ecol. Appl. 23: 777-790.

Castellanos, I., P. Barbosa, I. Zuria, and A. Caldas. 2015. Quantifying insect predation with predator exclusion cages: The role of prey antipredator behavior as a source of bias. Entomol. Exp. Appl. 157: 360-364.

Cheah, C. A. S.-J. 2011. *Sasajiscymnus* (=*Pseudoscymnus*) *tsugae*, a ladybeetle from Japan, pp. 43-52. In B. Onken and R. Reardon (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service FHTET-2011-04, Morgantown, WV.

Cheah, C. A. S.-J., and M. S. McClure. 1995. Exotic natural enemies of *Adelges tsugae* and their potential for biological control, pp. 103-112. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.

Clark, R. C., and N. R. Brown. 1960. Studies of predators of the balsam woolly aphid, *Adelges piceae* (Ratz.) (Homoptera: Adelgidae), VII. *Laricobius rubidus* (Lec.) (Coleoptera: Derodontidae), a predator of *Pineus strobi* (Htg.) (Homoptera: Adelgidae). Can. Entomol. 92: 237-240.

Cowles, R. S. 2009. Optimizing dosage and preventing leaching of imidacloprid for management of hemlock woolly adelgid in forests. For. Ecol. Manage. 257: 1026-1033.

Culliney, T. W., J. W. Beardsley, and J. J. Drea. 1988. Population regulation of the Eurasian pine adelgid (Homoptera, Adelgidae) in Hawaii. J. Econ. Entomol. 81: 142-147.

Davis, G. A., S. M. Salom, C. C. Brewster, B. P. Onken, and L. T. Kok. 2012. Spatiotemporal distribution of the hemlock woolly adelgid predator *Laricobius nigrinus* after release in eastern hemlock forests. Agric. For. Entomol. 14: 408-418.

Dharmadi, S. N., K. J. Elliott, and C. F. Miniat. 2019. Lack of forest tree seedling recruitment and enhanced tree and shrub growth characterizes post-*Tsuga* *canadensis* mortality forests in the southern Appalachians. For. Ecol. Manage. 440: 122-130.

Duan, J. J., L. S. Bauer, K. J. Abell, M. D. Ulyshen, and R. G. Van Driesche. 2015. Population dynamics of an invasive forest insect and associated natural enemies in the aftermath of invasion: Implications for biological control. J. Appl. Ecol. 52: 1246-1254.

Evans, R. A., E. Johnson, J. Shreiner, A. Ambler, B. Battles, N. Cleavitt, T. Fahey, J. Sciascia, and E. Pehek. 1995. Potential Impacts of hemlock woolly adelgid (*Adelges tsugae*) on eastern hemlock (*Tsuga canadensis*) ecosystems, pp. 42-57. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.

Fender, K. M. 1945. A new *Laricobius* from Oregon. Pan-Pac. Entomol. 21: 152.

Fidgen, J. G., D. E. Legg, and S. M. Salom. 2013. A sequential sampling plan for counts of *Adelges tsugae* on individual eastern hemlock trees. South. J. Appl. For. 37: 75-80.

Fischer, M. J., N. P. Havill, C. C. Brewster, G. A. Davis, S. M. Salom, and L. T. Kok. 2015. Field assessment of hybridization between *Laricobius nigrinus* and *L. rubidus*, predators of Adelgidae. Biol. Control 82: 1-6.

Hatch, M. H. 1962. The Beetles of the Pacific Northwest: Part III Pselaphidae and Diversicornia I, University of Washington Press, Seattle, Washington.

Havill, N. P., M. E. Montgomery, G. Y. Yu, S. Shiyake, and A. Caccone. 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Ann. Entomol. Soc. Am. 99: 195-203.

Havill, N. P., G. Davis, M. Fischer, S. Salom, D. Mausel, and B. P. Onken. 2011. The introduction of *Laricobius nigrinus* as a biological control agent for the hemlock woolly adelgid: Is there a threat to the native congener: *L. rubidus*, pp. 212-221. In B. Onken and C. Reardon (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service FHTET-2011-04, Morgantown, WV.

Havill, N. P., G. Davis, D. L. Mausel, J. Klein, R. McDonald, C. Jones, M. Fischer, S. Salom, and A. Caccone. 2012. Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control. Biol. Control 63: 359-369.

Havill, N. P., S. Shiyake, A. L. Galloway, R. G. Foottit, G. Y. Yu, A. Paradis, J. Elkinton, M. E. Montgomery, M. Sano, and A. Caccone. 2016. Ancient and modern colonization of North America by hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), an invasive insect from East Asia. Mol. Ecol. 25: 2065-2080.

Heminger, A. R. 2017. 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. M.S. L.F.S Thesis, Virginia Tech, Blacksburg, VA. 93 pp.

Jenkins, J. C., J. D. Aber, and C. D. Canham. 1999. Hemlock woolly adelgid impacts on community structure and N cycling rates in eastern hemlock forests. Can. J. Forest. Res. 29: 630-645.

Jetton, R. M., W. S. Dvorak, and W. A. Whittier. 2008. Ecological and genetic factors that define the natural distribution of Carolina hemlock in the southeastern United States and their role in ex situ conservation. For. Ecol. Manage. 255: 3212-3221.

Kohler, G. R., V. L. Stiefel, K. F. Wallin, and D. W. Ross. 2008. Predators associated with the hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific Northwest. Environ. Entomol. 37: 494-504.

Lamb, A. B., S. M. Salom, and L. T. Kok. 2005a. Survival and reproduction of *Laricobius nigrinus* Fender (Coleoptera : Derodontidae), a predator of hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera : Adelgidae) in field cages. Biol. Control 32: 200-207.

Lamb, A. B., S. M. Salom, and L. T. Kok. 2005b. Guidelines for rearing *Laricobius nigrinus* Fender, pp. 309-318. *In* B. Onken and R. Reardon [eds.], Proceedings of the Third Symposium on Hemlock Woolly Adelgid in the Eastern United States. USDA Forest Service FHTET-2005-01.

Lawrence, J. F. 1989. A catalog of the Coleoptera of America north of Mexico, Family: Derodontidae. USDA Agriculture Handbook 529-65.

Leschen, R. A. B. 2011. World Review of *Laricobius* (Coleoptera: Derodontidae). Zootaxa 2908: 1-44.

Lovett, G. M., C. D. Canham, M. A. Arthur, K. C. Weathers, and R. D. Fitzhugh. 2006. Forest ecosystem responses to exotic pests and pathogens in eastern North America. Bioscience 56: 395-405.

Luck, R. F., B. M. Shepard, and P. E. Kenmore. 1988. Experimental methods for evaluating arthropod natural enemies. Annu. Rev. Entomol. 33: 367-389.

Luck, R. F., B. M. Shepard, and P. E. Kenmore. 1999. Evaluation of biological control with experimental methods, pp. 17-44. In T. W. Fisher, T. S. Bellows, L. E. Caltagirone, D. L. Dahlsten, C. B. Huffaker and G. Gordh (eds.), Handbook of Biological Control. Academic Press.

Mausel, D. L., L. T. Kok, and S. M. Salom. 2017. Numerical response and impact of *Laricobius nigrinus* (Coleoptera: Derodontidae) on *Adelges tsugae* (Hemiptera: Adelgidae) in their native range. Environ. Entomol. 46: 544-551.

Mausel, D. L., S. M. Salom, L. T. Kok, and J. G. Fidgen. 2008. Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera: Derodontidae) on hemlock woolly adelgid in Virginia. Environ. Entomol. 37: 1498-1507.

Mausel, D. L., S. M. Salom, L. T. Kok, and G. A. Davis. 2010. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. Environ. Entomol. 39: 440-448.

Mayfield, A. E., B. C. Reynolds, C. I. Coots, N. P. Havill, C. Brownie, A. R. Tait, J. L. Hanula, S. V. Joseph, and A. B. Galloway. 2015. Establishment, hybridization and impact of *Laricobius* predators on insecticide-treated hemlocks: Exploring

integrated management of the hemlock woolly adelgid. For. Ecol. Manage. 335: 1-10.
McClure, M. S. 1989. Evidence of a polymorphic life-cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). Ann. Entomol. Soc. Am. 82: 50-54.
McClure, M. S. 1990. Role of wind, birds, deer, and humans in the dispersal of hemlock woolly adelgid (Homoptera: Adelgidae). Environ. Entomol. 19: 36-43.
McClure, M. S., and C. A. S.-J. Cheah. 1999. Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. Biol. Invasions 1: 247-254.
McClure, M. S., S. M. Salom, and K. S. Shields. 2001. Hemlock Woolly Adelgid. USDA Forest Service FHTET-2001-03, Morgantown, WV.
Montgomery, M. E., and S. M. Lyon. 1995. Natural enemies of adelgids in North America: their prospect for biological control of *Adelges tsugae* (Homoptera: Adelgidae), pp. 89-102. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.
Montgomery, M. E., S. Shiyake, N. P. Havill, and R. A. B. Leschen. 2011. A new species of *Laricobius* (Coleoptera: Derodontidae) from Japan with phylogeny and a key for native and introduced congeners in North America. Ann. Entomol. Soc. Am. 104: 389-401.
Nelson, L. A., and L. K. Rieske. 2014. Microclimatic variation within sleeve cages used in ecological studies. J. Insect Sci. 14: 1-5.
Orwig, D. A., and D. R. Foster. 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. J. Torrey Bot. Soc. 125: 60-73.
Peattie, D. C. 1950. A Natural History of Trees of Eastern and Central North America, Houghton Mifflin, Boston.
Potter, K. M., R. M. Jetton, W. S. Dvorak, V. D. Hipkins, R. Rhea, and W. A. Whittier. 2012. Widespread inbreeding and unexpected geographic patterns of genetic variation in eastern hemlock (*Tsuga canadensis*), an imperiled North American conifer. Conserv. Genet. 13: 475-498.
Salom, S. M., L. T. Kok, A. B. Lamb, and C. S. Jubb. 2012. Laboratory rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A predator of the hemlock woolly adelgid (Hemiptera: Adelgidae). Psyche 2012: 9.
Shepherd, W. P., M. E. Montgomery, B. T. Sullivan, and A. E. Mayfield. 2014. Novel method for determining sex of live adult *Laricobius nigrinus* (Coleoptera: Derodontidae). Can. Entomol. 146: 693-696.
Snyder, C. D., J. A. Young, D. P. Lemarle, and D. R. Smith. 2002. Influence of eastern hemlock (*Tsuga canadensis*) forests on aquatic invertebrate assemblages in headwater streams. Can. J. Fish. Aquat. Sci. 59: 262-275.
Souto, D., T. Luther, and B. Chianense. 1995. Past and current status of HWA in eastern and Carolina hemlock stands, pp. 9-15. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.

Tingley, M. W., D. A. Orwig, R. Field, and G. Motzkin. 2002. Avian response to removal of a forest dominant: Consequences of hemlock woolly adelgid infestations. J. Biogeogr. 29: 1505.

Tobin, P. C., R. M. Turcotte, L. M. Blackburn, J. A. Juracko, and B. T. Simpson. 2017. The big chill: Quantifying the effect of the 2014 North American cold wave on hemlock woolly adelgid populations in the central Appalachian Mountains. Popul. Ecol. 59: 251-258.

Van Driesche, R. 2014. Biological control of forest pests in North America: A reflection, pp. 1-8. In R. Van Driesche and R. C. Reardon (eds.), The Use of Classical Biological Control to Preserve Forests in North America, vol. FHTET-2013-2. USDA Forest Service.

Virginia Tech. 2019. HWA Predator Database, http://hiro.ento.vt.edu/pdb/. Virginia Tech.

Vose, J. M., D. N. Wear, A. E. Mayfield, and C. D. Nelson. 2013. Hemlock woolly adelgid in the southern Appalachians: Control strategies, ecological impacts, and potential management responses. For. Ecol. Manage. 291: 209-219.

Wallace, M. S., and F. P. Hain. 2000. Field surveys and evaluation of native and established predators of the hemlock woolly adelgid (Homoptera: Adelgidae) in the southeastern United States. Environ. Entomol. 29: 638-644.

Wantuch, H. A., N. P. Havill, E. R. Hoebeke, T. P. Kuhar, and S. M. Salom. 2019. Predators associated with the pine bark adelgid (Hemiptera: Adelgidae), a native insect in Appalachian forests, United States of America, in its southern range. Can. Entomol. 151: 73-84.

Waugh, D. W., A. H. Sobel, and L. M. Polvani. 2017. What is the polar vortex and how does it influence weather? Bul. Amer. Meteor. Soc. 98: 37.

Young, R. F., K. S. Shields, and G. P. Berlyn. 1995. Hemlock woolly adelgid (Homoptera: Adelgidae) - stylet bundle insertion and feeding sites. Ann. Entomol. Soc. Am. 88: 827-835.

Zilahi-Balogh, G. M. G., L. T. Kok, and S. M. Salom. 2002. Host specificity of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a potential biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). Biol. Control 24: 192-198.

Zilahi-Balogh, G. M. G., S. M. Salom, and L. T. Kok. 2003a. Temperature-dependent development of the specialist predator *Laricobius nigrinus* (Coleoptera: Derodontidae). Environ. Entomol. 32: 1322-1328.

Zilahi-Balogh, G. M. G., S. M. Salom, and L. T. Kok. 2003b. Development and reproductive biology of *Laricobius nigrinus*, a potential biological control agent of *Adelges tsugae*. BioControl 48: 293-306.

Zilahi-Balogh, G. M. G., C. D. Broeckling, L. T. Kok, and S. M. Salom. 2005. Comparison between a native and exotic adelgid as hosts for *Laricobius rubidus* (Coleoptera: Derodontidae). Biocontrol Sci. Technol. 15: 165-171.

Zilahi-Balogh, G. M. G., L. M. Humble, L. T. Kok, and S. M. Salom. 2006. Morphology of *Laricobius nigrinus* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid. Can. Entomol. 138: 595-601.

Zilahi-Balogh, G. M. G., L. M. Humble, A. B. Lamb, S. M. Salom, and L. T. Kok. 2003c. Seasonal abundance and synchrony between *Laricobius nigrinus* (Coleoptera: Derodontidae) and its prey, the hemlock woolly adelgid (Hemiptera: Adelgidae). Can. Entomol. 135: 103-115.

Chapter 2: Establishment of *Laricobius nigrinus* at release sites in Virginia

Abstract

Post-release assessment of natural enemy establishment is a critical component of classical biological control program evaluations. The ability for agents to colonize in an introduced region can be a predictor for future success against the pest and can help guide management decisions. *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a predatory beetle native to western North America, has been released since 2003 for management of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), a threat to native hemlocks in eastern North America. Over 400,000 beetles have been released in the eastern U.S. from field and lab-reared sources, 14,000 of which were deployed in the Commonwealth of Virginia at 26 sites. Prior studies in Virginia evaluated only a portion of these sites and demonstrated establishment at six locations. In this study, all sites were surveyed in 2017 and 2018 to determine if *L. nigrinus* were present. During the study, stand-level HWA densities were estimated, hemlock tree health and predator-prey ratios were quantified, and identification of *Laricobius* recoveries was assessed. Although HWA is not its primary host, a native species, *Laricobius rubidus* Leconte, is frequently found feeding on HWA, and can produce viable hybrid offspring with *L. nigrinus*. As a result, microsatellite analysis was used to determine which species or hybrid was recovered. During the period of our study, we determined that *L. nigrinus* was able to establish at 82% of Virginia release sites and were the primary species recovered (80%). Both *L. rubidus*

(18%) and hybrids (2%) were also recovered. Stand level HWA densities varied greatly over sites and years but showed a general decline in year two of the study. The mean predator-prey ratio ± S.E. for both years was 0.03 ± 0.01 *L. nigrinus* larvae per HWA which was lower than indicated during investigations of this species in its native range. Data indicated that tree health improved in Virginia, however, longer and more consistent studies are needed to determine if *L. nigrinus* can impact the health of hemlocks.

2.1 Introduction

Evaluating post-release establishment of predators released in classical biological control programs is a critical effort which helps guide future management of invasive pests (Van Driesche and Bellows 1996). There are many factors that can affect the ability of a natural enemy to successfully establish in an introduced range including climate, host suitability, timing of release, and quantity of natural enemies released (Van Driesche and Bellows 1996). Several of these factors were evaluated by Mausel et al. (2010) for *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a predatory beetle of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). *Laricobius nigrinus* has been released in the eastern U.S. since 2003 as a part of a robust biological control program implemented for the management of this invasive pest. HWA is native to Asia and western North America but was inadvertently introduced into the eastern U.S. from Japan (Havill et al. 2006). It is currently causing widespread dieback and mortality to two native hemlock species in eastern forests;

Tsuga canadensis (L.) Carriere (eastern hemlock), and *Tsuga caroliniana* Engelmann (Carolina hemlock) (Havill and Foottit 2007).

HWA have two anholocyclic generations per year (sistens and progrediens) (McClure 1989, Havill and Foottit 2007), while *L. nigrinus* is univoltine (Zilahi-Balogh et al. 2003). Both species are uniquely winter-active. During extensive testing of the suitability of *L. nigrinus* as a biological control agent, it was found to be phenologically synchronous with the sistens generation of HWA and was very host-specific, requiring HWA to complete its development (Zilahi-Balogh et al. 2002). To date over 400,000 *L. nigrinus* beetles have been released from field and lab-reared sources throughout the range of HWA infested eastern hemlock (Virginia Tech 2019). Mausel et al. (2010) demonstrated establishment of *L. nigrinus* in a study across a wide range of early release sites from Massachusetts to Georgia, suggesting that *L. nigrinus* is adaptable to environments outside of its native range. During previous studies, a native species, *Laricobius rubidus* Leconte was also collected from HWA infested hemlock during sampling (Mausel et al. 2008, Mausel et al. 2010, Davis et al. 2012). The primary host for this species is pine bark adelgid (PBA), *Pineus strobi* Hartig (Hemiptera: Adelgidae) on white pine, *Pinus stobus* L. (Clark and Brown 1960). *Laricobius nigrinus* and *L. rubidus* are closely related sister species that are able to mate and produce viable hybrid progeny (Havill et al. 2012, Fischer et al. 2015). Observations by Fischer et al. (2015) showed both species preferred to remain on their primary hosts, while hybrids appeared more frequently on HWA.

The first releases of *L. nigrinus* were made in Virginia in 2003 (Virginia Tech 2019). Since that time, approximately 14,000 adults have been released at 26 locations within the Blue Ridge or Valley and Ridge regions of Virginia with just a few releases occurring in the Piedmont and Coastal Plain/Tidewater areas. Prior efforts to evaluate establishment in Virginia included those indicated by Mausel et al. (2010), where establishment was confirmed at two of five sites evaluated. Heminger (2017) surveyed, 14 Virginia sites, and four of those were positive for *L. nigrinus*. In the study reported here, we sampled for *L. nigrinus* at all existing Virginia release sites to have a more concise idea of this beetle's ability to survive under a variety of conditions state-wide. During those efforts we also classified stand-level HWA densities, tree health, *Laricobius* spp. species composition, and predator-prey ratios at each site. The results from this study will help guide future releases of *L. nigrinus* in Virginia and offer insight to its ability to impact HWA populations and hemlock tree health.

2.2 Materials and Methods

2.2.1 Field sites

The HWA Predator Release and Monitoring Database (Virginia Tech 2019) was queried in spring of 2017 to identify all known *L. nigrinus* release sites in Virginia. A total of 26 sites were noted, however, five of those occurred within a one-kilometer radius in the Mountain Lake area and were therefore treated as one site during these investigations. Therefore, we utilized a total of 22 sites to evaluate establishment of *L. nigrinus* in Virginia (Table 2.1). All releases made in Virginia were of the coastal strain biotype, which was unique from an interior strain found in Idaho, Montana, and interior British

Columbia (Davis et al. 2011, Mausel et al. 2011, Havill et al. 2012). Release sites were situated within four distinct USDA Plant Hardiness Zones; 6a (-23.3 to -20.5°C), 6b (-20.6 to -17.8°C), 7a (-17.8 to -15°C) and 7b (-15 to -12.2°C), with the majority being within 6b (USDA 2012). Sites were visited from March to April of both 2017 and 2018 for sampling and coincided with the period of peak HWA egg abundance, *L. nigrinus* oviposition, and just prior to *L. nigrinus* larval presence. The exact timing of each site visit varied depending on USDA Plant Hardiness Zone and local temperatures leading up to the sampling period. Colder temperatures delayed *L. nigrinus* ovipositional periods. For all locations, visits were planned so that sampling occurred on days where precipitation was not forecasted, and temperatures were not below 0°C, as *L. nigrinus* would likely not be active (Zilahi-Balogh et al. 2003, Mausel et al. 2010). Due to distance to many release sites and limited timeframe available, visiting during optimal weather conditions was not always possible.

2.2.2 HWA density assessment

Approximately 20-30 branches were randomly sampled from the lower canopy (0-2 meters) at each site. Current years' growth on a 30 cm distal portion of each chosen branch was evaluated. The number of HWA/cm was approximated for each branch, and an overall mean density was then estimated to achieve a stand level density assessment. Categories used included:

1. No HWA – HWA not present.
2. Low HWA – An average less than 1 HWA per 30 cm of current years' growth.
3. Moderate HWA – An average between 1-10 HWA per 30 cm current years' growth.

4. High HWA – An average greater than 10 HWA per 30 cm current years' growth.

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

2.2.3 Tree health assessment

At each site, hemlock tree health was assessed using USFS Forest Inventory and Analysis (FIA) crown condition parameters (crown density, transparency, dieback, and live crown ratio) (USDA Forest Service 2011). Crown density is measure of the proportion of branches and foliage blocking light penetration through the tree crown. Transparency measures skylight visible through micro-holes in the live portion of the crown. Dieback is a measure of recent mortality of fine twigs beginning at the terminal portion of the tree. Live crown ratio evaluates the length of the tree that supports live foliage relative to the total length of the tree. A percentage value was assigned for each parameter, and collectively, these values served as a broad indicator of overall tree health. For both crown density and live crown ratio, higher percentages indicated better health. For both transparency and dieback, higher percentages indicated poorer health. Ten trees were selected at each site each year, and assessments were performed. A mean percentage for each parameter was then calculated for each site. For all parameters, data were pooled for all sites in each year during analysis. Tree health was not evaluated at Big Cherry #2 in 2017, or in either year at Sandy Point State Forest

2.2.4 *Laricobius* spp. beat sheet sampling

Adult *L. nigrinus* densities were quantified using beat sheet sampling techniques. Sampling was performed for approximately 20-30 min on HWA infested branches in the lower canopy (0-2 meters above ground-level) of hemlock trees throughout the site. Beat sheets were PVC framed, and constructed of 1 m^2, ripstop nylon (Bioquip Products, Rancho Dominguez, CA). Sheets were placed under selected HWA infested branches and the upper portion of the branch was then tapped approximately 10 times using a 60 cm section of PVC pipe to dislodge adult *Laricobius* spp. present on the branch. All adults found on the sheet were collected via aspirator, and then transferred to vials containing 95% EtOH for genetic analysis. The total number of adults collected per site was recorded. Beat sheet sampling was not conducted in either year at Mountain Lake or Sandy Point State Forest due to lack of HWA.

2.2.5 *Laricobius* spp. branch clip sampling

Laricobius nigrinus larval densities were quantified using branch clip sampling techniques (Mausel et al. 2010). Approximately 20-25 branches with varying densities of HWA were selected at each site from the lower canopy of hemlock trees. The distal 30 cm of each selected branch was cut from the tree and placed into a 3.8 L zip closure plastic bag and transported back to the Virginia Tech insect mass rearing facility. Branches were removed from the bags and the number of HWA per branch was estimated. Basal tips of each branch were re-clipped to improve water uptake and were then stuck into wetted Instant Deluxe Floral Foam (Smithers-Oasis North America, Kent, OH) which was wrapped in Parafilm M (Beemis N.A., Neemah, WI). Blocks with branches were placed into rearing funnels in conditions known to be appropriate for developing *Laricobius* spp. larvae (12h:12H (L:D), 13-15°C) (Lamb et al. 2005, Salom et al. 2012). Larvae were permitted to feed and develop through four instars and were collected as pharate pre-pupae from jars attached at the base of funnels. Jars were checked daily for the presence of pre-pupae and, when recovered, were placed into vials filled with 95% EtOH for genetic analysis to determine species or hybrid designation. The total number of larvae recovered from each site was quantified. Branches were not collected at Sandy Point State Forest in 2017, or in either year at Mountain Lake due to the lack of HWA.

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

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

2.2.7 Statistical analyses

FIA tree health parameters (crown density, transparency, dieback, and live crown) were pooled by year and analyzed to look for differences between values in 2017 and 2018 using a paired t-test at a significance level of α = 0.05. Tree health parameter response variables were tested for goodness-of-fit to a normal distribution using skewness and kurtosis values (Thode 2002). Dieback variables did not fit a normal distribution and were square root transformed to meet the assumptions of the test. Spearman's Rank correlation analysis was used to determine the association between *Laricobius* spp. larval densities and HWA densities in funnels. Additionally, combined adult + larval recoveries were analyzed with stand-level HWA density and the four tree health parameters. All correlation analyses were run at a significance level of α = 0.05. Nonparametric analysis was used due to non-normality of some variable distributions.

2.3. Results

2.3.1. HWA density assessment

HWA densities varied greatly across sites and years but declined at 10 sites during the period of the study (Table 2.2). In 2017 the number of sites with no, low, moderate, or high HWA densities were 1, 8, 5, and 6, respectively. In 2018, the number of sites with no, low moderate, or high HWA densities were 1, 13, 7, and 1, respectively.

2.2.3 Tree health assessment

Collectively, tree health assessment results indicated a slight improvement in overall health of hemlocks at Virginia *L. nigrinus* release sites. Mean crown density in 2017 was 62% (range: 45.50-89.00), and in 2018 was 64% (range: 44.50-91.00), with no significant difference detected between the two years (F = 1.6430, df = 1, 451, p = 0.2006) (Fig. 2.1, Table 2.2). Mean transparency in 2017 was 38% (range: 9.50-57.50), and in 2018 was 32% (range: 5.00-56.50), with a significant decrease between the two years (F = 13.8263, df = 1, 451, p = 0.0002) (Fig. 2.1, Table 2.2). Mean dieback in 2017 was 34% (range: 8.50-60.00) and in 2018 was 25% (range: 5.00-49.50), with a significant decrease between the two years (F = 35.2886, df = 1, 451, p < 0.0001) (Fig. 2.1, Table 2.2). Mean live crown ratio in 2017 was 63% (range: 37.50-91.00) and in 2018 was 64% (range: 31.50-100.00), with no significant difference detected between the two years (F = 0.4642, df = 1, 451, p = 0.4960) (Fig. 2.1, Table 2.2).

2.2.4 *Laricobius* spp. beat sheet sampling

A total of 44 *Laricobius* spp. adults were collected at 8 out of 22 (36%) sites in the spring of 2017 (Table 2.3). Mean number of adults ± S.E. per site was 2.10 ± 1.26 (range: 0-26) with Poverty Creek having the highest number of recoveries. A total of 186 adult *Laricobius* spp. were collected at 15 out of 22 (68%) sites in 2018 (Table 2.3). Mean number of adults ± S.E. per site was 8.45 ± 3.65 (range: 0-80) with the Kentland Farm site having the greatest number of recoveries during the study. No adult recoveries were made at Mountain Lake, Rose Hill, and Sandy Point State Forest during the period of the study.

2.2.5 *Laricobius* spp. branch clip sampling

A total of 961 *Laricobius* spp. larvae were recovered at 15 out of 22 (68%) sites in 2017 (Table 2.3). Mean number of larvae ± S.E. per site was 45.76 ± 17.19 (range: 0-343). Mean predator-prey ratio ± S.E. for all sites was 0.03 ± 0.01 (range: 0-0.14) (Table 2.3). Kentland Farm had the greatest number of larval recoveries and the highest predator-prey ratio. A total of 1,503 *Laricobius* spp. larvae were recovered at 19 out of 22 (86%) sites in 2018 (Table 2.3). Mean number of larvae ± S.E. per site was 68.91 ± 14.52 (range: 0-211) with Highland having the highest number of recoveries. Mean predator-prey ratio ± S.E. for all sites pooled was 0.02 ± 0.01 (range: 0-0.10) with Kentland Farm having the greatest predator-prey ratio. No larval recoveries were made at Mountain Lake, Rose Hill, or Sandy Point State Forest during the period of the study.

There was no significant association between the number *Laricobius* spp. larvae and the number of HWA in funnels. There was a significant positive relationship between combined *Laricobius* spp. adults + larvae and stand-level HWA density, and a significant negative relationship with crown transparency (Table 2.4). No significant associations were noted between *Laricobius* spp. adults + larvae and crown density, dieback, and live crown ratio (Table 2.4).

2.2.6 Genetic Analysis of *Laricobius* spp. adult and larval recoveries

Laricobius nigrinus was the primary species recovered from Virginia release sites. *Laricobius rubidus* and hybrids of *L. nigrinus* and *L. rubidus* were also recovered. Recoveries of *L. nigrinus* were made at 82% of release sites and as such, establishment of this species at those locations can be confirmed (Fig. 2.2, Table 2.5). In both years combined, the mean percent *L. nigrinus* at release sites was 80% followed by 18% *L. rubidus*, and 2% hybrids (Fig. 2.3). In 2017, the mean percent *L. nigrinus* at release sites was 76%, followed by 17% *L. rubidus,* and 7% hybrids. In 2018, the mean percent *L. nigrinus* at release sites was 77% followed by 19% *L. rubidus* and 4 % hybrids. No recoveries of *L. nigrinus* were made at Nature Camp, Mountain Lake, Rose Hill, or Sandy Point State Forest in either year. Interestingly, only *L. rubidus* was recovered from Nature Camp. At three sites, Cherokee Flats, Devil's Fork, and North Fork, the adult and larval recovery sample size across both years was < 10 (n = 4, 3, 9, respectively). All other sites had sample sizes greater than 10 across both years.

Some samples were not identifiable due to unsuccessful DNA extraction, or issues with loci amplification as a result of poor sample quality, or molecular techniques.

2.3 Discussion

Clausen (1951) suggested that biological control agents should be able to show establishment and some level of impact within three generations in order to be successful. Previous studies evaluating establishment of *L. nigrinus* in the eastern U.S. did indicate recoveries at many locations and USDA Plant Hardiness Zones within three generations, suggesting that this species showed good promise as a biological control. *Laricobius nigrinus* was also able to adapt to new climate conditions not found in its native range (Mausel et al. 2010). Continued evaluation of biological control agents after release is important in order to be able to justify and guide future work with that particular agent (Stiling 1990). Our study was likely the first state-wide evaluation of establishment in the eastern U.S., the results of which indicated that *L. nigrinus* has colonized successfully at a majority of release sites in Virginia. *Laricobius nigrinus* has been able to persist long-term at many of these locations, and based on original release dates, F_{15} generation individuals were recovered. They were able to establish at a variety of locations within the Blue Ridge, Valley and Ridge, and Piedmont regions, and were able to persist at locations where HWA stand density was low.

Although there were four sites where *L. nigrinus* was not recovered in this study, there are several factors that may have negatively affected their ability to establish. At two of the four sites, where establishment was not confirmed (Mountain Lake and Sandy Point

S.F.), HWA populations were either not found or were extremely low during the two-year period of the study. The Mountain Lake sites are situated in Giles, Co., VA at an elevation of 1100-1200 m. Low winter temperatures frequently experienced at these elevations are likely a source of mortality for HWA (Paradis et al. 2008, Trotter and Shields 2009, McAvoy et al. 2017, Tobin et al. 2017) and *L. nigrinus* populations. Conversely, at Sandy Point (Zone 7b), summer mortality may be a possible cause for low HWA populations (Mech et al. 2018). At the time of release in 2010 at Sandy Point, HWA densities were much lower than originally observed when the site was initially identified for possible release (Virginia Tech 2019). Low densities of HWA at this site may not have supported the higher than typical release numbers of *L. nigrinus* (2,040 adults) (Table 2.1) and therefore may have been a limiting factor for their establishment. At Rose Hill, where no *L. nigrinus* recoveries were made, query of the HWA predator database suggested that adult releases were made in mid-April of 2014. The timing of release could be a cause for non-recovery here. Although Mausel et al. (2010) showed establishment of *L. nigrinus* at a site where a release was made in April, it was in Zone 6a and HWA were likely not as developed there as they would have been at Rose Hill (Zone 6b) during this time. In April, at most sites in Virginia, the primary food source for *L. nigrinus*; HWA progrediens eggs, (Zilahi-Balogh et al. 2003), have likely already hatched. At Nature Camp, the final site without *L. nigrinus* recoveries, HWA was present, trees were in fairly good health, and the timing of release was more appropriate (January 2012). Although it's possible that extreme low temperatures may have occurred after release causing winter mortality of *L. nigrinus*, it is still unclear why establishment did not occur in this location.

A lack of recoveries at these sites, however, does not necessarily indicate a lack of establishment; populations of *L. nigrinus* may have just been too low to detect. There were sampling limitations in this study that could be the cause for no or low recoveries. Both adult and larval sampling was limited to the lower canopy of hemlock trees at most sites due to ease of accessibility specifically with beat sheet sampling. We know that beat sheet sampling often provides false negatives due to habits of adults (Mausel et al. 2010) and in this study, we showed much lower recoveries of this life stage when compared to larvae (Table 2.4). Although larval sampling is more effective, and in this study, we selected branches with high HWA infestations to increase odds of *Laricobius* spp. recovery, Davis et al. (2012) suggests that *L. nigrinus* disperses vertically (>15 m) within release trees. Further investigations of *L. nigrinus* establishment at sites where they were not initially recovered should therefore include sampling in a variety of canopy positions within trees and should be primarily focused on branch clip sampling for larvae.

Although tree health data collected in this study showed a slight increase in overall health of hemlocks between the two years of the study, it is important to note that FIA sampling techniques remain relatively subjective and may be susceptible to researcher bias. Additionally, random trees were selected at each site each year which did not allow individual tree health to be followed over a period of time. Since tree health between hemlocks within a site can vary greatly, it was impossible to record consistent changes over time using our methods. There was a significant negative association between *Laricobius* spp. adult and larval recoveries when compared to the tree health parameter, transparency. This suggests that as transparency decreased, *Laricobius* spp. densities increased in 2018. Although this type of relationship may be indicative of partial success within a biological control program, we cannot confidently make assertions about the relationships of *Laricobius* spp. densities and tree health using the parameters of this study. In order to have a more consistent and thorough analysis of tree health at Virginia release sites, and an understanding of how *Laricobius* spp. populations interact with these parameters, future evaluations should be performed on the same trees in multi-year studies.

Genetic analysis of recovered *Laricobius* spp. suggests that *L. nigrinus* was the species recovered from Virginia release sites. The percentages of hybrid recoveries in this study (4-7%) were lower than in previous studies (11-28%) (Havill et al. 2012, Arsenault et al. 2015, Fischer et al. 2015, Mayfield et al. 2015, Wiggins et al. 2016). While it is possible that hybridization rates have decreased over time at release sites, microsatellite analysis using the six selected loci indicated in Klein et al. (2010) is limited to detecting only earlier generation hybrids. Offspring of prior hybrids could have backcrossed with either species and therefore may have been identified as *L. nigrinus* or *L. rubidus* by the software programs used in the analysis (Havill et al. 2012). Increasing the number of loci used in microsatellite analysis could improve our ability to detect hybrids and understand how populations of each species are changing spatially and temporally at release sites.

Predator-prey ratios in this study were lower than those indicated in a study conducted in the native range of *L. nigrinus* (Mausel et al. 2017). During that investigation conducted at four sites in the Seattle, WA area, *L. nigrinus* ratios ranged between 0.05 and 0.41 egg and larval *L. nigrinus* per HWA. In western North America, HWA does not reach populations levels comparable to those observed in eastern North America, likely due to host resistance and the presence of a natural enemy complex (Cheah and McClure 1995, McClure and Cheah 1999). Predator-prey ratios reported in Mausel et al. (2017) suggest suitable densities of *L. nigrinus* for effective management of HWA in the native range. The highest ratio recorded in our study (0.14 larvae/HWA), was found at Kentland Farm in 2017, however most fell well-below ranges found in the West. The disparity between the findings in the East and West were not explained by the parameters of this study and are therefore not well understood. Several key differences between the study areas do exist. First, evaluations in the West occurred in urban park settings where abiotic and biotic conditions may have been vastly different than the primarily forested sites evaluated in Virginia. Many factors such as climate, tree health, lack of tree host resistance and natural enemy complex likely affect predator-prey populations in the East. Although there was a significant positive relationship between adult + larval densities when compared to HWA stand-level density, further investigations are necessary to have a more thorough understanding of the dynamics involved with *L. nigrinus* and HWA populations.

Our results provide information that is critical to steering future management of predator and prey. In this study, we have confirmed *L. nigrinus* establishment at 82% of sites in

Virginia. Future management efforts here should include releases of *L. nigrinus* at Rose Hill and Nature Camp where recoveries were not made during the period of our study. Continued monitoring of HWA and *L. nigrinus* populations at both Mountain Lake and Sandy Point State Forest is important to determine establishment status, as populations of both species may have been too low to detect during the period of our study. Future releases must also be made at sites with adequate HWA populations to support the release size and must be made during the appropriate time-period in order to have a higher probability of establishment. The results of this study further support the assertions that *L. nigrinus* is adaptable to climatic and other environmental conditions outside of its native range (Mausel et al. 2010) and can persist in these environments in spite of low prey populations.

Table 2.1. Virginia *L. nigrinus* release site names, latitude and longitude, and USDA Plant Hardiness Zone of site location, and original release dates.

Site Name	Latitude	Longitude	Plant Hardiness Zone	*L. nigrinus* Release Date	No. Released
Bear Creek	36.911486	-84.401136	6b	2013	225
Big Cherry #1	36.827663	-82.702242	6b	2008	500
Big Cherry #2	36.832379	-82.702242	6b	2008	500
Burns Creek	36.924661	-82.536936	6b	2008	300
Channels S.F.	36.828643	-81.962809	6b	2010	1000
Cherokee Flats	37.414479	-80.583234	6b	2014	400
Devil's Fork	36.820031	-82.630216	6b	2008	300
Dickey Creek	36.736894	-81.432461	6b	2005	75
Gullion Fork	36.995914	-81.27317	6b	2013	225
Highland	36.692104	-81.517071	6b	2004	1200
Hurricane	36.721789	-81.487527	6b	2003	300
James River	37.640505	-78.79973	7a	2005	300
Kentland Farm	37.208931	-80.589822	6b	2003	258
Lick Creek	37.01072	-81.427409	6a	2004	150
McCoy	37.214902	-80.6015	6b	2013/2014	150/267
Mountain Lake	37.368654	-80.536671	6a	2009/2010	42/1800
Nature Camp	37.875946	-79.214285	6b	2012	430
North Fork	37.443668	-80.515333	6b	2003	600
Pinnacle	36.961556	-82.053298	6b	2006	310
Poverty Creek	37.252649	-80.533711	6b	2009/2010/2014	150/1000/539
Rose Hill	36.682086	-83.364423	6b	2014	275
Sandy Point	37.682968	-76.944674	7b	2010	2040

Table 2.2. HWA stand level density, mean depth at breast height (DBH), and mean FIA tree health parameters (crown density, transparency, dieback, and live crown ratio) for Virginia release sites in 2017 and 2018.

Site	Year	Stand HWA Density[a]	Mean DBH	Mean Crown Density	Mean Transparency	Mean Dieback	Mean Live Crown
Bear Creek	2017	Moderate	5.9 ± 1.0	50.5 ± 4.5	37.0 ± 2.7	34.5 ± 2.5	44.0 ± 4.2
	2018	Low	11.3 ± 2.9	62.5 ± 3.8	33.0 ± 5.1	24.0 ± 3.5	41.5 ± 5.2
Big Cherry #1	2017	Low	5.4 ± 1.0	52.5 ± 5.1	48.0 ± 4.1	29.5 ± 5.0	71.0 ± 8.5
	2018	Moderate	3.1 ± 0.4	57.0 ± 3.2	43.5 ± 4.0	28.5 ± 5.7	60.0 ± 5.3
Big Cherry #2	2017	--	--	--	--	--	--
	2018	Moderate	3.9 ± 0.3	72.5 ± 3.7	22.5 ± 3.2	16.0 ± 5.5	92.0 ± 2.2
Burns Creek	2017	Moderate	13.5 ± 2.5	45.5 ± 6.0	57.5 ± 3.4	39.5 ± 2.9	37.5 ± 4.6
	2018	Low	10.2 ± 1.7	59.5 ± 5.6	36.0 ± 6.2	30.0 ± 2.9	31.5 ± 2.7
Channels S.F.	2017	High	6.4 ± 3.0	49.0 ± 3.4	55.5 ± 5.5	60.0 ± 6.8	48.0 ± 6.2
	2018	Low	11.6 ± 3.3	52.0 ± 3.1	41.5 ± 5.0	35.5 ± 5.0	56.5 ± 3.5
Cherokee Flats	2017	Low	4.6 ± 0.8	53.0 ± 7.1	39.0 ± 4.3	34.0 ± 4.7	61.0 ± 8.5
	2018	Low	6.2 ± 0.4	59.0 ± 1.9	36.5 ± 3.2	28.0 ± 3.7	63.0 ± 6.7
Devil's Fork	2017	High	9.7 ± 2.7	53.5 ± 5.8	47.5 ± 5.8	52.0 ± 5.6	51.5 ± 7.0
	2018	Low	5.9 ± 1.5	44.5 ± 7.1	56.5 ± 5.0	49.5 ± 7.7	55.0 ± 5.1
Dickey Creek	2017	High	6.5 ± 1.5	52.0 ± 4.7	46.5 ± 4.4	48.5 ± 5.9	52.5 ± 7.0
	2018	Moderate	6.8 ± 1.3	50.5 ± 5.4	38.0 ± 2.2	36.5 ± 5.9	58.0 ± 4.6
Gullion Fork	2017	High	8.3 ± 2.8	46.0 ± 4.9	41.5 ± 4.7	28.0 ± 4.4	50.0 ± 5.5
	2018	Low	11.1 ± 2.0	52.0 ± 3.0	31.0 ± 1.6	25.5 ± 4.6	44.0 ± 4.8
Highland	2017	Low	12.0 ± 3.3	85.5 ± 2.9	19.0 ± 2.8	12.0 ± 2.7	81.0 ± 4.3
	2018	Moderate	8.0 ± 1.7	85.0 ± 2.6	14.0 ± 2.8	8.0 ± 2.2	63.5 ± 8.6
Hurricane	2017	Low	8.0 ± 1.2	55.5 ± 4.5	42.5 ± 3.1	39.0 ± 2.4	58.5 ± 4.5
	2018	Low	6.6 ± 0.9	65.0 ± 3.6	31.0 ± 2.6	34.0 ± 6.5	73.5 ± 5.4
James River S.P.	2017	High	4.3 ± 0.6	69.0 ± 4.2	36.5 ± 4.9	36.0 ± 4.3	70.0 ± 3.8
	2018	Moderate	6.9 ± 1.6	63.5 ± 3.1	28.5 ± 1.8	38.5 ± 6.7	71.5 ± 2.2
Kentland Farm	2017	Low	5.2 ± 0.6	89.0 ± 4.0	9.5 ± 3.2	8.5 ± 2.5	91.0 ± 3.0
	2018	High	4.4 ± 0.4	91.0 ± 0.8	5.0 ± 0.0	5.0 ± 0.00	100 ± 0.0
Lick Creek	2017	Moderate	9. 2 ± 1.7	56.0 ± 3.5	50.5 ± 1.8	34.5 ± 3.9	51.0 ± 4.0
	2018	Low	12.9 ± 1.0	62.0 ± 2.2	42.0 ± 3.5	30.0 ± 4.6	59.0 ± 5.9
McCoy	2017	Low	4.0 ± 0.4	66.0 ± 3.8	27.5 ± 2.8	30.0 ± 4.0	65.0 ± 3.7
	2018	Low	2.6 ± 0.4	58.5 ± 2.3	38.5 ± 3.2	28.7 ± 3.6	62.2 ± 3.1
Mountain Lake	2017	None	10.8 ± 1.0	69.0 ± 2.5	35.0 ± 2.6	36.5 ± 2.4	68.3 ± 2.4
	2018	None	10.9 ± 1.1	68.5 ± 1.8	29.0 ± 2.8	12.7 ± 3.1	72.0 ± 2.4

Nature Camp	2017	Moderate	5.3 ± 1.1	67.0 ± 3.5	30.0 ± 3.3	21 ± 2.45	69.0 ± 3.5
	2018	Moderate	7.0 ± 1.2	69.0 ± 2.0	26.0 ± 1.9	12.5 ± 2.01	72.0 ± 2.7
North Fork	2017	Low	9.5 ± 0.6	67.0 ± 3.7	32.5 ± 3.1	32 ± 1.86	69.0 ± 2.4
	2018	Low	7.0 ± 0.6	60.5 ± 2.2	43.0 ± 4.7	37 ± 4.90	51.0 ± 4.2
Pinnacle	2017	Low	7.5 ± 1.4	75.5 ± 1.8	23.5 ± 3.1	20.5 ± 2.41	71.0 ± 2.4
	2018	Low	5.7 ± 0.7	74.5 ± 2.4	26.0 ± 1.8	9.5 ± 2.52	67.5 ± 2.9
Poverty Creek	2017	High	7.4 ± 1.3	64.0 ± 7.0	45.0 ± 6.6	37 ± 4.23	63.5 ± 7.3
	2018	Moderate	3.0 ± 0.6	59.0 ± 4.0	33.5 ± 3.8	32 ± 3.35	70.0 ± 3.3
Rose Hill	2017	Moderate	9.0 ± 1.9	69.0 ± 5.3	36.0 ± 4.0	35 ± 4.08	74.5 ± 5.6
	2018	Low	11.2 ± 1.7	75.0 ± 2.1	29.5 ± 3.9	22 ± 3.74	58.5 ± 5.8
Sandy Point S.F.	2017	--	--	--	--	--	--
	2018	Low	--	--	--	--	--

[a]Stand-level HWA density categories:
None – HWA not present
Low – An average less than 1 HWA per 30 cm of current years' growth
Moderate – An average between 1-10 HWA per 30 cm current years' growth
High – An average greater than 10 HWA per 30 cm current years' growth
-- Data not collected at site

Table 2.3. The number of *Laricobius* spp. adult recoveries from beat sheet sampling, the number of larval recoveries from branch clip sampling, and the predator-prey ratio (larvae/HWA) in foliage funneled for each Virginia release site in 2017 and 2018.

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

--Data not collected at site

Table 2.4. Spearman's Rank correlation results for multiple factors tested at a significance level of $\alpha = 0.05$. A negative Spearman's ρ value indicates a negative correlation. Positive values indicate a positive correlation.

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

Table 2.5. Percentage of *Laricobius* recoveries identified as *L. nigrinus*, *L. rubidus*, and hybrids using microsatellite loci analysis for each Virginia release site in 2017 and 2018.

	2017			2018		
Site	% *L. nigrinus*	% *L. rubidus*	% Hybrids	% *L. nigrinus*	% *L. rubidus*	% Hybrids
Bear Creek	100	0	0	100	0	0
Big Cherry #1	100	0	0	4	88	8
Big Cherry #2	0	0	0	100	0	0
Burns Creek	0	0	0	100	0	0
Channels S.F.	72	28	0	88	0	13
Cherokee Flats	0	100	0	100	0	0
Devil's Fork	0	0	0	100	0	0
Dickey Creek	0	100	0	94	3	3
Gullion Fork	96	4	0	100	0	0
Highland	100	0	0	100	0	0
Hurricane	43	57	0	91	3	6
James River S.P.	100	0	0	100	0	0
Kentland Farm	100	0	0	100	0	0
Lick Creek	17	78	6	91	2	7
McCoy	76	17	7	77	19	4
Mountain Lake	--	--	--	--	--	--
Nature Camp	0	100	0	0	100	0
North Fork	33	67	0	*	*	*
Pinnacle	0	0	0	100	0	0
Poverty Creek	88	10	2	87	8	5
Rose Hill	0	0	0	0	0	
Sandy Point S.F.	--	--	--	--	--	--

--Data not collected at site

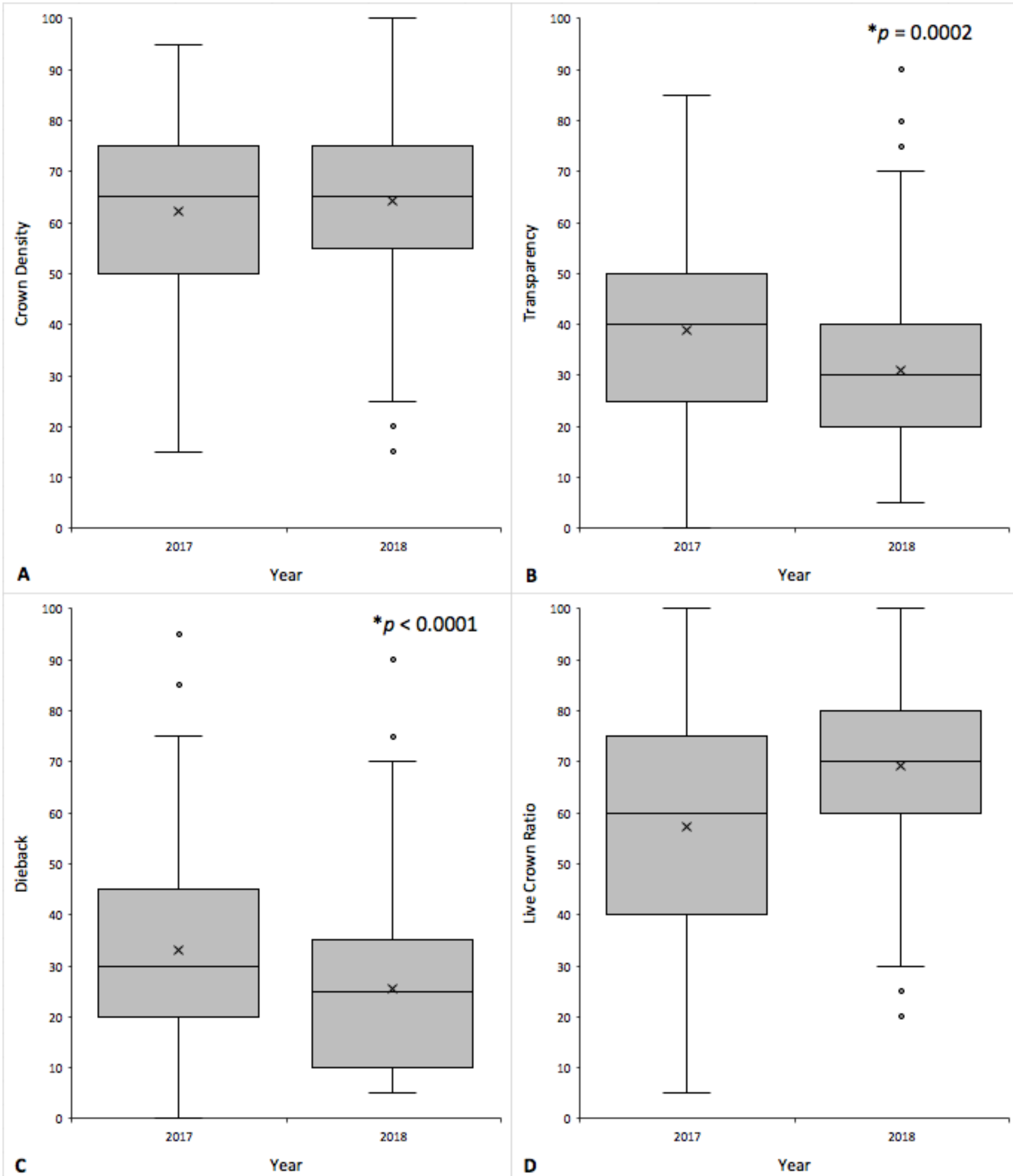

Figure 2.1. Box plots depicting 2017 and 2018 mean percent FIA tree health parameters: A. crown density, B. transparency, C. dieback, and D. live crown ratio from *L. nigrinus* release sites in Virginia. Significant differences in parameter values between the two years was tested using a paired t-test. * = significance at $\alpha = 0.05$, x = parameter mean, shaded boxes show interquartile range with median indicated by horizontal line, box whiskers extend to minimum and maximum values, circles indicate outliers.

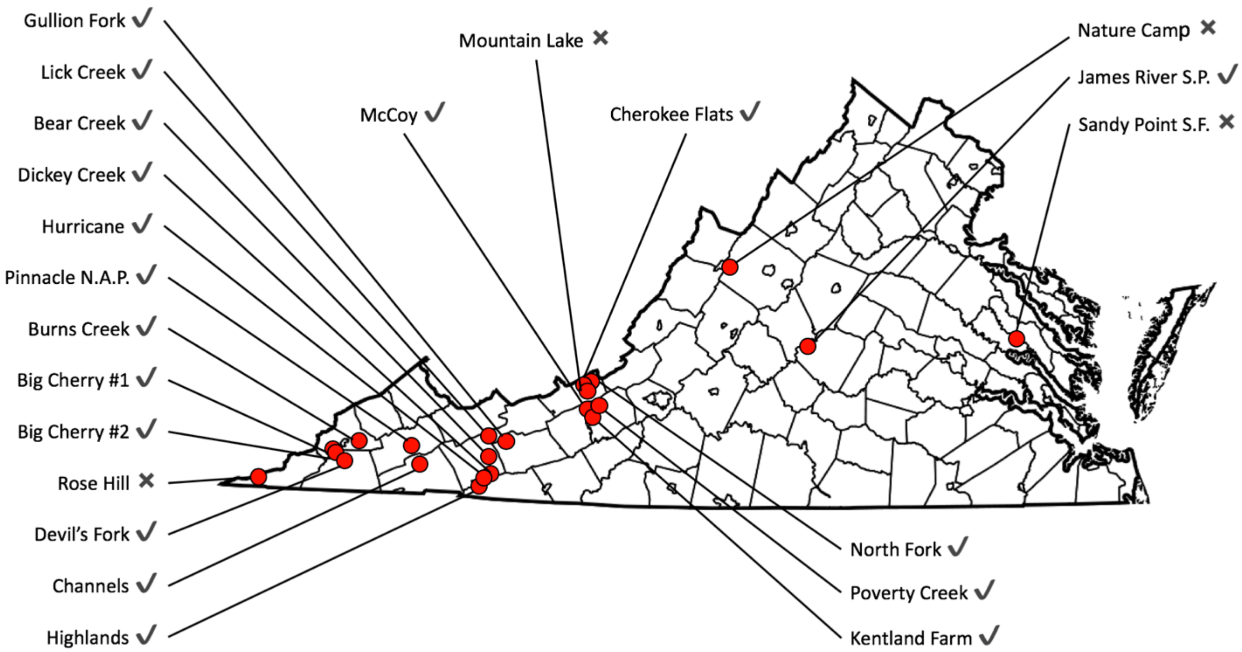

Figure 2.2. Establishment of *L. nigrinus* at Virginia release sites.
'✓' - Indicates sites where *L. nigrinus* was recovered
'X' - Indicates sites where no *L. nigrinus* recoveries were made

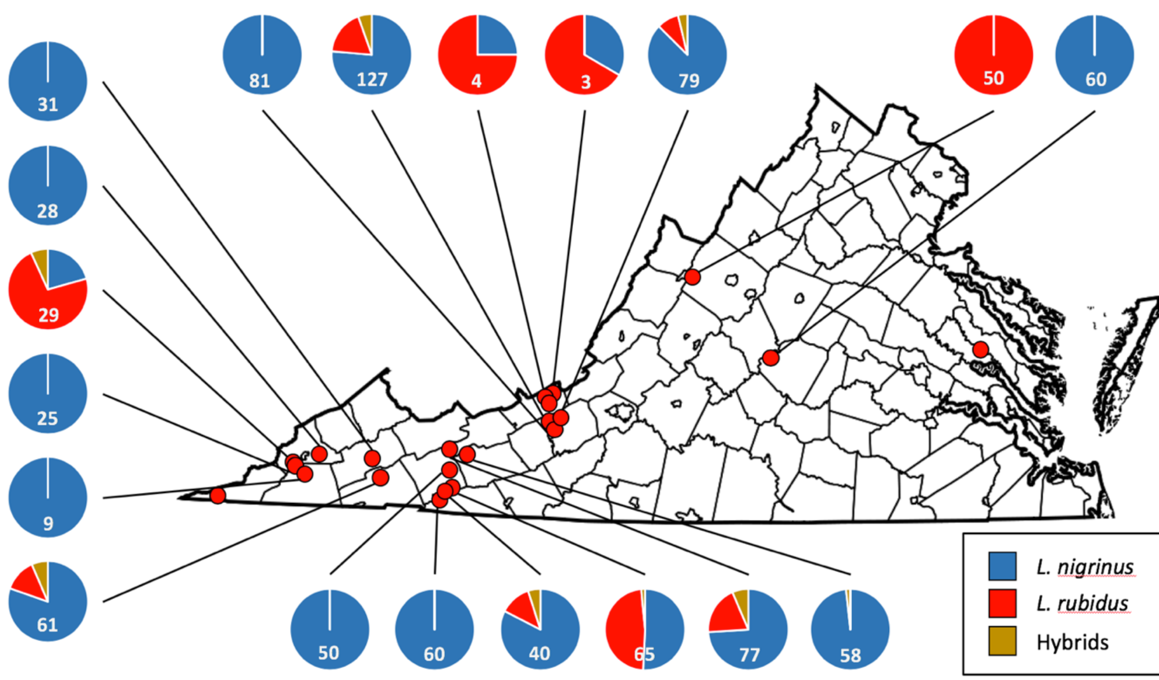

Figure 2.3. Percentage of *L. nigrinus*, *L. rubidus*, and hybrids, including sample size of individuals genetically tested, for each Virginia release site which had adult or larval recoveries. Percentage data reflects combined 2017 and 2018 recoveries.

2.4 References

Arsenault, A. L., N. P. Havill, A. E. Mayfield, and K. F. Wallin. 2015. Behavioral responses of *Laricobius* spp. and hybrids (Coleoptera: Derodontidae) to hemlock woolly adelgid and adelgid host tree odors in an olfactometer. Environ. Entomol. 44: 1562-1570.

Cheah, C. A. S.-J., and M. S. McClure. 1995. Exotic natural enemies of *Adelges tsugae* and their potential for biological control, pp. 103-112. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.

Clark, R. C., and N. R. Brown. 1960. Studies of predators of the balsam woolly aphid, *Adelges piceae* (Ratz.) (Homoptera: Adelgidae), VII. *Laricobius rubidus* (Lec.) (Coleoptera: Derodontidae), a predator of *Pineus strobi* (Htg.) (Homoptera: Adelgidae). Can. Entomol. 92: 237-240.

Clausen, C. P. 1951. The time factor in biological control - Presidential Address, 62nd Annual Meeting, American Association of Economic Entomologists. J. Econ. Entomol. 44: 1-9.

Davis, G. A., S. M. Salom, C. C. Brewster, B. P. Onken, and L. T. Kok. 2012. Spatiotemporal distribution of the hemlock woolly adelgid predator *Laricobius nigrinus* after release in eastern hemlock forests. Agric. For. Entomol. 14: 408-418.

Davis, G. A., N. P. Havill, Z. N. Adelman, A. Caccone, L. T. Kok, and S. M. Salom. 2011. DNA barcodes and molecular diagnostics to distinguish an introduced and native *Laricobius* (Coleoptera: Derodontidae) species in eastern North America. Biol. Control 58: 53-59.

Fischer, M. J., N. P. Havill, C. C. Brewster, G. A. Davis, S. M. Salom, and L. T. Kok. 2015. Field assessment of hybridization between *Laricobius nigrinus* and *L. rubidus*, predators of Adelgidae. Biol. Control 82: 1-6.

Havill, N. P., and R. G. Foottit. 2007. Biology and evolution of Adelgidae. Annu. Rev. Entomol. 52: 325-349.

Havill, N. P., M. E. Montgomery, G. Y. Yu, S. Shiyake, and A. Caccone. 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Ann. Entomol. Soc. Am. 99: 195-203.

Havill, N. P., G. Davis, D. L. Mausel, J. Klein, R. McDonald, C. Jones, M. Fischer, S. Salom, and A. Caccone. 2012. Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control. Biol. Control 63: 359-369.

Heminger, A. R. 2017. 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. M.S. L.F.S Thesis, Virginia Tech, Blacksburg, VA. 93 pp.

Klein, J. L., A. Caccone, and N. P. Havill. 2010. Polymorphic microsatellite loci for *Laricobius nigrinus* and *L. rubidus* (Coleoptera: Derodontidae), predators of the hemlock woolly adelgid. Mol. Ecol. Resour. 10: 751-754.

Lamb, A. B., S. M. Salom, and L. T. Kok. 2005. Guidelines for rearing *Laricobius nigrinus* Fender, pp. 309-318. *In* B. Onken and R. Reardon [eds.], Proceedings of the Third Symposium on Hemlock Woolly Adelgid in the Eastern United States. USDA Forest Service FHTET-2005-01.

Mausel, D. L., R. G. Van Driesche, and J. S. Elkinton. 2011. Comparative cold tolerance and climate matching of coastal and inland *Laricobius nigrinus* (Coleoptera: Derodontidae), a biological control agent of hemlock woolly adelgid. Biol. Control 58: 96-102.

Mausel, D. L., L. T. Kok, and S. M. Salom. 2017. Numerical response and impact of *Laricobius nigrinus* (Coleoptera: Derodontidae) on *Adelges tsugae* (Hemiptera: Adelgidae) in their native range. Environ. Entomol. 46: 544-551.

Mausel, D. L., S. M. Salom, L. T. Kok, and J. G. Fidgen. 2008. Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera: Derodontidae) on hemlock woolly adelgid in Virginia. Environ. Entomol. 37: 1498-1507.

Mausel, D. L., S. M. Salom, L. T. Kok, and G. A. Davis. 2010. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. Environ. Entomol. 39: 440-448.

Mayfield, A. E., B. C. Reynolds, C. I. Coots, N. P. Havill, C. Brownie, A. R. Tait, J. L. Hanula, S. V. Joseph, and A. B. Galloway. 2015. Establishment, hybridization and impact of *Laricobius* predators on insecticide-treated hemlocks: Exploring integrated management of the hemlock woolly adelgid. For. Ecol. Manage. 335: 1-10.

McAvoy, T. J., J. Régnière, R. St-Amant, N. F. Schneeberger, and S. M. Salom. 2017. Mortality and recovery of hemlock woolly adelgid (*Adelges tsugae*) in response to winter temperatures and predictions for the future. Forests 8: 21 pp.

McClure, M. S. 1989. Evidence of a polymorphic life-cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). Ann. Entomol. Soc. Am. 82: 50-54.

McClure, M. S., and C. A. S.-J. Cheah. 1999. Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. Biol. Invasions 1: 247-254.

Mech, A. M., P. C. Tobin, R. O. Teskey, J. R. Rhea, and K. J. K. Gandhi. 2018. Increases in summer temperatures decrease the survival of an invasive forest insect. Biol. Invasions 20: 365-374.

Paradis, A., J. Elkinton, K. Hayhoe, and J. Buonaccorsi. 2008. Role of winter temperature and climate change on the survival and future range expansion of the hemlock woolly adelgid (*Adelges tsugae*) in eastern North America. Mitig. Adapt. Strat. Gl. 13: 541-554.

Salom, S. M., L. T. Kok, A. B. Lamb, and C. S. Jubb. 2012. Laboratory rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A predator of the hemlock woolly adelgid (Hemiptera: Adelgidae). Psyche 2012: 9.

Stiling, P. 1990. Calculating the establishment rates of parasitoids in classical biological control. Am. Entomol. 36: 225-230.

Thode, H. C. 2002. Testing for Normality, Marcel Dekker, New York, NY.

Tobin, P. C., R. M. Turcotte, L. M. Blackburn, J. A. Juracko, and B. T. Simpson. 2017. The big chill: Quantifying the effect of the 2014 North American cold wave on hemlock woolly adelgid populations in the central Appalachian Mountains. Popul. Ecol. 59: 251-258.

Trotter, T. R., and K. S. Shields. 2009. Variation in winter survival of the invasive hemlock woolly adelgid (Hemiptera: Adelgidae) across the eastern United States. Environ. Entomol. 38: 557-587.

USDA. 2012. USDA Plant Hardiness Zone Map, https://planthardiness.ars.usda.gov/PHZMWeb/. United States Department of Agriculture.

USDA Forest Service. 2011. Crowns: Measurements and sampling, pp. 1-22, Phase 3 Field Guide, Version 5.1. USDA Forest Service.

Van Driesche, R., and T. S. Bellows. 1996. Biological Control, Chapman & Hall, New York.

Virginia Tech. 2019. HWA Predator Database, http://hiro.ento.vt.edu/pdb/. Virginia Tech.

Wiggins, G. J., J. F. Grant, J. R. Rhea, A. E. Mayfield, A. Hakeem, P. L. Lambdin, and A. B. L. Galloway. 2016. Emergence, seasonality, and hybridization of *Laricobius nigrinus* (Coleoptera: Derodontidae), an introduced predator of hemlock woolly adelgid (Hemiptera: Adelgidae), in the Tennessee Appalachians. Environ. Entomol. 45: 1371-1378.

Zilahi-Balogh, G. M. G., L. T. Kok, and S. M. Salom. 2002. Host specificity of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a potential biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). Biol. Control 24: 192-198.

Zilahi-Balogh, G. M. G., L. M. Humble, A. B. Lamb, S. M. Salom, and L. T. Kok. 2003. Seasonal abundance and synchrony between *Laricobius nigrinus* (Coleoptera: Derodontidae) and its prey, the hemlock woolly adelgid (Hemiptera: Adelgidae). Can. Entomol. 135: 103-115.

Chapter 3: Impact assessment of predatory beetle, *Laricobius nigrinus*, on hemlock woolly adelgid in the eastern United States

Abstract

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an invasive pest causing significant mortality to eastern and Carolina hemlock trees in the eastern U.S. Since 2003, management of HWA has included controlled release of the HWA predator *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), native to the Pacific Northwest. A two-phase study to assess the impact of *L. nigrinus* on HWA at nine release sites from New Jersey to Georgia was initiated in 2014 (Phase One). *L. nigrinus* was released at each of these sites at least 4 years prior to the study and was determined to have established at all of the sites. Significant reduction of HWA sistens ovisacs on study branches were noted during this phase of the study, therefore, it was continued in 2016 to provide longer-term evaluations of *L. nigrinus* impact (Phase Two). To evaluate the impact assessment of *L. nigrinus*, predator exclusion cages were used. Two paired treatments of either caged or no-cage branches were utilized to monitor predator and prey populations. Two assessments were made during the study period; one in October/November when treatment cages were applied, and one in the March/April when treatment cages were removed. In the first assessment, initial HWA density on study branches was determined. During the second assessment, HWA winter mortality, and cumulative predation of HWA by *L. nigrinus* was quantified. In Phase Two of the study, significantly more HWA sistens ovisacs were disturbed on no-

cage branches when compared to caged branches. Disturbance rates on no-cage branches were as high as 80%. Winter temperatures were also a significant factor in overall mortality of the sistens generation with rates as high as 92% at some sites. *Laricobius* spp. larval recoveries were made at all sites during Phase Two. When data were pooled for all sites, approximately 97% of larval recoveries were *L. nigrinus*, 2% were hybrids of *L. nigrinus* and *L. rubidus*, and 1% were *L. rubidus*. These data suggest that *L. nigrinus* significantly reduces the HWA sistens generation.

3.1 Introduction

Two native hemlock species in the eastern United States, eastern hemlock (*Tsuga canadensis* (L.) Carriere), and Carolina hemlock (*Tsuga caroliniana* Engelm), are currently being threatened by the invasive insect, hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). The first detection of HWA in eastern North America was in Richmond, VA where it likely arrived sometime prior to 1951 (Havill et al. 2006). Since its accidental introduction, HWA has spread throughout a significant portion of the native range of eastern hemlock. HWA is endemic to Asia and western North America and is associated with the nine other hemlock species worldwide (Havill et al. 2016). Populations in the native range however, remain at innocuous levels due to the co-evolution of resistance in host trees, and an assemblage of associated natural enemies (Cheah and McClure 1995, McClure and Cheah 1999). HWA has an anholocyclic life cycle in the eastern U.S, with two distinct asexual generations per year; the longer over-wintering sistens generation, and a shorter, spring progrediens generation (McClure 1989, Havill and Foottit 2007). HWA feed by inserting their stylet bundles into hemlock xylem ray parenchyma cells where they uptake plant nutrients (Mcclure 1987, Young et al. 1995). This feeding can cause branch dieback leading to tree mortality in 4-10 years (McClure 1991, Orwig et al. 2002).

Current management of HWA involves a suite of tactics. Chemical controls such as neonicotinoid formulations are highly effective and favored for urban environments (Silcox 2002, Webb et al. 2003, Cowles et al. 2005, Benton et al. 2015). In the forest setting, chemical controls have been a significant part of the management efforts, however they are not a long-term solution due to cost effectiveness and concerns with potential impacts on non-target organisms (McClure et al. 2001, Dilling et al. 2009). Some additional management techniques, currently in development include silvicultural practices, resistance breeding, and gene conservation, however; classical biological control has been the significant focus of HWA management in the forest setting (Jetton et al. 2008, Havill et al. 2011, Vose et al. 2013).

Studying predator complexes in the native and introduced ranges of invasive pests is a critical early step in the implementation of classical biological control programs (Rosen and DeBach 1992, Van Driesche et al. 2008). Investigations of potential natural enemies of HWA in the introduced range of the eastern U.S. indicated that although present, predators were often generalists and did not manage HWA populations to levels that would prevent hemlock mortality (Wallace and Hain 2000). Surveys were also conducted in areas where HWA was native, such as; Japan, China, and western

North America. The status and details of those efforts have been summarized by Onken and Reardon (2011). In western North America, the most abundant predator recovered in association with HWA on hemlock was *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) (Kohler et al. 2008). This species was imported to Virginia Tech from Victoria B.C. in 1997 for evaluation as a potential biological control agent under quarantine (Zilahi-Balogh et al. 2003). *Laricobius nigrinus,* like other species in the genus, are known to be specialists of family Adelgidae and are therefore the focus of biological control efforts (Lamb et al. 2011). During evaluations in quarantine, *L. nigrinus* was found to be univoltine (Zilahi-Balogh et al. 2003) and highly host-specific to HWA (Zilahi-Balogh et al. 2002), with both species having a coinciding summer aestivation. *Laricobius nigrinus* also showed ovipositional preference for HWA, and in field studies, significantly reduced HWA sistens ovisac densities when placed in caged branches compared to uncaged branches without these predators (Lamb et al. 2005b).

In addition to *L. nigrinus*, there are two other species of *Laricobius* that are of particular interest to HWA biological control efforts in the eastern U.S. *Laricobius osakensis* Shiyake and Montgomery was most recently imported from Japan and has been reared, released, and established at several sites (Mooneyham et al. 2016, Toland et al. 2018). The other, *Laricobius rubidus* LeConte, is an eastern North American native whose primary host is pine bark adelgid, *Pineus strobi* Hartig (Hemiptera: Adelgidae) on white pine, (*Pinus strobus* L.) (Clark and Brown 1960, Wantuch et al. 2019). When white pine and hemlock co-occur in forests, *L. rubidus* often capitalizes on the abundance of HWA and can be found feeding and completing development on this pest (Zilahi-Balogh et al. 2005). *L. nigrinus* and *L. rubidus* are sister species which have a recent divergence from a common ancestor and are capable of reproducing with each other. Resulting hybrid offspring are shown to be reproductively viable (Havill et al. 2012, Fischer et al. 2015).

Post-release evaluations of predators deployed in classical biological control programs are critical in determining the status of project objectives (Luck et al. 1999, Hajek 2004). The first operational releases of *L. nigrinus* began in 2003 and have been the focus of significant funding by various agencies. Over 400,000 beetles have since been released from field and lab-reared sources (Virginia Tech 2019), and have shown to establish and disperse (Mausel et al. 2010, Davis et al. 2012). This leads us to a next step in its assessment; their ability to manage HWA populations. The purpose of this study was to evaluate the impact of *L. nigrinus* on HWA sistens generation ovisacs at select sites where the beetle was introduced in the eastern U.S., to help inform future management goals for this pest. Impact was evaluated by making paired comparisons using predator exclusion cages. In this study, initial HWA densities, HWA winter mortality, and *L. nigrinus* predation of HWA ovisacs quantified. We hypothesize that study branches exposed to *L. nigrinus* would have higher rates of HWA ovisac disturbance than those branches where predators were excluded. Because prior studies indicated minimal presence and impact of existing native predators on HWA during the selected timing of this study, we are able to evaluate *L. nigrinus* activity on

HWA specifically (Montgomery and Lyon 1995, Wallace and Hain 2000). The study reported here (Phase Two - Fall 2016 to Spring 2018) is a continuation of Virginia Tech research conducted in Phase One - Fall 2014 to Spring 2016 (Heminger 2017). The eastern U.S. experienced two polar vortex events that caused extreme low winter temperatures in January 2014 and February 2015. Those temperatures caused high mortality of both HWA and *L. nigrinus* populations at study sites. Therefore, the study was extended for a second phase to better capture the cycling of HWA populations with the predator over time.

3.2 Materials and Methods

3.2.1 Field site and study tree selection

Nine field sites in six states (New Jersey, Maryland, Virginia, North Carolina, Tennessee, and Georgia) were selected and utilized in Phases One and Two of the study (Fig. 3.1). The sites are situated within four distinct USDA Plant Hardiness Zones that reflect average annual minimum temperatures experienced in the past 30 years (Table 3.1). The zones included in the study were 6a (-23.3 to -20.5°C), 6b (-20.6 to -17.8°C), 7a (-17.8 to -15°C) and 7b (-15 to -12.2°C) (USDA 2012). Site selection criteria were three-fold. First, releases of *L. nigrinus* were to have been made at least 4 years prior to the initiation of the study in 2014. Second, recoveries of *L. nigrinus* at these sites were made multiple years after the initial release which suggested this species established. Finally, HWA densities were moderate to heavy (2-3 HWA/cm) to allow for adequate prey populations to examine predation in the study.

3.2.2 Assessment 1: HWA density

The first assessment was made between October and early November after HWA instars had broken summer aestivation and begun development. During this time, adult *L. nigrinus* emerges from a subterranean summer aestivation period in soil and migrates to hemlock to begin feeding on HWA. The exact timing of Assessment 1 varied by geographic location of the site and average local temperatures leading up to this time period, as cessation of aestivation in both HWA and *L. nigrinus* was triggered by a decrease in temperature. Warmer temperatures often affected phenology and prolonged aestivation in both species. HWA populations were therefore monitored to assess the most appropriate time to initiate the assessment. During Assessment 1, *T. canadensis* trees were selected based on the presence of branches containing approximately 2-3 HWA/cm, however, these densities were not always present and therefore, branches with the highest densities were chosen. In year one, the number of trees used per site varied, and some trees hosted multiple paired treatments, however in year two, a total of 15 trees per site were used with one set of paired treatments per tree. Following tree selection, fifteen paired branches were randomly selected and tagged. After tagging, HWA densities on branches were recorded in the field by counting the number of HWA nymphs developing on new growth on each branchlet. Total HWA was divided by total length of new growth (cm) to obtain the number of HWA per cm on the study branch. Following these measurements, the treatments were assigned to the branch. The first treatment was an open branch (no-cage) which was completely exposed to HWA predators. The second treatment was a branch fully enclosed within a predator exclusion cage made of fine nylon mesh and measuring 1 m in length (MegaView Science Co. Ltd., Taichung, Taiwan). The cage was intended to exclude predators from HWA, allowing for comparisons of *L. nigrinus* predation levels with branches that were open to predators. During Phase One of the study, an additional treatment of an open cage was utilized in order to detect any possible cage effects (Heminger 2017). With this treatment, a predator exclusion cage was applied to the branch, but the zippered enclosure was left open to allow free movement of predators in and out of the cage. No open cage effects were observed during the Phase One study; therefore, this treatment was excluded in Phase Two. Cages were secured to branches using zip ties to cinch the open end of the bag over a 7.5 cm section of 1.27 cm thick foam pipe insulation placed around the branch (Thermwell Products Co. Inc., Mahwah, NJ, USA). Branches then remained in the field for ca. four to five months to allow both HWA and *Laricobius* spp. to feed, develop, and oviposit. In 2016, all nine sites were utilized in the study. In 2017, hemlocks at site TN2 did not support adequate populations of HWA with which to run the experiment, so it was not utilized in either Assessment 1 or 2 of the study.

3.2.3 Assessment 2: HWA winter mortality and ovisac disturbance

A second assessment was performed in late-March to early-April, during peak *L. nigrinus* oviposition and when *L. nigrinus* larvae were first present on branches. Again, the timing of this assessment was based on geographic location and average local temperatures leading up to the time-period. This assessment evaluated rates of winter mortality, quantified predation of HWA sistens ovisacs, and determined densities of *L.*

nigrinus larvae present on study branches. Sites were visited, and sample branches were removed from study trees and were placed into 3.8 L plastic zip closure bags for transport to laboratory facilities for further analysis. The location of facilities used varied by site. Bags were allowed to passively fill with air prior to closure in an effort to minimize mechanical disturbance of ovisacs against the bag during transport. Upon arrival at the laboratory, branches were removed from bags and the proximal tips of stems were cut to allow for improved water uptake. The cut ends were inserted into saturated blocks of Instant Deluxe floral foam (Smithers-Oasis North America, Kent, OH) wrapped in Parafilm M (Beemis N.A., Neemah, WI), or other similar vessels for hydration. Blocks containing the study branches were then placed into funnels modified for *Laricobius* spp. larval rearing and were held in the following environmental conditions known to be appropriate for developing *L. nigrinus* larvae: 12h:12:h (L:D), 13-15°C (Lamb et al. 2005a, Salom et al. 2012). Funnels remained in place for approximately 4-6 weeks to allow *Laricobius* spp. larvae present on study branches to feed on HWA and develop through 4 instars. At maturity, *Laricobius* spp. larvae dropped from branches to a small collection jar placed at the bottom of the funnel. Jars were checked once daily to collect dropped mature larvae and this process was repeated until no further larvae were observed. Collected larvae were preserved in vials containing 95% EtOH to allow for downstream genetic analysis to make species or hybrid identifications. In both years of Phase One, some *Laricobius* spp. larval recoveries were made in caged samples which indicated that adults were not dislodged from branches when the study was initially set-up in Assessment 1. Those branches were removed from the study analysis and associated larvae were not counted as part of the total recovered.

In 2017 at the completion of larval development, branch samples were removed, and HWA winter mortality and ovisac predation was assessed using a dissecting microscope. Adelgids within undisturbed HWA ovisacs, which were smaller in size, shriveled, and were found to be hardened when pressure was applied using a straight tip teasing needle, were counted as dead due to winter temperatures. The smaller size of these adelgids indicated that mortality occurred while in an earlier instar when temperatures may have been more extreme. Winter mortality was not assessed in 2017 at TN1, TN3, NC1, and GA1. In 2018, winter mortality assessments were made prior to branches being placed into funnels. For these assessments, only a non-destructive visual analysis of ovisacs was made. Adelgids which were much smaller when compared to other ovisacs on the study branch were counted as dead due to winter temperatures. These counts were then repeated during the assessment of ovisac disturbance to confirm accuracy. Adelgids with intact wool that produced red hemolymph when pressure was applied using a straight tip teasing needle, were considered alive and their ovisacs were counted as undisturbed. Adelgids with ovisacs that had a blown-out appearance with shredded wool were considered preyed upon and were counted as disturbed. This method of counting ovisac disturbance as a means for quantifying predation has been used in several prior studies (Lamb et al. 2005b, Mausel et al. 2008, Vieira et al. 2011, Vieira et al. 2013a, Mayfield et al. 2015, Mausel et al.

2017). Percent winter mortality was calculated as the total number of winter-killed adelgid divided by the total number of adelgid (live + dead) on the branch. Percent ovisac disturbance was counted as the total number of disturbed ovisacs divided by the total number of adelgids (live + dead).

3.2.4 Genetic analysis of recovered *Laricobius* spp.

Genetic analysis was performed on *Laricobius* spp. larval samples recovered at study sites to identify species and potential hybrids of *L. nigrinus* and the native species, *L. rubidus*. DNA was extracted from larvae using the Omega Bio-tek E.Z.N.A.® Tissue DNA kit and its associated protocols (Omega Bio-tek, Inc., Norcross, GA). Six microsatellite loci (LaGT01, LaCA04, LaGT07, LaGT13, LaCA14, LaCA16) (Klein et al. 2010, Havill et al. 2012) were amplified using techniques described in Klein et al. (2010). Fragments were analyzed using a 3730xl 96-Capillary Genetic Analyzer at the DNA Analysis Facility at Science Hill, New Haven, CT. Alleles were called using Geneious Prime 2019 (Biomatters, Inc., Newark, NJ). Hybrids were distinguished from *L. nigrinus* and *L. rubidus* using the software programs Structure 2.3.2 (Stanford University) and New Hybrids 1.1 (University of California).

3.2.5 Statistical analyses

All statistical analyses were performed using JMP Pro 13.0 (SAS Pro, Inc. 2018). The effects of treatment (cage or no-cage) on initial HWA density, percent HWA winter mortality and percent HWA ovisac disturbance were tested within sites using a one-way analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$. The distributions of each of the response variables (HWA density, winter mortality, and ovisac disturbance) were tested for normality using the goodness-of-fit Shapiro-Wilk W test statistic, or by analysis of skewness and kurtosis values (Thode 2002, Zar 2010). HWA density data did not fit a normal distribution and were therefore square root transformed to meet the assumptions of the test. For winter mortality and ovisac disturbance, a constant of 0.001 was added to both data sets to remove zeroes (Zar 2010). The data were then Box-Cox transformed to achieve normality. Winter mortality and ovisac disturbance data from some sites still did not meet the assumptions of normality, leading to the use of Levene's test to assess homogeneity of variances between treatments. For those sites which had heterogeneity of variances, a Welch's ANOVA was used to assess differences between treatments. Original data values are reported.

3.3 Results

3.3.1 Assessment 1: HWA density

HWA density data were collected in the fall of 2016 and 2017 during Assessment 1. HWA densities varied considerably among sites with means ranging from 1-10 HWA/cm in 2016 and 1-7 HWA/cm in 2017 (Fig 3.2). There were no significant effects of treatment (cage or no-cage) on HWA densities when data were analyzed within sites in 2016 and 2017 (Table 3.2). Hemlocks at site TN2 did not support adequate populations of HWA with which to run the experiment in 2017, and therefore, was not used.

3.3.2 Assessment 2: HWA winter mortality and ovisac disturbance

In 2017, mean HWA winter mortality ranged from 2-92% at study sites (Fig. 3.3). Treatment had a significant effect on winter mortality with caged branches having higher rates of mortality at NJ1, MD1 and VA2, but not at other sites (Table 3.2, Fig. 3.3). In 2018, mean winter mortality ranged from 20-84% at study sites. Treatment had a significant effect on winter mortality with caged branches having higher rates of mortality at MD1, VA1 and VA2, but not at other sites (Table 3.2, Fig. 3.3). Data indicated that mean winter mortality decreased as USDA Plant Hardiness Zone increased (Table 3.3).

Mean percent disturbance at study sites in 2017 ranged from 1-16% in caged samples and 12-80% on no-cage samples (Fig. 3.4). In both 2017 and 2018, there was a significant effect of treatment on percent ovisac disturbance. No-cage branches had higher mean percent ovisac disturbance at all sites except VA1 in 2017 and NJ1 in 2018 (Table 3.2, Fig. 3.4). In 2018, mean percent ovisac disturbance ranged from 0-16% in caged samples, and from 9-57% in no-cage samples (Fig. 3.4).

3.3.3 *Laricobius* spp. larval recoveries and genetic analysis

Genetic analysis revealed that *L. nigrinus* was the dominant species on study branches. *L. rubidus* and hybrids of *L. rubidus* and *L. nigrinus* were also recovered. Recoveries of *Laricobius* spp. larvae occurred at all sites during the period of the study (Fig. 3.4). In 2017, larvae were recovered at 8 of 9 sites (Table 3.4). The mean percent *L. nigrinus*, *L. rubidus*, and hybrid recoveries for all sites were 85%, 14%, and 1%, respectively. Larvae were recovered at 8 of 8 sites in 2018 (Table 3.4). The mean percent *L. nigrinus*, *L. rubidus*, and hybrid recoveries for all sites were 92%, 0%, 8% respectively.

3.4 Discussion

This four-year study represents the first assessment of the impact of *L. nigrinus* on HWA at multiple release sites and USDA Plant Hardiness Zones in the eastern U.S. This approach evaluated HWA sistens ovisac disturbance as a measure of predation. The results of this study demonstrated higher rates of HWA ovisac disturbance on no-cage branches when compared to those where predators were excluded, and that *L. nigrinus* was the primary species responsible for the disturbance. Details of prior studies using ovisac disturbance as a measure of predation are reported by Mausel et al. (2008) and Mayfield et al. (2015). Both of these studies showed significantly higher rates of HWA ovisac disturbance on no-cage branches when compared to those that were caged. The VA2 site used in the study reported here, was also utilized by Mausel et al. (2008), and the GA1 location in our study, was also used by Mayfield et al. (2015). The results of our study are consistent with the results of these two studies and demonstrate continued predatory impact of *L. nigrinus* on HWA populations at multiple sites for multiple years. Our evaluations of impact assessment build on data observed in these prior studies and are an important step in the development of this classical biological control program. Knowing the status of released predators and their ability to control the target pest, informs future management decisions and can save implementing agencies time and help direct allocation of future funding efforts (Luck et al. 1999, Hajek 2004).

Pre-treatment densities in Assessment 1 varied greatly between sites and years over the duration of the study, however, they remained consistent between treatments at each site which was ideal for analyzing treatment effects in Assessment 2. At several sites, HWA densities showed a distinct period of gradual increase after sustained low temperatures experienced during the polar vortex events in January 2014 and February 2015. These temperatures caused high mortality to HWA (Tobin et al. 2017) and *L. nigrinus* populations (Heminger 2017). In general, both HWA and *L. nigrinus* populations appeared to recover in the years following these damaging temperatures. At some sites, such as GA1, HWA density remained more consistent throughout the study. This could be attributed to the southern latitude of the site which may provide less drastic winter temperatures than those seen at more northern latitudes (McAvoy et al. 2017). Some sites experienced a decline in HWA densities and new growth on branches in 2018. This decline may be related to density-dependent HWA population changes. High density HWA infestations on hemlock have been shown to cause a deterioration in tree health, which can then in turn cause a subsequent decline in HWA populations. Trees will often make a partial recovery from HWA feeding during this decline, however HWA eventually returns, and their populations build again (McClure 1991, Sumpter et al. 2018). It is possible we observed some of these effects after a rebound of HWA following the polar vortex events. Decreased HWA populations could also have occurred due to certain abiotic factors unique to each site such as drought or excessively moist conditions, or temperature extremes in both the summer and winter (McAvoy et al. 2017).

Winter temperatures experienced by the HWA sistens generation in the field setting proved to be an important factor in overall mortality of this insect with rates as high as 97% seen on branches throughout the four years of the study. In both 2017 and 2018 there were cage effects indicated at some sites. This effect was unique to Phase Two of the study, as an open cage treatment was implemented in Phase One to detect such effects, and none were noted (Heminger 2017). Similar prior studies showed no effects of temperature caused by the use of exclusion cages on lower canopy branches (Lamb et al. 2005b, Lamb et al. 2006, Mausel et al. 2008), however, there may be site specific factors that spur the occurrence of a cage effect such as variation of microclimates at branch locations, or cage materials used. Although sleeve cages have been reported to affect branch microclimates by altering temperature, light intensity, and wind, research has shown that these effects are inconsistent and highly variable (Smith and De Bach 1942, Luck et al. 1988, Nelson and Rieske 2014). Efforts were taken by research collaborators to randomize selection of treatment branches, so that a variety of microclimate conditions would be represented in the study, so it is difficult to identify a cause for this cage effect.

Rates of sistens generation ovisac disturbance in no-cage samples in both years of Phase Two indicate that *Laricobius* spp. can have a significant impact on the eggs of progrediens generation laid by these adults. *Laricobius* spp. larval recovery totals followed closely with the patterns of disturbance at many sites in most years with recoveries increasing as ovisac disturbance increased. Although one coccinellid larva and one syrphid larva were collected in Phase One of the study, no alternate predators were recovered on study branches during Phase Two, indicating that disturbance of HWA ovisacs was primarily the result of feeding by *Laricobius* spp. Other common generalist predators such as *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) typically become active after the period of our study, and therefore, were likely not responsible for ovisac disturbance observed (Koch 2003). Ovisacs disturbed by *Laricobius* spp. larvae lost their spherical form, and had a shredded appearance caused by the larvae displacing the wool from the branch. The HWA eggs originally contained within ovisacs were often completely consumed by *Laricobius* spp. larvae and those that remained likely became desiccated as a result of this disturbance and did not hatch. This type of feeding is consistent with the genus *Laricobius* as noted in Brown and Clark (1962) and has been used as a proxy for *Laricobius* spp. predation in several prior studies (Lamb et al. 2005b, Mausel et al. 2008, Vieira et al. 2011, Vieira et al. 2013b, Mayfield et al. 2015, Mausel et al. 2017).

There were low recoveries of *Laricobius* spp. at all three TN sites in 2017, however, the data show that significant ovisac disturbance occurred. The low larval recoveries at these sites is remarkable, however, the ability of *Laricobius* spp. adults to create disturbance prior to larval abundance should not be discounted. Prior field cage studies showed significant impact by *L. nigrinus* adults on HWA nymphs during the pre-oviposition period (Lamb et al. 2005b). In our study, early feeding on HWA nymphs by

Laricobius spp. adults could have occurred prior to both oviposition and branches being removed for Assessment 2. It is possible that these adults dispersed from study branches to other areas within the site or tree. *Laricobius* spp. has been shown to migrate vertically within trees after initial release on lower-canopy branches. Davis et al. (2012) reported that *L. nigrinus* beetles dispersed to the upper crown (>15 m) for oviposition, and a large proportion of subsequent larvae were collected within these crown strata at some sites. Selection of treatment branches in the present study was limited to lower crown strata (< 2 m) due to the challenges involved with the application of predator exclusion cages to branches in the upper crown. An alternative explanation to impact without the recovery of *Laricobius* spp. larvae, could be due to mechanical disturbance. Although care was taken not to create disturbance during transport of branches to the laboratory, this disturbance could have been produced by branch-to-branch or branch-to-cage abrasion caused wind or animals in the field.

Development of a portion of the larval phase of *L. nigrinus* during Assessment 2 occurred in the laboratory setting which simulates select abiotic conditions present in the field such as temperature and daylength. Rearing conditions used in this study were selected based on those developed as a result of extensive testing detailed in Salom et al. (2012). These methods have been successfully used by the Virginia Tech insectary and other rearing facilities for mass production of *L. nigrinus* utilized for biological control. To date, approximately 300,000 *L. nigrinus* have been released from lab-reared sources (Virginia Tech 2019). We believe that although the laboratory rearing environment simulates only a portion of conditions experienced by the predator and the prey in the field, this provides an important initial assessment of impact on which future studies can build.

Results from the genetic analysis indicated that *L. nigrinus* was the primary *Laricobius* species present on study branches and therefore, most ovisac disturbance could be attributed their feeding activity. Subsamples of total larval recoveries were taken at some sites to reduce time and costs associated with the analysis. Some samples could not be identified due to issues with either DNA extraction or loci amplification during PCR. Hybridization rates in Phase Two of this study (1-8%), were lower than those indicated in previous studies which ranged from 11-28% (Havill et al. 2012, Arsenault et al. 2015, Fischer et al. 2015, Mayfield et al. 2015, Wiggins et al. 2016). We cannot however, confidently assert that hybridization rates are declining throughout the region because methods used this study are limited in their ability to detect later generation hybrids. Some hybrids that may have backcrossed with either *L. nigrinus* or *L. rubidus* may not have been detected (Havill et al. 2012). It is apparent however that some introgression continues to occur between species at several sites used in this study, and although limiting, identification through the use of microsatellite techniques provides a general indication of *Laricobius* population distribution. In order to improve hybridization results, additional microsatellite loci should be identified to make the analysis more robust (Nathan Havill, personal communication, February 1, 2019).

This study represents a sizeable snapshot of *L. nigrinus* impact over a variety of seasonal temperatures and USDA Plant Hardiness Zones. *Laricobius nigrinus* had a significant negative impact on the density of intact sistens ovisacs on study branches. In order to more fully understand the scope of *L. nigrinus* impact, future work should investigate how hemlock tree health is affected by *L. nigrinus* predation of HWA. The response of the HWA progrediens generation to *L. nigrinus* predation of sistens ovisacs is also an important component of overall impact on HWA populations. Recent work by researchers at the University of Massachusetts have investigated the ability of HWA to overcome predation by *L. nigrinus* on sistens ovisacs in their second, progrediens generation. Data from this study suggest that due to the parthenogenic reproductive biology of this species, rebound is likely possible. Although HWA populations appear to be able to recover, it is reasonable to assert that *L. nigrinus* plays a critical role in the overall predation of the sistens generation and progrediens eggs, and that effective management of this species in the eastern U.S. may only be possible with a suite of predators. This hypothesis is supported by the range of predators recovered during surveys on hemlock in the Pacific Northwest (Kohler et al. 2008). Evaluations investigating western strains of *Leucopis argenticollis* and *L. piniperda* in the eastern U.S. are in progress (Ross et al. 2011). These organisms may complement *L. nigrinus* by feeding during late-spring when *Laricobius* is in its inactive subterranean life stage. (Motley et al. 2017). The data presented here supports the continued use of *Laricobius nigrinus* for biological control of HWA, with the ultimate goal of reducing damage to native hemlocks in the eastern U.S.

Table 3.1. Phase One and Two impact assessment study sites, USDA Plant Hardiness Zones, and year of *L. nigrinus* release.

Site	Coordinates	Plant Hardiness Zone[a]	Release Year
NJ1	41.12 N, -74.91 W	6a	2007, 2008
MD1	39.70 N, -78.67 W	6b	2004
VA1	37.64 N, -78.80 W	7a	2005
VA2	37.21 N, -80.59 W	6b	2003
NC1	35.82 N, -82.21 W	6b	2005
TN1	35.76 N, -83.30 W	7a	2007
TN2	35.69 N, -83.87 W	7a	2008
TN3	35.66 N, -83.59 W	7a	2006
GA1	34.79 N, -83.76 W	7b	2008, 2010

[a]Plant Hardiness Zones are based on average annual minimum temperature and acquired from planthardiness.ars.usda.gov. 6a (-23.3 to -20.5°C), 6b (-20.6 to -17.8°C), 7a (-17.8 to -15°C) and 7b (-15 to -12.2°C).

Table 3.2. One-way ANOVA results comparing HWA density, winter mortality, and ovisac disturbance in 2017 (A) and 2018 (B) for all nine sample sites.

A.	Assessment 1 HWA/cm			Assessment 2 % Winter Mortality			Assessment 2 % Ovisac Disturbance		
Site	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*
NJ1	0.67	1, 34	0.42	5.32	1, 34	0.0274*	27.27	1, 18.52	0.0001*†
MD1	0.15	1, 28	0.70	20.21	1, 28	0.0001*	78.17	1, 28	0.0001*
VA1	0.04	1, 28	0.84	2.22	1, 28	0.15	1.92	1, 28	0.18
VA2	1.24	1, 26	0.28	49.63	1, 26	0.0001*	39.40	1, 26	0.0001*
NC1	0.17	1, 26	0.68	--	--	--	52.38	1, 14.20	0.0001*†
TN1	0.90	1, 22	0.35	--	--	--	240.76	1, 22	0.0001*
TN2	0.05	1, 26	0.82	--	--	--	42.94	1, 13.86	0.0001*†
TN3	1.31	1, 24	0.26	--	--	--	52.17	1, 15.41	0.0001*†
GA1	0.76	1, 24	0.39	--	--	--	65.43	1, 14.12	0.0001*†

B.	Assessment 1 HWA/cm			Assessment 2 % Winter Mortality			Assessment 2 % Ovisac Disturbance		
Site	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*
NJ1	0.07	1, 22	0.79	0.44	1, 22	0.52	1.70	1, 11.34	0.22†
MD1	0.14	1, 24	0.71	8.44	1, 24	0.0078*	22.61	1, 24	0.0001*
VA1	0.05	1, 20	0.82	26.74	1, 12.19	0.0002*†	72.65	1, 14.92	0.0001*†
VA2	1.18	1, 26	0.29	12.54	1, 18.25	0.0023*†	50.23	1, 14.24	0.0001*†
NC1	1.5	1, 28	0.24	0.17	1, 28	0.69	14.28	1, 14	0.0020*†
TN1	3.85	1, 28	0.06	0.17	1, 28	0.69	13.21	1, 16.22	0.0011*†
TN2	--	--	--	--	--	--	--	--	--
TN3	0.93	1, 22	0.35	2.42	1, 22	0.13	18.03	1, 16.21	0.0006*
GA1	2.50	1, 10	0.14	1.00	1, 10	0.34	12.86	1, 10	0.0050*

[1]A one-way ANOVA was used to determine the effects of treatment (cage or no-cage) on response variables. To address non-normality of data, HWA densities were square root transformed, and winter mortality and ovisac disturbance data were Box-Cox transformed.
*Indicates statistical significance at $p < 0.05$
--Data not collected at site
†Indicates analysis using Welch's ANOVA

Table 3.3. Mean HWA/cm, % winter mortality, % ovisac disturbance, and *Laricobius* spp. larval recoveries for study sites in each USDA Plant Hardiness Zone in Phase Two.

Plant Hardiness Zone[a]	Mean HWA/cm	Mean % Winter Mortality	Mean % HWA Ovisac Disturbance	Mean *Laricobius* spp. Larval Recoveries
6a	4.5 ± 0.5	67.1 ± 11.4	19.67 ± 8.9	15.2 ± 10.3
6b	2.9 ± 0.4	45.6 ± 4.7	50.1 ± 8.0	44.4 ± 10.6
7a	3.6 ± 0.5	38.9 ± 5.2	33.8± 5.4	6.5 ± 3.3
7b	4.4± 0.4	37.3 ± 2.4	37.0 ± 9.6	51.2 ± 19.8

[a]Plant Hardiness Zones: 6a (NJ1), 6b (MD1, VA2, NC1), 7a (VA1, TN1, TN2, TN3), 7b (GA1)

Table 3.4. Total larval recoveries, total larvae successfully tested using microsatellite analysis, and resulting percentages of *L. nigrinus*, *L. rubidus*, and hybrids at each site in Phase Two.

Year	Site	No. Larvae Recovered	No. Larvae Successfully Tested	% *L. nigrinus*	% *L. rubidus*	% Hybrid
2017	NJ1	44	40	100%	0%	0%
	MD1	51	25	96%	0%	4%
	VA1	**	**	**	**	**
	VA2	119	30	100%	0%	0%
	NC1	92	78	100%	0%	0%
	TN1	1	1	100%	0%	0%
	TN2	3	2	0%	100%	0%
	TN3	1	0	0%	0%	0%
	GA1	99	76	100%	0%	0%
2018	NJ1	17	16	100%	0%	0%
	MD1	31	29	100%	0%	0%
	VA1	50	30	100%	0%	0%
	VA2	73	30	100%	0%	0%
	NC1	63	60	93%	2%	5%
	TN1	17	15	93%	0%	7%
	TN2	--	--	--	--	--
	TN3	13	8	50%	0%	50%
	GA1	26	25	96%	0%	4%

**Larvae not recovered at site
--Data not collected at site

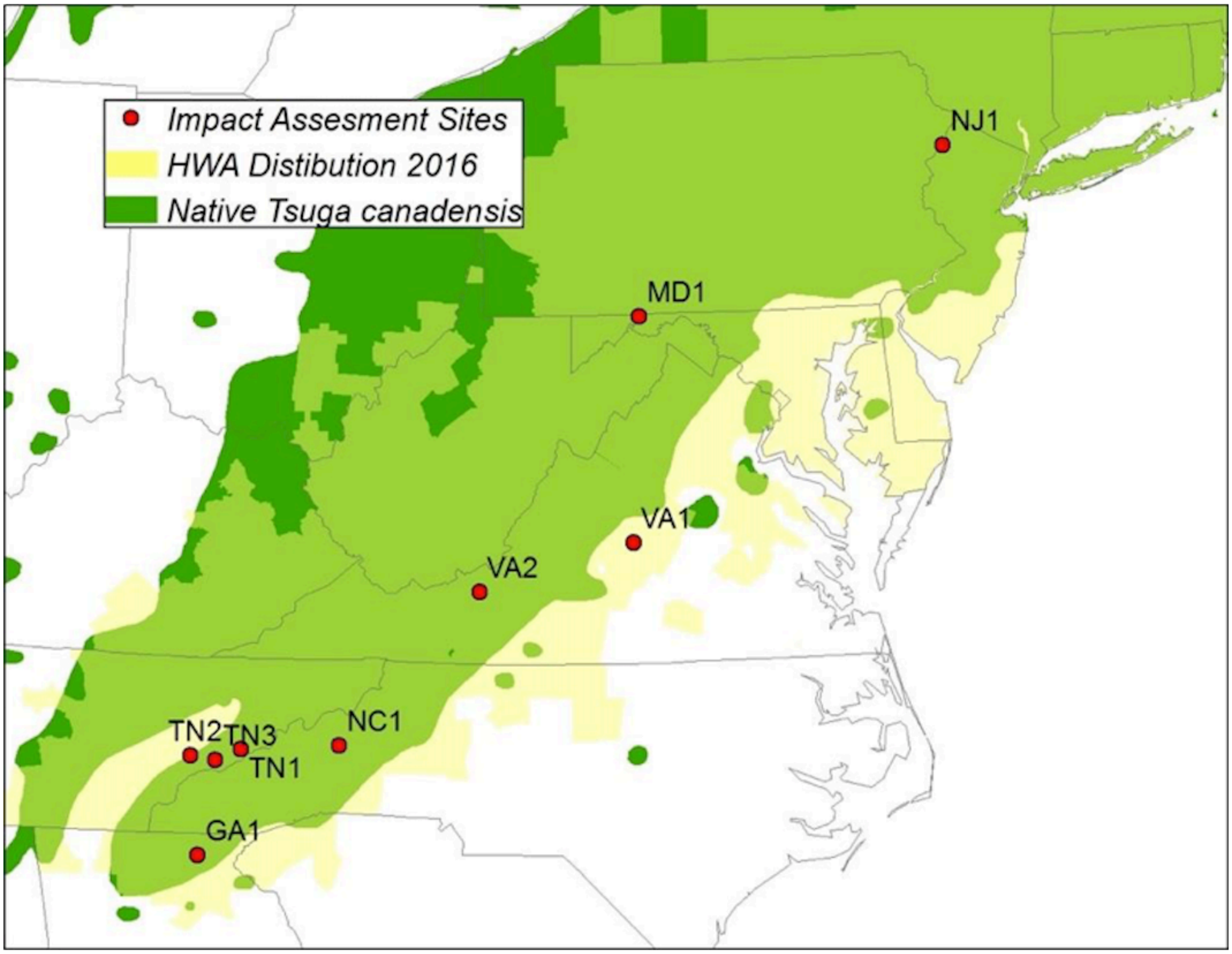

Figure 3.1. Locations of the nine *Laricobius nigrinus* impact assessment study sites used in the eastern U.S.

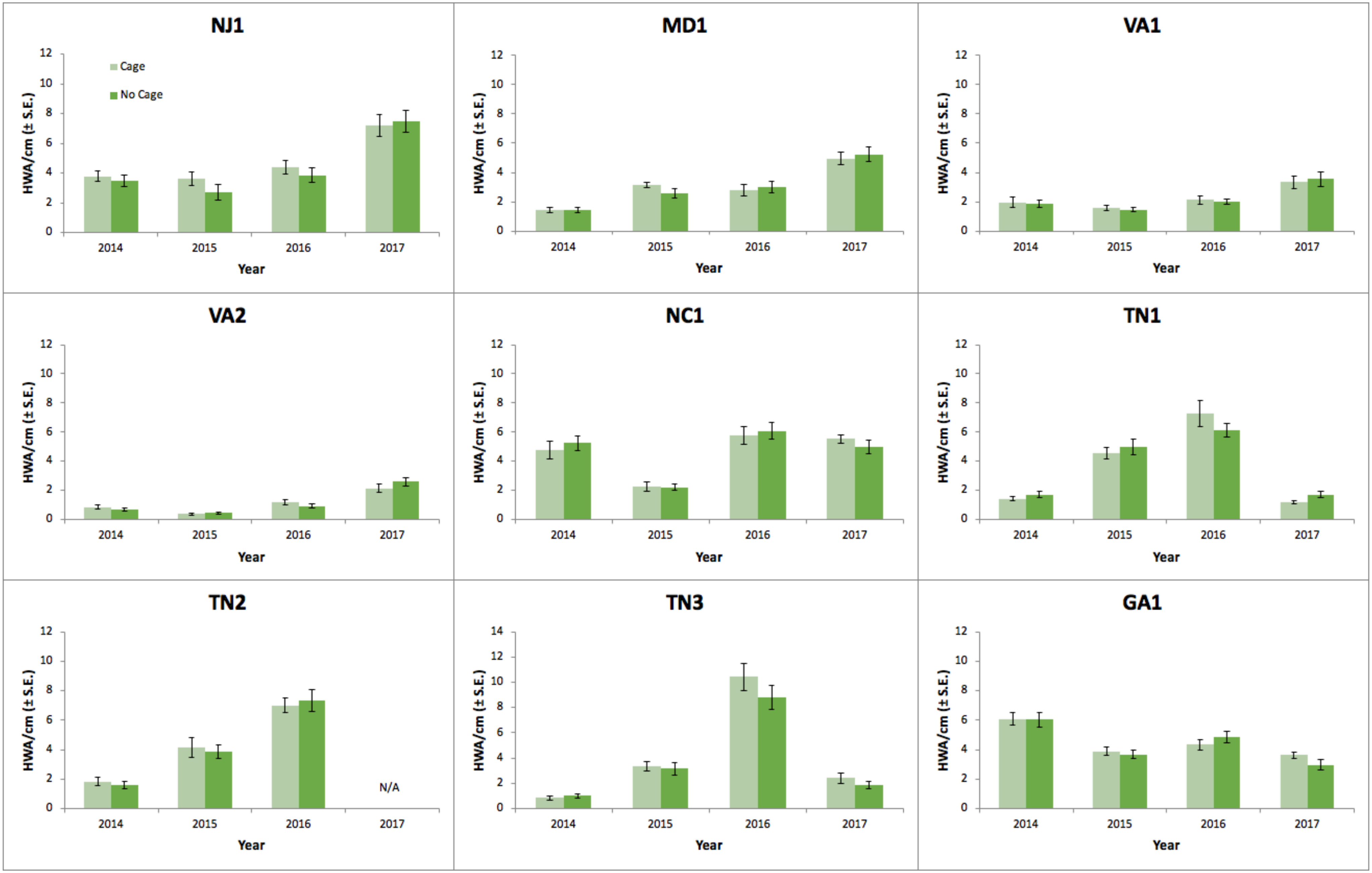

Figure 3.2. Assessment 1 – Mean (± S.E.) HWA sistens density on branches from 2014-2017 at the nine test sites. Data were collected at sites each year from October to November.
N/A - Indicates data not collected at site.

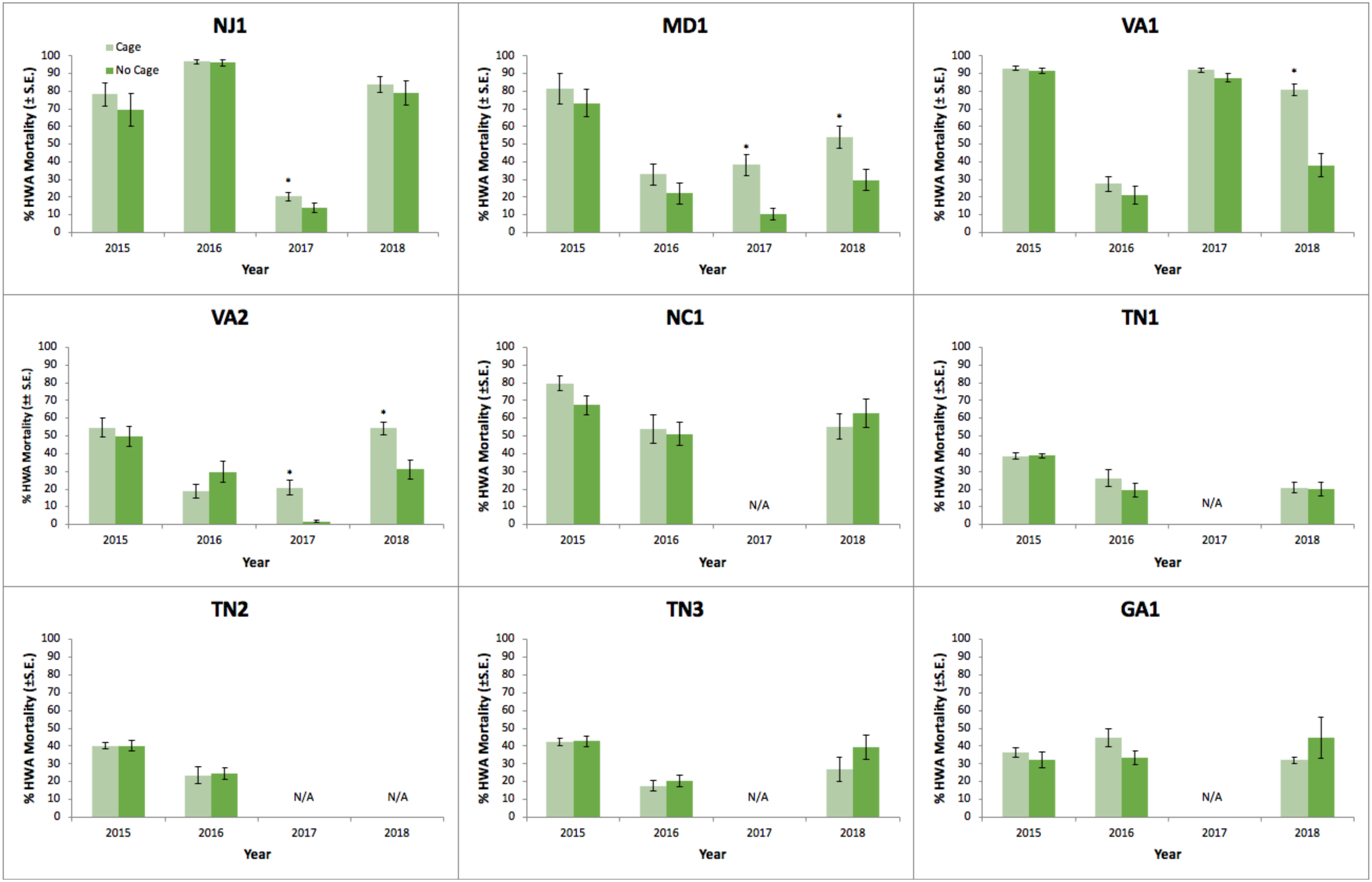

Figure 3.3. Assessment 2 - Mean (± S.E.) percent winter mortality of HWA sistens from 2015-2018. Data were collected at sites each year from March to April.
* - Indicates statistical significance between treatments using one-way, or Welch's ANOVA, $p < 0.05$.
N/A - Indicates data not collected at site

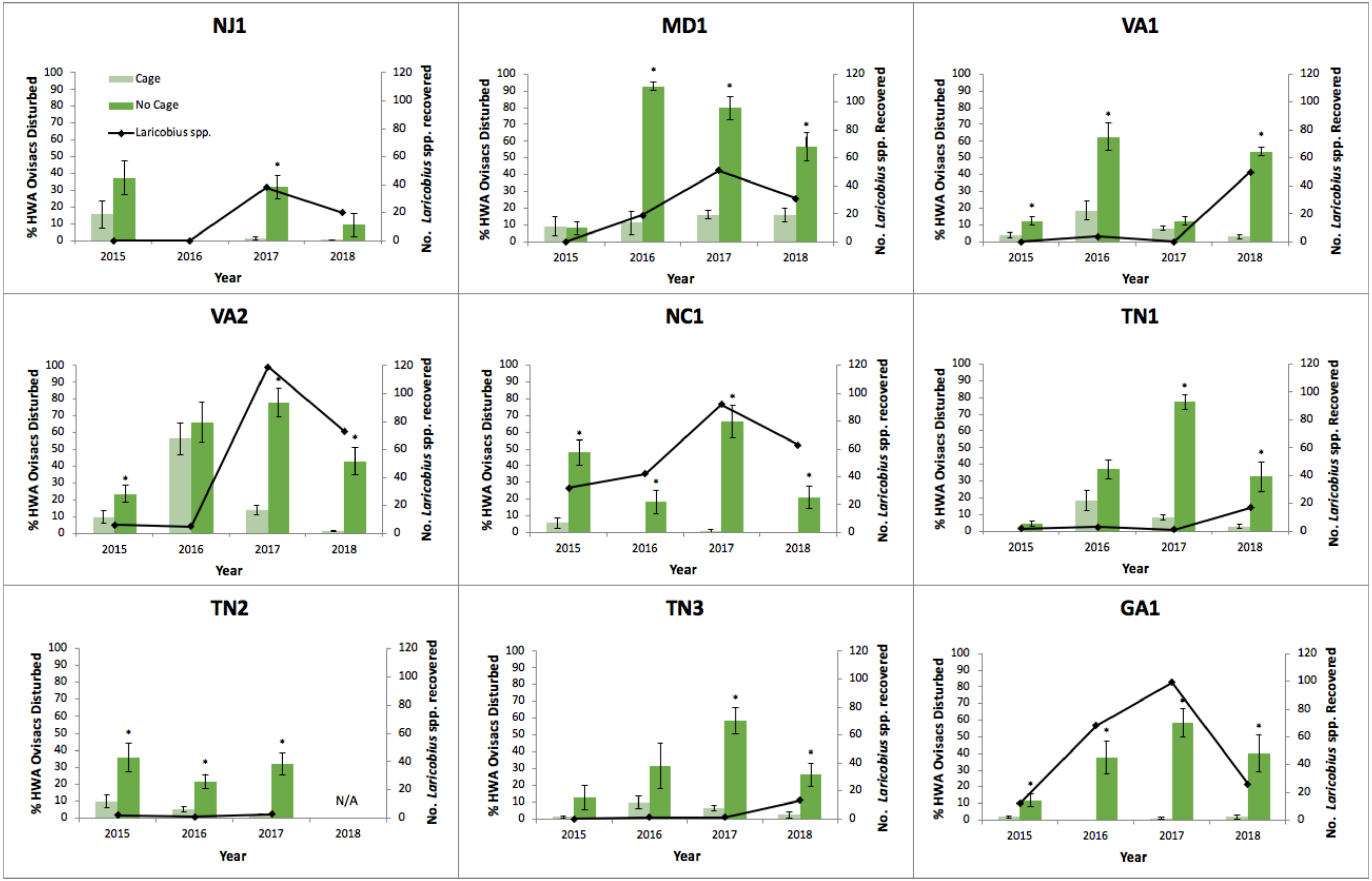

Figure 3.4. Assessment 2 - Mean ± S.E. percent HWA sistens ovisac disturbance and *Laricobius* spp. larval recoveries from 2015-2018
* - Indicates statistical significance between caged and uncaged treatments using one-way, or Welch's ANOVA, p < 0.05
N/A - Indicates data not collected at site

3.5 References

Arsenault, A. L., N. P. Havill, A. E. Mayfield, and K. F. Wallin. 2015. Behavioral responses of *Laricobius* spp. and hybrids (Coleoptera: Derodontidae) to hemlock woolly adelgid and adelgid host tree odors in an olfactometer. Environ. Entomol. 44: 1562-1570.

Benton, E. P., J. F. Grant, R. J. Webster, R. J. Nichols, R. S. Cowles, A. F. Lagalante, and C. I. Coots. 2015. Assessment of imidacloprid and its metabolites in foliage of eastern hemlock multiple years following treatment for hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in forested conditions. J. Econ. Entomol. 108: 2672-2682.

Brown, N. R., and R. C. Clark. 1962. Studies of predators of the balsam woolly aphid, *Adelges piceae* (Ratz.) (Homoptera: Adelgidae) X. Field identification of *Laricobius erichsonii* Rosen. (Coleoptera: Derodontidae). Can. Entomol. 94: 191-193.

Cheah, C. A. S.-J., and M. S. McClure. 1995. Exotic natural enemies of *Adelges tsugae* and their potential for biological control, pp. 103-112. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.

Clark, R. C., and N. R. Brown. 1960. Studies of predators of the balsam woolly aphid, *Adelges piceae* (Ratz.) (Homoptera: Adelgidae), VII. *Laricobius rubidus* (Lec.) (Coleoptera: Derodontidae), a predator of *Pineus strobi* (Htg.) (Homoptera: Adelgidae). Can. Entomol. 92: 237-240.

Cowles, R. S., C. A. S.-J. Cheah, and M. E. Montgomery. 2005. Comparing systemic imidacloprid application methods for controlling hemlock woolly adelgid, pp. 4-6. *In* K. W. Gottschalk [ed.], Proceedings of the 16th USDA Interagency Research Forum on Gypsy Moth and Other Invasive Species USDA Forest Service.

Davis, G. A., S. M. Salom, C. C. Brewster, B. P. Onken, and L. T. Kok. 2012. Spatiotemporal distribution of the hemlock woolly adelgid predator *Laricobius nigrinus* after release in eastern hemlock forests. Agric. For. Entomol. 14: 408-418.

Dilling, C., P. Lambdin, J. Grant, and R. Rhea. 2009. Community response of insects associated with eastern hemlock to imidacloprid and horticultural oil treatments. Environ. Entomol. 38: 53-66.

Fischer, M. J., N. P. Havill, C. C. Brewster, G. A. Davis, S. M. Salom, and L. T. Kok. 2015. Field assessment of hybridization between *Laricobius nigrinus* and *L. rubidus*, predators of Adelgidae. Biol. Control 82: 1-6.

Hajek, A. E. 2004. Natural Enemies: An Introduction to Biological Control, Cambridge University Press, Cambridge, UK, New York, NY.

Havill, N. P., and R. G. Foottit. 2007. Biology and evolution of Adelgidae. Annu. Rev. Entomol. 52: 325-349.

Havill, N. P., M. E. Montgomery, G. Y. Yu, S. Shiyake, and A. Caccone. 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Ann. Entomol. Soc. Am. 99: 195-203.

Havill, N. P., G. Davis, M. Fischer, S. Salom, D. Mausel, and B. P. Onken. 2011. The introduction of *Laricobius nigrinus* as a biological control agent for the hemlock woolly adelgid: Is there a threat to the native congener: *L. rubidus*, pp. 212-221. In B. Onken and C. Reardon (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service FHTET-2011-04, Morgantown, WV.

Havill, N. P., G. Davis, D. L. Mausel, J. Klein, R. McDonald, C. Jones, M. Fischer, S. Salom, and A. Caccone. 2012. Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control. Biol. Control 63: 359-369.

Havill, N. P., S. Shiyake, A. L. Galloway, R. G. Foottit, G. Y. Yu, A. Paradis, J. Elkinton, M. E. Montgomery, M. Sano, and A. Caccone. 2016. Ancient and modern colonization of North America by hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), an invasive insect from East Asia. Mol. Ecol. 25: 2065-2080.

Heminger, A. R. 2017. 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. M.S. L.F.S Thesis, Virginia Tech, Blacksburg, VA. 93 pp.

Jetton, R. M., W. S. Dvorak, and W. A. Whittier. 2008. Ecological and genetic factors that define the natural distribution of Carolina hemlock in the southeastern United States and their role in ex situ conservation. For. Ecol. Manage. 255: 3212-3221.

Klein, J. L., A. Caccone, and N. P. Havill. 2010. Polymorphic microsatellite loci for *Laricobius nigrinus* and *L. rubidus* (Coleoptera: Derodontidae), predators of the hemlock woolly adelgid. Mol. Ecol. Resour. 10: 751-754.

Koch, R. L. 2003. The multicolored Asian lady beetle, *Harmonia axyridis:* a review of its biology, uses in biological control, and non-target impacts. J. Insect Sci. 3: 32-32.

Kohler, G. R., V. L. Stiefel, K. F. Wallin, and D. W. Ross. 2008. Predators associated with the hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific Northwest. Environ. Entomol. 37: 494-504.

Lamb, A., M. E. Montgomery, L. C. Vieria, S. Shiyake, and S. Salom. 2011. *Laricobius osakensi*s, a hemlock woolly adelgid predator from Japan, pp. 90-96. In B. Onken and C. Reardon (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service FHTET-2011-04, Morgantown, WV.

Lamb, A. B., S. M. Salom, and L. T. Kok. 2005a. Guidelines for rearing *Laricobius nigrinus* Fender, pp. 309-318. *In* B. Onken and R. Reardon [eds.], Proceedings of the Third Symposium on Hemlock Woolly Adelgid in the Eastern United States. USDA Forest Service FHTET-2005-01.

Lamb, A. B., S. M. Salom, and L. T. Kok. 2005b. Survival and reproduction of *Laricobius nigrinus* Fender (Coleoptera : Derodontidae), a predator of hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera : Adelgidae) in field cages. Biol. Control 32: 200-207.

Lamb, A. B., S. M. Salom, L. T. Kok, and D. L. Mausel. 2006. Confined field release of *Laricobius nigrinus* (Coleoptera : Derodontidae), a predator of the hemlock woolly adelgid, *Adelges tsugae* (Hemiptera : Adelgidae), in Virginia. Can. J. For. Res. 36: 369-375.

Luck, R. F., B. M. Shepard, and P. E. Kenmore. 1988. Experimental methods for evaluating arthropod natural enemies. Annu. Rev. Entomol. 33: 367-389.

Luck, R. F., B. M. Shepard, and P. E. Kenmore. 1999. Evaluation of biological control with experimental methods, pp. 17-44. In T. W. Fisher, T. S. Bellows, L. E. Caltagirone, D. L. Dahlsten, C. B. Huffaker and G. Gordh (eds.), Handbook of Biological Control. Academic Press.

Mausel, D. L., L. T. Kok, and S. M. Salom. 2017. Numerical response and impact of *Laricobius nigrinus* (Coleoptera: Derodontidae) on *Adelges tsugae* (Hemiptera: Adelgidae) in their native range. Environ. Entomol. 46: 544-551.

Mausel, D. L., S. M. Salom, L. T. Kok, and J. G. Fidgen. 2008. Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera: Derodontidae) on hemlock woolly adelgid in Virginia. Environ. Entomol. 37: 1498-1507.

Mausel, D. L., S. M. Salom, L. T. Kok, and G. A. Davis. 2010. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. Environ. Entomol. 39: 440-448.

Mayfield, A. E., B. C. Reynolds, C. I. Coots, N. P. Havill, C. Brownie, A. R. Tait, J. L. Hanula, S. V. Joseph, and A. B. Galloway. 2015. Establishment, hybridization and impact of *Laricobius* predators on insecticide-treated hemlocks: Exploring integrated management of the hemlock woolly adelgid. For. Ecol. Manage. 335: 1-10.

McAvoy, T. J., J. Régnière, R. St-Amant, N. F. Schneeberger, and S. M. Salom. 2017. Mortality and recovery of hemlock woolly adelgid (*Adelges tsugae*) in response to winter temperatures and predictions for the future. Forests 8: 21 pp.

Mcclure, M. S. 1987. Biology and Control of Hemlock Woolly Adelgid, Bulletin 851, pp. 1-9. Connecticut Agricultural Experimet Station, New Haven, CT.

McClure, M. S. 1989. Evidence of a polymorphic life-cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). Ann. Entomol. Soc. Am. 82: 50-54.

McClure, M. S. 1991. Density-dependent feedback and population-cycles in *Adelges tsugae* (Homoptera: Adelgidae) on *Tsuga canadensis*. Environ. Entomol. 20: 258-264.

McClure, M. S., and C. A. S.-J. Cheah. 1999. Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. Biol. Invasions 1: 247-254.

McClure, M. S., S. M. Salom, and K. S. Shields. 2001. Hemlock Woolly Adelgid. USDA Forest Service FHTET-2001-03, Morgantown, WV.

Montgomery, M. E., and S. M. Lyon. 1995. Natural enemies of adelgids in North America: their prospect for biological control of *Adelges tsugae* (Homoptera: Adelgidae), pp. 89-102. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.

Mooneyham, K. L., S. M. Salom, and L. T. Kok. 2016. Release and colonization of *Laricobius osakensis* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid, *Adelges tsugae*. Northeast. Nat. 23: 141-150.

Motley, K., N. P. Havill, A. L. Arsenault-Benoit, A. E. Mayfield, D. S. Ott, D. Ross, M. C. Whitmore, and K. F. Wallin. 2017. Feeding by *Leucopis argenticollis* and *Leucopis piniperda* (Diptera: Chamaemyiidae) from the western USA on *Adelges tsugae* (Hemiptera: Adelgidae) in the eastern USA. Bull. Entomol. Res. 107: 699-704.

Nelson, L. A., and L. K. Rieske. 2014. Microclimatic variation within sleeve cages used in ecological studies. J. Insect Sci. 14: 1-5.

Onken, B., and C. Reardon. 2011. An overview and outlook for biological control of hemlock woolly adelgid, pp. 222-228. In B. Onken and C. Reardon (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service FHTET-2011-04, Morgantown, WV.

Orwig, D. A., D. R. Foster, and D. L. Mausel. 2002. Landscape patterns of hemlock decline in New England due to the introduced hemlock woolly adelgid. J. Biogeogr. 29: 1475-1487.

Rosen, D., and P. DeBach. 1992. Foreign exploration: The key to classical biological control. Fla. Entomol. 75: 409-413.

Ross, D. W., S. D. Gaimari, G. R. Kohler, K. F. Wallin, and S. M. Grubin. 2011. Chamaemyiid predators of the hemlock woolly adelgid from the Pacific Northwest, pp. 97-106. In B. Onken and C. Reardon (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service FHTET-2011-04, Morgantown, WV.

Salom, S. M., L. T. Kok, A. B. Lamb, and C. S. Jubb. 2012. Laboratory rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A predator of the hemlock woolly adelgid (Hemiptera: Adelgidae). Psyche 2012: 9.

Silcox, C. A. 2002. Using imidacloprid to control hemlock woolly adelgid, pp. 280-287. *In* B. Onken, R. Reardon and J. Lashomb [eds.], Proceedings: Hemlock Woolly Adelgid in the Eastern Untied States Symposium. New Jersey Agricultural Experiment Station, Rutgers - The State University of New Jersey.

Smith, H. S., and P. De Bach. 1942. The measurement of the effect of entomophagous insects on population densities of their hosts. J. Econ. Entomol. 35: 845-849.

Sumpter, K. L., T. J. McAvoy, C. C. Brewster, A. E. Mayfield, and S. M. Salom. 2018. Assessing an integrated biological and chemical control strategy for managing hemlock woolly adelgid in southern Appalachian forests. For. Ecol. Manage. 411: 12-19.

Thode, H. C. 2002. Testing for Normality, Marcel Dekker, New York, NY.

Tobin, P. C., R. M. Turcotte, L. M. Blackburn, J. A. Juracko, and B. T. Simpson. 2017. The big chill: Quantifying the effect of the 2014 North American cold wave on hemlock woolly adelgid populations in the central Appalachian Mountains. Popul. Ecol. 59: 251-258.

Toland, A., C. Brewster, K. L. Mooneyham, and S. M. Salom. 2018. First report on establishment of *Laricobius osakensis* (Coleoptera: Derodontidae), a biological control agent for hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in the eastern U.S. Forests 9: 13 pp.

USDA. 2012. USDA Plant Hardiness Zone Map, https://planthardiness.ars.usda.gov/PHZMWeb/. United States Department of Agriculture.

Van Driesche, R., M. Hoddle, and T. D. Center. 2008. Control of Pests and Weeds by Natural Enemies: An Introduction to Biological Control, Blackwell Publishing, Malden, MA.

Vieira, L. C., S. M. Salom, M. E. Montgomery, and L. T. Kok. 2013a. Field-cage evaluation of the survival, feeding and reproduction of *Laricobius osakensis* (Coleoptera: Derodontidae), a predator of *Adelges tsugae* (Hemiptera: Adelgidae). Biol. Control 66: 195-203.

Vieira, L. C., A. B. Lamb, S. Shiyake, S. M. Salom, and L. T. Kok. 2013b. Seasonal abundance and synchrony between *Laricobius osakensis* (Coleoptera: Derodontidae) and its prey, *Adelges tsugae* (Hemiptera: Adelgidae), in Japan. Ann. Entomol. Soc. Am. 106: 249-257.

Vieira, L. C., T. J. McAvoy, J. Chantos, A. B. Lamb, S. M. Salom, and L. T. Kok. 2011. Host range of *Laricobius osakensis* (Coleoptera: Derodontidae), a new biological control agent of hemlock woolly adelgid (Hemiptera: Adelgidae). Environ. Entomol. 40: 324-332.

Virginia Tech. 2019. HWA Predator Database, http://hiro.ento.vt.edu/pdb/. Virginia Tech.

Vose, J. M., D. N. Wear, A. E. Mayfield, and C. D. Nelson. 2013. Hemlock woolly adelgid in the southern Appalachians: Control strategies, ecological impacts, and potential management responses. For. Ecol. Manage. 291: 209-219.

Wallace, M. S., and F. P. Hain. 2000. Field surveys and evaluation of native and established predators of the hemlock woolly adelgid (Homoptera: Adelgidae) in the southeastern United States. Environ. Entomol. 29: 638-644.

Wantuch, H. A., N. P. Havill, E. R. Hoebeke, T. P. Kuhar, and S. M. Salom. 2019. Predators associated with the pine bark adelgid (Hemiptera: Adelgidae), a native insect in Appalachian forests, United States of America, in its southern range. Can. Entomol. 151: 73-84.

Webb, R. E., R. J. Frank, and M. Raupp. 2003. Eastern hemlock recovery from hemlock wooly adelgid damage following imidaclopid therapy. Aboric. Urban. For. 29: 298-302.

Wiggins, G. J., J. F. Grant, J. R. Rhea, A. E. Mayfield, A. Hakeem, P. L. Lambdin, and A. B. L. Galloway. 2016. Emergence, seasonality, and hybridization of *Laricobius nigrinus* (Coleoptera: Derodontidae), an introduced predator of hemlock woolly adelgid (Hemiptera: Adelgidae), in the Tennessee Appalachians. Environ. Entomol. 45: 1371-1378.

Young, R. F., K. S. Shields, and G. P. Berlyn. 1995. Hemlock woolly adelgid (Homoptera: Adelgidae) - stylet bundle insertion and feeding sites. Ann. Entomol. Soc. Am. 88: 827-835.

Zar, J. H. 2010. Biostatistical Analysis, Prentice-Hall/Pearson, Upper Saddle River, N.J.

Zilahi-Balogh, G. M. G., L. T. Kok, and S. M. Salom. 2002. Host specificity of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a potential biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). Biol. Control 24: 192-198.

Zilahi-Balogh, G. M. G., S. M. Salom, and L. T. Kok. 2003. Development and reproductive biology of *Laricobius nigrinus*, a potential biological control agent of Adelges tsugae. BioControl 48: 293-306.

Zilahi-Balogh, G. M. G., C. D. Broeckling, L. T. Kok, and S. M. Salom. 2005. Comparison between a native and exotic adelgid as hosts for *Laricobius rubidus* (Coleoptera: Derodontidae). Biocontrol Sci. Technol. 15: 165-171.

Chapter 4: Summary

4.1. Background

Hemlock woolly adelgid is a serious pest of both eastern and Carolina hemlock in the eastern U.S. A loss of these species will negatively affect forest ecosystems by causing an alteration of species composition and microclimates (Lovett et al. 2006). A robust classical biological program was implemented in the late-1990's for HWA management in the forest setting. A predatory beetle, *Laricobius nigrinus,* has been the primary focus of those efforts and releases of this species have occurred over the range of HWA infested hemlock in the eastern U.S. Studies have shown that *L. nigrinus* is a promising management tool in field settings (Mausel et al. 2008, Mayfield et al. 2015), however comprehensive studies regarding its ability to establish in Virginia and create impact on HWA in a variety of locations and USDA Plant Hardiness Zones has not been evaluated. In this study, our goal was to determine if *L. nigrinus* had established at all previous release sites in the state of Virginia, and to determine their level of impact at sites across a 500 mile transect of the eastern U.S.

4.2. Establishment of *L. nigrinus* at release sites in Virginia

Since its initial release in 2003, over 14,000 *L. nigrinus* have been deployed in Virginia. Previous studies have only evaluated establishment at some of these sites (Mausel et al. 2010, Heminger 2017). In our study, we assessed all 26 release sites to determine presence or absence of *L. nigrinus*. Within each site we also estimated HWA stand-level density, assessed tree health, and quantified predator-prey ratios. Our findings indicated that *L. nigrinus* had established at 82% of sites and at some locations, persisted to the F_{15} generation in spite of low HWA densities. Microsatellite analysis suggested that *L. nigrinus* was the primary species recovered from release sites. HWA stand-level densities appeared to decline overall in year two of the study, with more sites having low or moderate infestation levels. Although it appeared that tree health improved in year two of the study, and that there were significant associations between the number of *Laricobius* spp. recovered and the crown class conditions, dieback and transparency, longer-term evaluation of these interactions are necessary to elucidate possible relationships between predator abundance and tree health. Lag effects in the response of both tree health and *L. nigrinus* to varying densities of HWA are likely present in these systems, but observable only over multi-year studies. Predator-prey ratios were lower than those demonstrated in a study of *L. nigrinus* in the native range, however, there is much left to be understood about relationships of predator, prey, and hemlock health in the eastern U.S. The results of this study emphasize the ability of *L. nigrinus* to establish and persist in a variety of locations and will guide future releases in Virginia as well as support protocols indicated for suitable release timing and predator release numbers.

4.3. Impact assessment of *L. nigrinus* on HWA

In 2003, the first controlled releases of *L. nigrinus* began and to date, over 400,000 individuals have been released from field and lab-reared sources in the eastern U.S.

Previous studies evaluating impact of *L. nigrinus* on HWA showed promising results (Mausel et al. 2008, Mayfield et al. 2015), however, in order to investigate impact on a larger scale and in a variety of locations this study was initiated at nine sites in the eastern U.S. from New Jersey to Georgia within USDA Plant Hardiness Zones 6a-7b. In this study we utilized predator exclusion cages to monitor predator and prey populations. Two treatments (caged and no-cage branches) helped quantify the level of predation by *L. nigrinus* on HWA and used ovisac disturbance as a proxy for predation. In Phase One of this study conducted from 2014-2016, mean predation rates were as high as 66% on no-cage branches at some sites. In Phase Two conducted from 2016-2018, predation rates were significantly higher on no-cage branches when compared to those that were caged in both years, with rates as high as 80%. Microsatellite analysis used to determine species of *Laricobius* recovered from study branches indicated that *L. nigrinus* was the dominant species on study branches. No alternate predators were recovered in Phase Two indicating that *L. nigrinus* was likely responsible for the predation observed. These predation levels are promising; however, the impact of *L. nigrinus* on HWA is limited to only sistens ovisacs. They leave a feeding gap during a subterranean period of pupation and aestivation while progrediens ovisacs are developing. Evaluation of natural enemies in western North America suggested that there were a variety of predators managing HWA populations. It is therefore suspected that *L. nigrinus* plays a critical role in the overall management of HWA, however a complex of predators is likely necessary for effective management in the eastern U.S.

4.4. Future Work

Our studies have demonstrated that *L. nigrinus* is an impactful predator on HWA sistens ovisacs. They appeared to easily establish at most sites in Virginia and persisted over many generations. Further investigations are required to understand the dynamics of this system. Specifically, the effects of *L. nigrinus* on tree health should be evaluated in longer-term studies. Lag effects in the response of *L. nigrinus* to varying HWA densities similar to those shown with tree health in Sumpter et al. (2018) may not adequately be captured in shorter-term studies such as the one presented here. Although much is left to be understood about their ability to establish and create impact, two species of *Leucopis* native to western North America are currently being evaluated for possible biological control in the eastern U.S. Future investigations should include the potential interactions between *L. nigrinus*, *Leucopis* spp., HWA, and tree health in multi-year investigations. Additionally, more robust microsatellite loci testing should be included to increase our ability to detect later generation *Laricobius* hybrids at *L. nigrinus* release sites.

4.5. References

Heminger, A. R. 2017. 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. M.S. L.F.S Thesis, Virginia Tech, Blacksburg, VA. 93 pp.

Lovett, G. M., C. D. Canham, M. A. Arthur, K. C. Weathers, and R. D. Fitzhugh. 2006. Forest ecosystem responses to exotic pests and pathogens in eastern North America. Bioscience 56: 395-405.

Mausel, D. L., S. M. Salom, L. T. Kok, and J. G. Fidgen. 2008. Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera:

Derodontidae) on hemlock woolly adelgid in Virginia. Environ. Entomol. 37: 1498-1507.

Mausel, D. L., S. M. Salom, L. T. Kok, and G. A. Davis. 2010. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. Environ. Entomol. 39: 440-448.

Mayfield, A. E., B. C. Reynolds, C. I. Coots, N. P. Havill, C. Brownie, A. R. Tait, J. L. Hanula, S. V. Joseph, and A. B. Galloway. 2015. Establishment, hybridization and impact of *Laricobius* predators on insecticide-treated hemlocks: Exploring integrated management of the hemlock woolly adelgid. For. Ecol. Manage. 335: 1-10.

Sumpter, K. L., T. J. McAvoy, C. C. Brewster, A. E. Mayfield, and S. M. Salom. 2018. Assessing an integrated biological and chemical control strategy for managing hemlock woolly adelgid in southern Appalachian forests. For. Ecol. Manage. 411: 12-19.

Appendix 1: Numerical response of *Laricobius nigrinus* on hemlock woolly adelgid in the eastern United States

Abstract

Assessing the numerical response of predatory insects to varying prey densities can be an effective method for evaluating the suitability of introduced biological control agents. Since 2003, the predatory beetle, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), has been released in the eastern U.S. in an effort to manage the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). HWA is an invasive pest causing significant dieback and mortality to native hemlocks in eastern North America. Previous studies evaluating numerical response of *L. nigrinus* to HWA in their native range indicated that they strongly aggregated as adults and increased their reproduction in response to increasing densities of HWA. In an effort to understand the numerical response of *L. nigrinus* in the introduced range, a study was initiated at nine *L. nigrinus* release sites in the eastern U.S. The study examined the aggregation response of adults to varying densities of HWA in the fall of both 2016 and 2017. In year one, there was a significant negative relationship between *Laricobius* spp. adult aggregation and increasing HWA densities. In year two, no significant relationships were found. Predator-prey ratios pooled for both years ranged from 0.00-0.03 *Laricobius* spp. adults per HWA. The reproductive response of *L. nigrinus* to varying densities of HWA was assessed in the spring of 2017 and 2018. No significant relationships were noted between *Laricobius* spp. reproductive response to different

densities of HWA. Predator-prey ratios pooled for both years ranged from 0.00-0.08 *Laricobius* spp. larvae per HWA. Identification of adult *Laricobius* spp. was performed using visual morphological analysis. Identifications of recovered larval *Laricobius* spp. was performed using microsatellite analysis. The primary species from both adult and larval recoveries was *L. nigrinus*. Although the adult aggregation and reproductive responses of this predator in the introduced range did not coincide with those found in the native range, this study provides important information about the population dynamics of predator and prey in the introduced range. Key differences between the native and introduced range which potentially drive the disparity in numerical response results are explored.

A1.1 Introduction

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an exotic, invasive pest in the eastern U.S. that is native to Asia and western North America (Havill et al. 2016). It is a serious threat to native hemlocks found in eastern forests; *Tsuga canadensis* (L.) Carriere (eastern hemlock), and *Tsuga caroliniana* Engelmann (Carolina hemlock) (Orwig and Foster 1998, Siderhurst et al. 2010, Brantley et al. 2013). HWA is associated with all other known species of hemlock worldwide including *Tsuga heterophylla* (Raf.) Sargent in the western North America (Havill et al. 2014), though it only threatens the health and longevity of those in eastern North America due to the lack of natural enemies and host resistance afforded by co-evolution (Cheah and McClure 1995, McClure and Cheah 1999, Havill et al. 2006).

In the eastern U.S., HWA have two asexual generations per year (McClure 1989, Havill and Foottit 2007). Because of their parthenogenic reproductive biology, populations of these insects can build quickly to high densities which can cause significant decline in tree health. Poor tree health can then cause an eventual reduction in HWA populations due to declining availability of tree resources. Eventually, tree health rebounds slightly which encourages re-infestation by HWA. This density-dependent population fluctuation can occur several times before trees succumb (McClure 1991). Adult HWA densities of 0.4HWA/cm serve as a threshold above which will cause damage to trees (Fidgen et al. 2013).

A small, univoltine beetle, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) was one of the most abundant predators present during natural enemy surveys in western North America (Kohler et al. 2008) and has since been a significant focus of a classical biological control program in the eastern U.S. The first releases were made in 2003, and to date over 400,000 beetles have been released from field and lab-reared sources (Virginia Tech 2019). Since that time, studies have shown that it can successful establish (Mausel et al. 2010) and disperse (Davis et al. 2012) from release locations. Other studies, including the one detailed in Chapter 2 of this thesis suggest that this species can have a significant impact on HWA sistens adults and progrediens eggs (Mausel et al. 2008, Mayfield et al. 2015). A second species, *Laricobius rubidus* LeConte, is of particular interest to HWA biological control efforts in eastern North

America. It is native in the East and its primary host is pine bark adelgid, *Pineus strobi* Hartig (Hemiptera: Adelgidae) on white pine, *Pinus strobus* L. (Clark and Brown 1960). This species can often be found feeding and completing development on HWA where white pine and hemlock co-occur (Zilahi-Balogh et al. 2005). Both *L. nigrinus* and *L. rubidus* are very closely related sister species which are capable of hybridizing and producing reproductively viable offspring (Havill et al. 2012, Fischer et al. 2015).

Huffaker et al. (1969) described several important characteristics of effective natural enemies; 1) They must be able to adapt to a variety of biotic and abiotic conditions, 2) have an ability to search for and travel to prey, and then increase or decrease populations relative to prey, 3) have synchronicity with and specificity to the host, and 4) and have the ability to survive when population densities of the host are low. A significant challenge in classical biological control programs is accurately predicting the effectiveness of introduced predators (DeBach and Rosen 1991, Van Driesche and Bellows 1996, Kimberling 2004). Ranges into which they are imported may offer vastly different ecological and climatic conditions which may complicate the ability of predators to be effective (DeBach and Rosen 1991, Gerling et al. 2004). Assessing predators in their native habitat may help predict their ability to successfully manage prey populations in introduced ranges (Gerling et al. 2004). Analysis of functional and numerical response is an important evaluation when assessing the performance of natural enemies (Huffaker et al. 1969). These two conditions were first described by Solomon (1949) and are characterized as the change in prey consumption (functional response) and the change in predator density (numerical response) in response to changing prey abundance. Evaluation of numerical response is especially important when a pest develops rapidly, as the natural enemy must be able to respond effectively (Hajek 2004). Numerical response can be further categorized into both aggregation and reproductive responses. Positive aggregation response indicates an increase in the predator population due to immigration stimulated by increased prey. Reproductive responses are changes in the reproductive rates of predators in relation to changes in prey densities (Crawley 1975). These numerical response changes are not always immediate and may exhibit a time-lag effect due to fecundity, handling time, and life cycles (Huffaker et al. 1969, Crawley 1975).

Numerical response relationships of *L. nigrinus* to HWA were first evaluated in laboratory studies, which showed that *L. nigrinus* laid more eggs as HWA ovisac densities increased (Vieira et al. 2012). During initial studies of this predator in its native range, Kohler et al. (2008) noted a positive correlation between *L. nigrinus* and HWA abundance. Further evaluation of *L. nigrinus* in the coastal temperate rainforests of western North America confirmed that there was a significant aggregation response and increased reproductive rate when HWA densities increased (Mausel et al. 2017). Collectively, these results suggest that *L. nigrinus* is able to respond to changing prey densities; an attribute which is critically important in classical biological control programs. These studies also provide important benchmarks for evaluations of *L. nigrinus* in its introduced range. In the study reported here, we evaluated *L. nigrinus* adult aggregation and larval abundance as a measure of their numerical response to varying levels of HWA in the eastern U.S. at sites where *L. nigrinus* had previously been released and was known to have established. Comparison of these responses to those found in prior studies in their native range, will help us understand their effectiveness in the introduced range. We hypothesize that as HWA levels increase, that *L. nigrinus* adults will aggregate in higher numbers and will respond with higher reproductive rates.

A1.2 Materials and Methods

A1.2.1 Field site and study tree selection

The field sites selected for the study reported here, were also utilized in Chapter 2 of this thesis, however unique trees were selected and used for adult aggregation. Branches utilized in Assessment #1 and #2 in the impact study provided data on the reproductive response of *L. nigrinus* for this study. The nine sites used included six states (New Jersey, Maryland, Virginia, North Carolina, Tennessee, and Georgia). Sites were located within four unique USDA Plant Hardiness Zones (6a-7b) providing a diversity of growing environments. These zones are partitioned based on average minimum temperature data collected yearly (Table A1.1). The zones included in the study were 6a (-23.3 to -20.5°C), 6b (-20.6 to -17.8°C), 7a (-17.8 to -15°C) and 7b (-15 to -12.2°C) (USDA 2012). Sites were selected based on several criteria which included length of time since initial *L. nigrinus* release (at least 4 years), establishment of *L. nigrinus* was confirmed, and adequate populations of HWA (2-3/cm) were present in order to support *L. nigrinus* populations. In year two of the study, the TN2 site was not utilized because HWA populations were too low.

A1.2.2 Aggregation response of *L. nigrinus* adults on HWA

The numerical response of *L. nigrinus* to varying densities of HWA was quantified by evaluating adult aggregation. Sites were visited in late-October to early-November after both adult *L. nigrinus* and first instar HWA nymphs had broken their respective summer aestivation periods and had begun feeding and developing. The exact timing of the site visit was dependent on geographical location, USDA Plant Hardiness Zone, and local temperatures leading up to this time period. Higher temperatures prolonged aestivation in both species. At each site, thirty trees were randomly selected for the study. Although each tree had differing densities of HWA, it was at least present in order for the tree to be selected. Four branches at each cardinal point of the tree were beat-sheet sampled to collect *Laricobius* spp. adults. A 1 m^2 beat sheet made of ripstop nylon with PVC plastic framing (Bioquip Products, Rancho Dominguez, CA) was placed under a portion of the selected HWA infested tree branch, and the top of the branch was tapped approximately 10 times using a 60 cm section of straight PVC plastic pipe to dislodge any adult *Laricobius* spp. present. Coarse identification of collected *Laricobius* spp. beetles was made based on elytral color and recorded prior to aspirating adults into a collection vial. *Laricobius* spp. with distinct black elytra were identified as *L. nigrinus* and those with reddish elytra were identified as *L. rubidus.* Hybrids of *L. nigrinus* and *L. rubidus* if present, were not identified in this study. The distal 30 cm of the sample branch was then cut and placed into a 3.8 L zip enclosure plastic bag for transport back to the laboratory. Branches at the remaining cardinal points of the tree were then sampled using the same protocols. If branches were not present at the exact cardinal point, the next closest branch was chosen. When all branches were sampled, the recovered *Laricobius* spp. adults were placed back onto the tree. After transport back to the laboratory, adelgid densities and branchlet lengths for all twigs on the 30 cm branch were counted on both first- and second-year growth. Branchlet lengths (cm) were measured, and the number of live adelgids were counted to determine the number of HWA per cm. Predator-prey ratios were determined by calculating the total number of *Laricobius* spp. adults recovered per study branch divided by the number of live HWA sistens nymphs present on the branch.

A1.2.3 Reproductive response of *L. nigrinus* on HWA

The reproductive response of *L. nigrinus* to varying HWA densities was measured at the same nine field sites. Fifteen branches from multiple trees were selected at each site in late-October to early-November during numerical response assessments. Branches were selected based on the presence of at least 2-3 HWA per cm. If those densities were not available, the next highest were selected. A 30 cm section of the branch was tagged with flagging tape and first-year growth was measured (cm), along with the corresponding number of live HWA sistens ovisacs. The branches remained intact on trees and open to predation for 4-5 months. In late-March to early-April, after the period of peak *L. nigrinus* oviposition, and prior to maximum larval abundance, sites were revisited. At this time, the branches were clipped from the trees and were placed into 3.8 L zip closure plastic bags for transport back to the Virginia Tech rearing insectary. Branches were removed from bags, and basal tips were cut to allow for improved water uptake after transport. Branches were stuck into wetted Instant Deluxe floral foam blocks (Smithers-Oasis North America, Kent, OH)) which were previously wrapped in Parafilm M (Beemis N.A., Neemah, WI). Blocks with branches were then placed into larval rearing funnels and were held in environmental conditions known to be appropriate for developing *L. nigrinus* larvae: 12h:12:h (L:D), 13-15°C (Salom et al. 2012). Each funnel was equipped at its base with a collection jar to receive mature *L. nigrinus* larvae. After their development through four instars, their natural habit is to drop from tree branches to the soil where they burrow into the duff layer and create a pupation chamber. Jars served to intercept larvae as they initiated this behavior. Each jar was checked daily for the presence of larvae. Total number were counted, and larvae were then collected and preserved in vials containing 95% EtOH to allow for species or hybrid identification using genetic analysis. Funnels remained in place until larvae had stopped dropping (approx. 4-6 weeks). Predator-prey ratios were determined by calculating the total number of *L. nigrinus* larvae recovered per study branch divided by the number of live HWA sistens adults present on the branch.

A1.2.4 Genetic analysis of *Laricobius* spp. larval recoveries

Microsatellite loci for recovered *Laricobius* spp. larvae were analyzed to determine species designation or hybridization between *L. nigrinus* and *L. rubidus*. The Omega Bio-tek E.Z.N.A.® Tissue DNA kit and its associated protocols were used to extract DNA from larval samples. Six microsatellite loci (LaGT01, LaCA04, LaGT07, LaGT13, LaCA14, LaCA16) (Klein et al. 2010, Havill et al. 2012) were amplified utilizing techniques described in Klein et al. (2010). Fragments were analyzed at the DNA

Analysis Facility at Science Hill using a 3730xl 96-Capillary Genetic Analyzer. Genotypes were scored using Geneious Prime 2019 (Biomatters, Inc., Newark, NJ). Final species and hybrid designations were made using the software programs Structure 2.3.2 (Stanford University) and New Hybrids 1.1. (University of California).

A1.2.5 Statistical analyses

Simple linear regressions were used to evaluate relationships between mean HWA per cm (independent variable) and mean *Laricobius* spp. densities (dependent variable) for both years one and two with data from all sites pooled. Both adult (aggregation response) and larval (reproductive response) densities were assessed. Upon evaluation of residuals, year one adult and larvae and year two adult *Laricobius* spp. densities were square root transformed to remedy non-normality. The distributions for HWA density data were tested for normality using the goodness-of-fit Shapiro-Wilk W test statistic. Year one HWA densities for both the aggregation and reproductive response portions of the study did not fit a normal distribution and were therefore square root transformed to meet the assumptions of the test. All statistical analyses were completed using JMP Pro 13.0 (SAS Pro, Inc. 2018).

A1.3 Results

A1.3.1 Numerical response of *L. nigrinus* adults on HWA

In year one, *Laricobius* spp. adults were recovered at eight out of the nine sites. The mean density ± S.E. of adult *Laricobius* spp. recoveries was 0.65 ± 0.19 per tree (range: 0.00-1.63) and the mean ± S.E. density of HWA was 3.26 ± 1.05 HWA/cm (range: 0.44-

11.06). The mean percent trees with *Laricobius* spp. adults was 27% (range: 0-57%) (Table A1.2). Simple linear regression indicated that there was a significant negative relationship between mean HWA/cm and mean adults recovered (r^2 = 0.6640; $\sqrt{Y}$ = 1.47 - 0.47$\sqrt{X}$; $F_{1,7}$ = 13.84, P = 0.0075 (Fig. A1.1). Morphological identification of recovered adult *Laricobius* spp. beetles indicated that *L. nigrinus* was the primary species recovered (Table A1.3). Predator-prey ratios ranged from 0.00-0.03 *Laricobius* spp. per HWA in year one (Fig. A1.2). In year two, *Laricobius* spp. adults were recovered at seven out of eight sites. The mean density ± S.E. of adult *Laricobius* spp. recoveries was 1.24 ± 0.66 per tree (range: 0.00-5.57), and the mean density ± S.E. of HWA/cm was 2.04 ± 0.44 (range: 0.40-3.98). The mean percentage of trees with *Laricobius* spp. adult recoveries was 42% (range: 0-93%) (Table A1.2). Simple linear regression did not indicate a significant relationship between mean HWA/cm and mean adults recovered (r^2 = 0.0367; Y = 0.64 + 0.11$\sqrt{X}$; $F_{1,6}$ = 0.23, P = 0.06494). Morphological identification of recovered adult *Laricobius* spp. beetles suggested that *L. nigrinus* was the primary species recovered (97%) followed by *L. rubidus* (3%) (Table A1.3). Predator-prey ratios ranged from 0.00-0.01 *Laricobius* spp. adults per HWA in year two (Fig. A1.3).

A1.3.2 Reproductive response of *L. nigrinus* on HWA

The reproductive response of *Laricobius* spp. was measured at all eight sites in the spring of 2017 and 2018. In year one, *Laricobius* spp. larvae were recovered at eight of nine sites. The mean ± S.E. density of larval recoveries was 3.04 ± 1.14 (range: 0.00-8.50) and the mean ± S.E. density of HWA was 4.77 ± 0.85 (range: 0.94-8.79). The mean percent trees with *Laricobius* spp. larvae was 47% (range: 0-92%) (Table A1.2). Simple linear regression did not indicate a significant relationship between mean HWA/cm and mean larvae recovered ($r^2 = 0.1564$, $\sqrt{Y} = 2.86 - 0.72\sqrt{X}$; $F_{1,7} = 1.30$, $P = 0.2920$). Predator-prey ratios ranged from 0.00-0.08 *Laricobius* spp. per HWA in year one (Fig. A1.2). In year two, larvae were recovered at all eight sites used in the study. The mean ± S.E. density of larval recoveries was 2.85 ± 0.66 (range: 0.17-5.21), and the mean ± S.E. density of HWA was 3.71 ± 0.68 (range: 1.73-7.48). The mean percent trees with *Laricobius* spp. larval recoveries was 50% (range: 8-83%) (Table A1.2). Simple linear regression did not indicate a significant relationship between mean HWA/cm and mean larvae recovered ($r^2 = 0.0001$, $Y = 2.893 - 0.012X$; $F_{1,6} = 0.0010$, $P = 0.9758$. Predator-prey ratios ranged from 0.00-0.08 *Laricobius* spp. per HWA in year two (Fig. A1.3).

A1.3.3 Genetic analysis of *Laricobius* spp. larval recoveries

Genetic analysis revealed that *L. nigrinus* was the primary species recovered from the reproductive response study branches. *Laricobius rubidus* and hybrids of *L. rubidus* and *L. nigrinus* were also recovered. Recoveries of *Laricobius* spp. larvae occurred at all sites during the period of the study (Table A1.3). In year one, larvae were recovered at 8 of 9 sites. The mean percent *L. nigrinus*, *L. rubidus*, and hybrid recoveries for all sites pooled were 98.8%, 0.8%, 0.4%, respectively. Larvae were recovered at 8 of 8 sites in year two (Table A1.3). The mean percent *L. nigrinus*, *L. rubidus*, and hybrid recoveries for all sites pooled were 95.3%, 0.5%, 4.2%, respectively.

A1.4 Discussion

Numerical responses of predators to varying rates of prey are often difficult to understand and many factors can affect abundance within these systems (Crawley 1975). The results of our analysis of the numerical response of *L. nigrinus* to HWA densities in the introduced range of the eastern U.S. were inconsistent with those found in previous studies (Kohler et al. 2008, Mausel et al. 2017). Additionally, predator-prey ratios were lower than those reported by Mausel et al. (2017). There are many possible reasons for lack of adequate numerical response in some species. Crawley (1975) proposed that predator egg-limits are destabilizing to overall numerical response models in that females have a finite number of eggs available to be laid regardless of the number of prey consumed. In some instances, negative numerical responses were seen in systems where prey formed high density aggregates. This was observed with aphidophagous predators; however, these responses are not well investigated or understood (Crawley 1975). Additionally, Holling (1959) presents an alternate "null case" in numerical response where predator densities were not affected by prey densities and therefore must be influenced by other factors. Based on our findings in the eastern U.S., it is likely that there are complex interactions occurring between predator and prey, tree health, and climate that the parameters of this study were unable to capture. Comparing parameters from eastern and western North America point to several critical differences that may be steering the results found in this study.

In the West *T. heterophylla* has co-evolved with HWA and has developed host resistance in order to withstand HWA infestations. As HWA has only made a relatively recent invasion into eastern North America, eastern hemlock does not benefit from this same co-evolved resistance, and this likely contributes to the significant outbreak levels observed (Cheah and McClure 1995, McClure and Cheah 1999, Havill et al. 2006). Additionally, as indicated in Kohler et al. (2008), there is a diverse predator complex existing in the western North America which may contribute towards regulating HWA populations at low levels. Although generalist natural enemies are present in the eastern U.S., their impact is considered minimal (Wallace and Hain 2000). Lack of host resistance and a natural enemy complex coupled with the parthenogenic reproductive biology of this species are major driving factors that allow HWA to build significant populations within hemlock stands in eastern North America. The volume of these infestations, specifically in more southern states where HWA mortality is less affected by winter temperatures (Evans and Gregoire 2007), is a stark contrast to populations of HWA on *T. heterophylla.* In western North America, populations are often patchy within and among trees in forested stands or urban groupings, however, field studies support the ability of *L. nigrinus* to locate low-density HWA populations in this region (Mausel 2005).

A density-dependent cycle spurred by the interactions of tree health and HWA populations may also cause downstream effects for *L. nigrinus* which could have implications for their ability to respond to increasing HWA populations. As hemlock health declines due to HWA pressure, it is presumed that their physiological condition in turn negatively effects HWA's ability to survive and reproduce (McClure 1991). Although Jones et al. (2015) investigated relationships between hemlock tree health and HWA fitness and physiology, results suggested that interactions between host health and insect fitness were complex and further investigation was needed. Additional testing of these complexities could elucidate possible carry-over effects from poor hemlock health to HWA and *L. nigrinus* populations.

Population fluctuations of both HWA and *L. nigrinus* in the eastern U.S. are also likely driven by climatic conditions, specifically, significant temperature fluctuations. The range of eastern hemlock extends from southern Ontario and Quebec westward to Michigan and Minnesota and south along the Appalachian Mountains and Plateau to Georgia and Alabama (Kessell 1979). These regions encompass a wide range of USDA Plant Hardiness Zones (3b-7b). Most *L. nigrinus* released in the eastern United States originated from the coastal temperate area in western North America, much of which is situated in Zones 8a-8b. The climate of this area is highly moderated, including mild, wet winters and dry, warm summers. Conversely, in the eastern U.S., winter temperatures are often subject to dramatic fluctuations such as those observed in the winter of 2014 and 2015. During this time, there was a southward shift of the northern polar vortex, which produced extreme low temperatures throughout the HWA-infested range of eastern hemlock. These fluctuations caused widespread mortality to HWA

(Tobin et al. 2017) and *L. nigrinus* populations (Heminger 2017) further adding to the complexities of population dynamics in this system in the eastern U.S.

In addition to these speculations regarding the cause for the lack of positive numerical response findings, it is worth noting that the design of this study also had limitations. The most notable of which was that it provided only a snapshot analysis of *L. nigrinus* numerical response. Adult aggregation sampling was completed as a direct, single event in the fall of both 2016 and 2017, and certain factors could have confounded our ability to locate aggregations of predators such as weather and branch location within selected trees. The adult beat sheet sampling technique used in this and other studies have been shown to be less effective than larval branch clip sampling and can produce false negatives during sampling events (Mausel et al. 2010). High winds can cause beetles to fly from sheets, and beetles may not be as abundant on branches during periods of precipitation and/or lower temperatures. Due to distance of some collaborators to study sites, and parameters for timing of the study, this often necessitated that sites be visited in less suitable weather conditions. Anecdotal evidence from years of beat sheet sampling efforts suggest that this beetle is more active and easily collected on dry, warmer days on branches exposed to direct sunlight (personal observation). Sampling location within the tree could also be a factor as only lower canopy branches were selected due to difficulties with beat sheet sampling in the upper canopy. Although larval sampling was a more passive, longer-term effort with selected branches being exposed to *L. nigrinus* for several months in the field, it was also subjected snapshot effects. Because of the difficulties of tracking predators in the field, the design of the reproductive response portion of this study necessitated that branches be clipped from trees for evaluation in the laboratory setting, potentially before *L. nigrinus* completed oviposition. A snapshot study such as this may not be a fully accurate representation of what is actually occurring in the system, and a more extended study may reveal different results.

The significant negative relationship found between adult aggregation and HWA density in year one is notable, however, it was not replicated in year two, so it is not possible to make assertions about predator population dynamics without a longer-term study. A longer-term study would incorporate possible lag effects that occur within the predator-prey population relationship. Similar to lag effects noted in Sumpter et al. (2018) in relation to tree health and HWA populations, it is highly likely that based on the reproductive biology of *L. nigrinus* in the eastern U.S., their population increases may lag behind those of their prey. This study was initiated in 2017 as HWA populations were rebuilding following the two polar vortex events in 2014 and 2015. If lag-effects are indeed present in *L. nigrinus* populations, it is possible that the timing of our study occurred during that lag. Following *L. nigrinus* populations for an extended period of time may more accurately reflect their numerical response capabilities in eastern North America.

Although this study did not indicate significant positive relationships of *L. nigrinus* to HWA densities, it provides critical information regarding these predator-prey relationships in the introduced range of *L. nigrinus* and HWA. It builds our awareness about this complex system and informs us of knowledge gaps in this classical biological control program. Ongoing studies are investigating additional predators of HWA, including *Leucopis* spp. which may complement the predation efforts of *L. nigrinus* (Ross et al. 2011, Motley et al. 2017). These predators may have an important impact on HWA progrediens nymphs, adults, and sistens eggs which are left unchecked by *L. nigrinus*. Although further investigation is needed to understand the ability of *Leucopis* spp. to establish, disperse, and impact HWA in the eastern U.S., future numerical response studies should include these two species working in conjunction with one another and should be a multi-year effort in order to capture lag effects often present in density-dependent predator-prey systems.

Table A4.1. *Laricobius nigrinus* numerical response study site locations in the eastern U.S., year of *L. nigrinus* release, and USDA Plant Hardiness Zone.

Site	Coordinates	*L.n.* Release Year	Plant Hardiness Zone[a]
NJ1	41.12 N, -74.91 W	2007, 2008	6a
MD1	39.70 N, -78.67 W	2004	6b
VA1	37.64 N, -78.80 W	2005	7a
VA2	37.21 N, -80.59 W	2003	6b
NC1	35.82 N, -82.21 W	2005	6b
TN1	35.76 N, -83.30 W	2007	7a
TN2	35.69 N, -83.87 W	2008	7a
TN3	35.66 N, -83.59 W	2006	7a
GA1	34.79 N, -83.76 W	2008, 2010	7b

[a]Plant hardiness zones are based on average annual minimum temperature and acquired from planthardiness.ars.usda.gov. 6a (-23.3 to -20.5°C), 6b (-20.6 to -17.8°C), 7a (-17.8 to -15°C) and 7b (-15 to -12.2°C)

Table A4.2. Mean ± S.E *Laricobius* spp. per tree., mean ± S.E. HWA/cm, and % trees with *Laricobius* spp. in years one and two for both adults (aggregation response) and larvae (reproductive response) at nine study sites in the eastern U.S.

	Adults		Larvae	
Site	Year 1	Year 2	Year 1	Year 2
NJ1				
Mean *Laricobius* spp. per tree ± S.E.	0.26 ± 0.14	1.93 ± 0.35	1.78 ± 0.66	1.00 ± 0.44
Mean HWA/cm ± S.E.	3.05 ± 0.33	3.45 ± 0.27	3.86 ± 0.55	7.48 ± 0.70
% trees with *Laricobius* spp.	17%	70%	56%	42%
MD1				
Mean *Laricobius* spp. per tree ± S.E.	1.37 ± 0.30	1.17 ± 0.21	3.40 ± 1.49	2.84 ± 1.06
Mean HWA/cm ± S.E.	1.27 ± 0.15	2.16 ± 0.16	2.99 ± 0.43	4.52 ± 0.55
% trees with *Laricobius* spp.	57%	77%	80%	47%
VA1				
Mean *Laricobius* spp. per tree ± S.E.	0.50 ± 0.22	0.37 ± 0.21	0.00 ± 0.00	4.45 ± 1.72
Mean HWA/cm ± S.E.	1.39 ± 0.16	3.98 ± 0.21	2.00 ± 0.19	3.55 ± 0.47
% trees with *Laricobius* spp.	23%	30%	0%	73%
VA2				
Mean *Laricobius* spp. per tree ± S.E.	1.63 ± 0.54	5.57 ± 0.90	8.50 ± 2.27	5.21 ± 2.04
Mean HWA/cm ± S.E.	0.44 ± 0.05	1.65 ± 0.10	0.94 ± 0.12	2.60 ± 0.32
% trees with *Laricobius* spp.	46%	93%	86%	57%
NC1				
Mean *Laricobius* spp. per tree ± S.E.	0.88 ± 0.35	0.27 ± 0.10	6.57 ± 1.29	4.20 ± 1.76
Mean HWA/cm ± S.E.	2.72 ± 0.26	2.33 ± 0.15	6.07 ± 0.60	4.98 ± 0.48
% trees with *Laricobius* spp.	36%	23%	86%	53%
TN1				
Mean *Laricobius* spp. per tree ± S.E.	0.30 ± 0.15	0.00 ± 0.00	0.08 ± 0.08	1.07 ± 0.50
Mean HWA/cm ± S.E.	2.79 ± 0.35	0.54 ± 0.08	6.12 ± 0.47	1.73 ± 0.25
% trees with *Laricobius* spp.	17%	0%	8%	33%
TN2				
Mean *Laricobius* spp. per tree ± S.E.	0.00 ± 0.00	--	0.14 ± 0.15	--
Mean HWA/cm ± S.E.	4.65 ± 0.38	--	7.33 ± 0.78	--
% trees with *Laricobius* spp.	0%	--	7%	--

TN3				
Mean *Laricobius* spp. per tree ± S.E.	0.03 ± 0.03	0.07 ± 0.18	0.08 ± 0.08	0.17 ± 0.16
Mean HWA/cm ± S.E.	11.06 ± 0.51	1.80 ± 0.23	8.79 ± 0.98	1.83 ± 0.26
% trees with *Laricobius* spp.	3%	6%	8%	8%
GA1				
Mean *Laricobius* spp. per tree ± S.E.	0.88 ± 0.26	0.53 ± 0.13	6.85 ± 2.02	3.83 ± 0.83
Mean HWA/cm ± S.E.	1.99 ± 0.23	0.40 ± 0.07	4.86 ± 0.41	2.95 ± 0.25
% trees with *Laricobius* spp.	48%	40%	92%	83%

--Data not collected at site

Table A4.3 Percent *L. nigrinus*, *L. rubidus*, and hybrid adults and larvae recovered in years one and two at nine study sites in the eastern U.S.

	Adults[a]		Larvae[b]	
Site	Year 1	Year 2	Year 1	Year 2
NJ1				
% *L. nigrinus*	100%	97%	100%	100%
% *L. rubidus*	0%	3%	0%	0%
% Hybrids	*	*	0%	0%
MD1				
% *L. nigrinus*	100%	97%	96%	100%
% *L. rubidus*	0%	3%	0%	0%
% Hybrids	*	*	4%	0%
VA1				
% *L. nigrinus*	100%	100%	**	100%
% *L. rubidus*	0%	0%	**	0%
% Hybrids	*	*	**	0%
VA2				
% *L. nigrinus*	100%	96%	100%	100%
% *L. rubidus*	0%	4%	0%	0%
% Hybrids	*	*	0%	0%
NC1				
% *L. nigrinus*	100%	100%	100%	93%
% *L. rubidus*	0%	0%	0%	2%
% Hybrids	*	*	0%	5%
TN1				
% *L. nigrinus*	100%	**	100%	93%
% *L. rubidus*	0%	**	0%	0%
% Hybrids	*	*	0%	7%
TN2				
% *L. nigrinus*	**	--	0%	--
% *L. rubidus*	**	--	100%	--
% Hybrids	*	--	0%	--
TN3				
% *L. nigrinus*	100%	100%	0%	50%
% *L. rubidus*	0%	0%	0%	0%
% Hybrids	*	*	0%	50%
GA1				
% *L. nigrinus*	100%	100%	100%	96%
% *L. rubidus*	0%	0%	0%	0%
% Hybrids	*	*	0%	4%

[a]Adults identified using morphological characteristics
[b]Larvae identified by analyzing amplified microsatellite loci
*Hybrids not identified using adult morphological characteristics
**No Laricobius spp. recoveries were made
--Data not collected at site

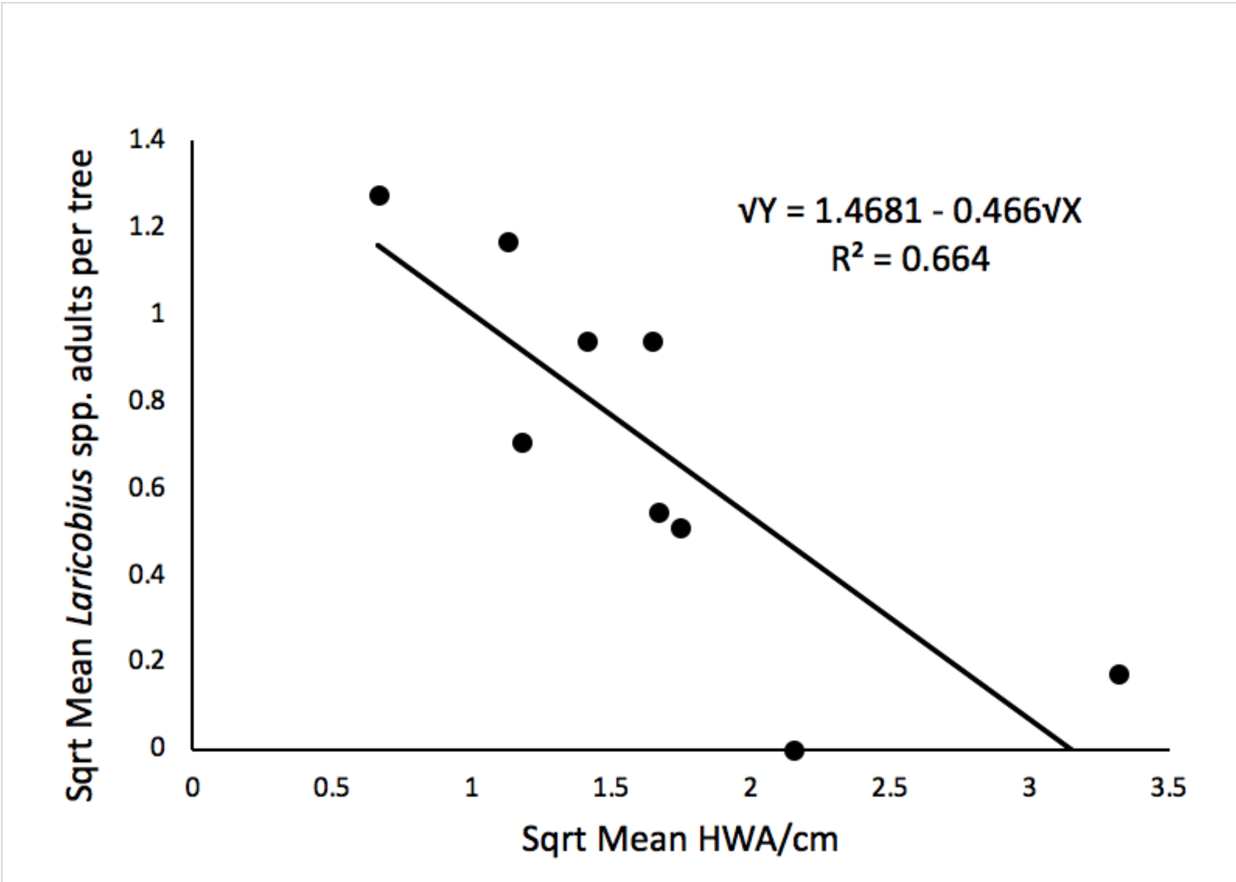

Figure A4.1. The relationship between the square root mean HWA/cm and the square root mean *Laricobius* spp. adults recovered in year one using simple linear regression with data pooled for all nine sites.

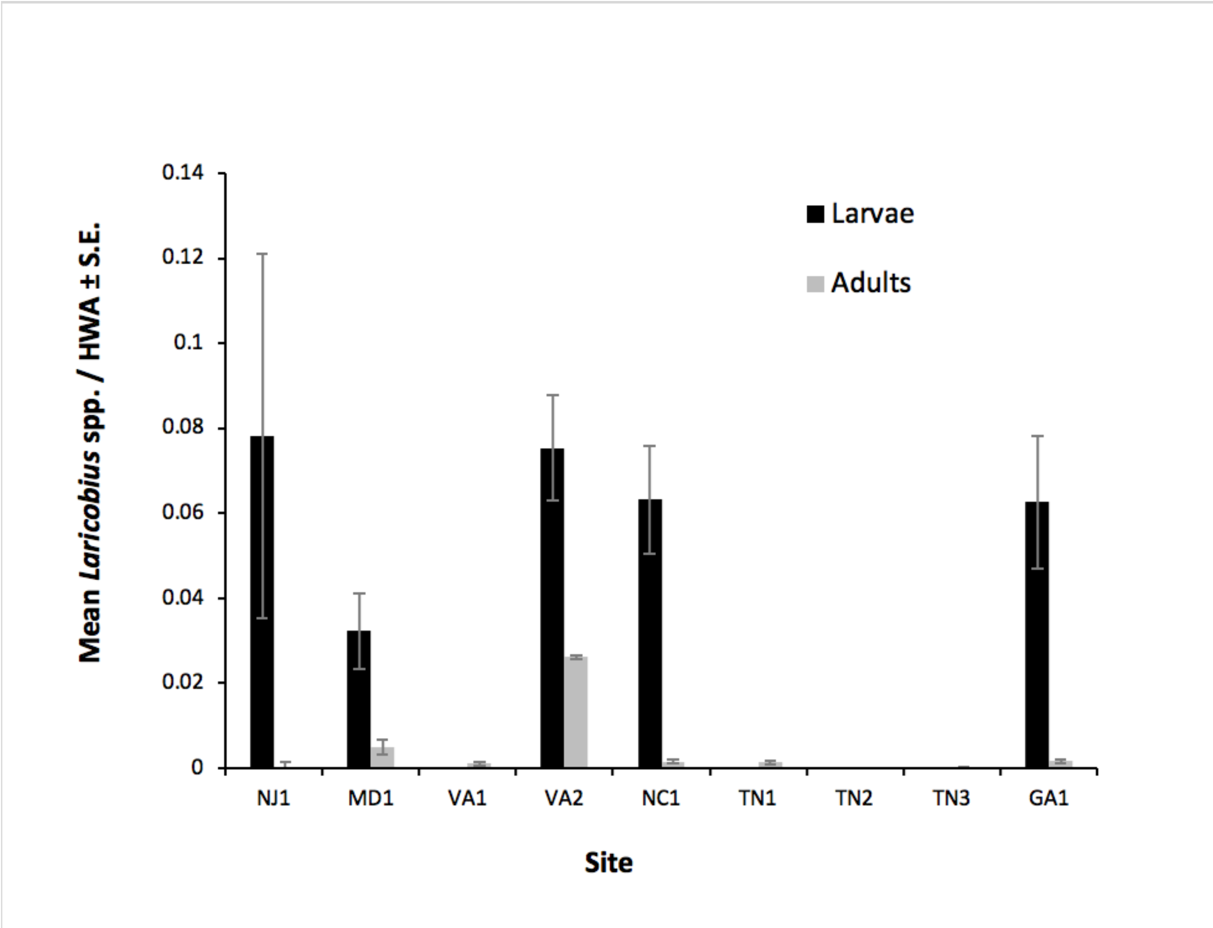

Figure A4.2. Mean ± S.E. adult and larval *Laricobius* spp. per HWA for year one at nine field sites in the eastern U.S. Results were calculated by dividing the mean number of *Laricobius* spp. adults or larvae by the mean number of living HWA nymphs present on study branches.

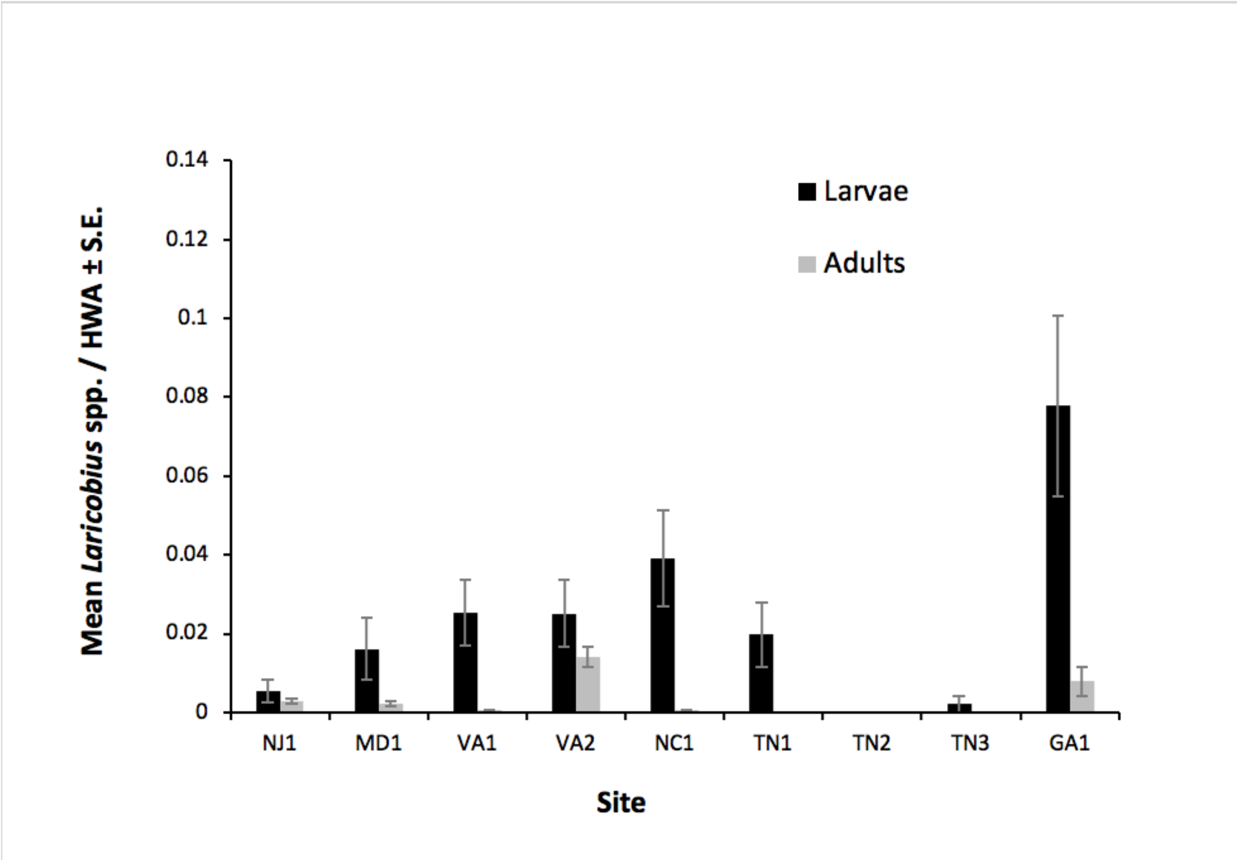

Figure A4.3. Mean ± S.E. adult and larval *Laricobius* spp. per HWA for year two at nine field sites in the eastern U.S. Results were calculated by dividing the mean number of *Laricobius* spp. adults or larvae by the mean number of living HWA nymphs present on study branches.

A1.5 References

Brantley, S., C. R. Ford, and J. M. Vose. 2013. Future species composition will affect forest water use after loss of eastern hemlock from southern Appalachian forests. Ecol. Appl. 23: 777-790.

Cheah, C. A. S.-J., and M. S. McClure. 1995. Exotic natural enemies of *Adelges tsugae* and their potential for biological control, pp. 103-112. *In* S. M. Salom, T. C. Tigner and R. C. Reardon [eds.], Proceedings of the First Hemlock Woolly Adelgid Review. USDA Forest Service FHTET-96-10.

Clark, R. C., and N. R. Brown. 1960. Studies of predators of the balsam woolly aphid, *Adelges piceae* (Ratz.) (Homoptera: Adelgidae), VII. *Laricobius rubidus* (Lec.) (Coleoptera: Derodontidae), a predator of *Pineus strobi* (Htg.) (Homoptera: Adelgidae). Can. Entomol. 92: 237-240.

Crawley, M. J. 1975. The numerical responses of insect predators to changes in prey density. J. Anim. Ecol. 44: 877-892.

Davis, G. A., S. M. Salom, C. C. Brewster, B. P. Onken, and L. T. Kok. 2012. Spatiotemporal distribution of the hemlock woolly adelgid predator *Laricobius nigrinus* after release in eastern hemlock forests. Agric. For. Entomol. 14: 408-418.

DeBach, P., and D. Rosen. 1991. Biological control by natural enemies, Cambridge University Press, Cambridge, UK, New York, NY.

Evans, A. M., and T. G. Gregoire. 2007. A geographically variable model of hemlock woolly adelgid spread. Biol. Invasions 9: 369-382.

Fidgen, J. G., D. E. Legg, and S. M. Salom. 2013. A sequential sampling plan for counts of *Adelges tsugae* on individual eastern hemlock trees. South. J. Appl. For. 37: 75-80.

Fischer, M. J., N. P. Havill, C. C. Brewster, G. A. Davis, S. M. Salom, and L. T. Kok. 2015. Field assessment of hybridization between *Laricobius nigrinus* and *L. rubidus*, predators of Adelgidae. Biol. Control 82: 1-6.

Gerling, D., O. Rottenberg, and T. S. Bellows. 2004. Role of natural enemies and other factors in the dynamics of field populations of the whitefly *Siphoninus phillyreae* (Haliday) in introduced and native environments. Biol. Control 31: 199-209.

Hajek, A. E. 2004. Natural Enemies: An Introduction to Biological Control, Cambridge University Press, Cambridge, UK, New York, NY.

Havill, N. P., and R. G. Foottit. 2007. Biology and evolution of Adelgidae. Annu. Rev. Entomol. 52: 325-349.

Havill, N. P., L. C. Vieira, and S. M. Salom. 2014. Biology and Control of Hemlock Woolly Adelgid, pp. 21. USDA Forest Service FHTET-2014-05, Morgantown, WV.

Havill, N. P., M. E. Montgomery, G. Y. Yu, S. Shiyake, and A. Caccone. 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Ann. Entomol. Soc. Am. 99: 195-203.

Havill, N. P., G. Davis, D. L. Mausel, J. Klein, R. McDonald, C. Jones, M. Fischer, S. Salom, and A. Caccone. 2012. Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control. Biol. Control 63: 359-369.

Heminger, A. R. 2017. 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. M.S. L.F.S Thesis, Virginia Tech, Blacksburg, VA. 93 pp.

Holling, C. S. 1959. The components of predation as revealed by a study of small-mammal predation of the European pine sawfly. Can. Entomol. 91: 293-320.

Huffaker, C. B., P. S. Messenger, and P. Debach. 1969. The natural enemy component in natural control and the theory of biological control. *In* C. B. Huffaker [ed.], AAAS Symposium on Biological Control. Plenum Press, Boston, MA.

Jones, A. C., D. E. Mullins, C. Brewster, J. P. Rhea, and S. M. Salom. 2015. Fitness and physiology of *Adelges tsugae* (Hemiptera: Adelgidae) in relation to the health of the eastern hemlock. Insect Sci. 23: 843-853.

Kessell, S. R. 1979. Adaptation and dimorphism in eastern hemlock, *Tsuga canadensis* (L.) Carr. Am. Nat. 113: 333.

Kimberling, D. N. 2004. Lessons from history: Predicting successes and risks of intentional introductions for arthropod biological control. Biol. Invasions 6: 301-318.

Kohler, G. R., V. L. Stiefel, K. F. Wallin, and D. W. Ross. 2008. Predators associated with the hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific Northwest. Environ. Entomol. 37: 494-504.

Mausel, D. L. 2005. Observations on fecundity and natural enemies of hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) in Seattle, Washington. Pan-Pac. Entomol. 81: 97-98.

Mausel, D. L., L. T. Kok, and S. M. Salom. 2017. Numerical response and impact of *Laricobius nigrinus* (Coleoptera: Derodontidae) on *Adelges tsugae* (Hemiptera: Adelgidae) in their native range. Environ. Entomol. 46: 544-551.

Mausel, D. L., S. M. Salom, L. T. Kok, and J. G. Fidgen. 2008. Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera: Derodontidae) on hemlock woolly adelgid in Virginia. Environ. Entomol. 37: 1498-1507.

Mausel, D. L., S. M. Salom, L. T. Kok, and G. A. Davis. 2010. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. Environ. Entomol. 39: 440-448.

Mayfield, A. E., B. C. Reynolds, C. I. Coots, N. P. Havill, C. Brownie, A. R. Tait, J. L. Hanula, S. V. Joseph, and A. B. Galloway. 2015. Establishment, hybridization and impact of *Laricobius* predators on insecticide-treated hemlocks: Exploring integrated management of the hemlock woolly adelgid. For. Ecol. Manage. 335: 1-10.

McClure, M. S. 1989. Evidence of a polymorphic life-cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). Ann. Entomol. Soc. Am. 82: 50-54.

McClure, M. S. 1991. Density-dependent feedback and population-cycles in *Adelges tsugae* (Homoptera: Adelgidae) on *Tsuga canadensis*. Environ. Entomol. 20: 258-264.

McClure, M. S., and C. A. S.-J. Cheah. 1999. Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. Biol. Invasions 1: 247-254.

Motley, K., N. P. Havill, A. L. Arsenault-Benoit, A. E. Mayfield, D. S. Ott, D. Ross, M. C. Whitmore, and K. F. Wallin. 2017. Feeding by *Leucopis argenticollis* and *Leucopis piniperda* (Diptera: Chamaemyiidae) from the western USA on *Adelges tsugae* (Hemiptera: Adelgidae) in the eastern USA. Bull. Entomol. Res. 107: 699-704.

Orwig, D. A., and D. R. Foster. 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. J. Torrey Bot. Soc. 125: 60-73.

Ross, D. W., S. D. Gaimari, G. R. Kohler, K. F. Wallin, and S. M. Grubin. 2011. Chamaemyiid predators of the hemlock woolly adelgid from the Pacific Northwest, pp. 97-106. In B. Onken and C. Reardon (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service FHTET-2011-04, Morgantown, WV.

Salom, S. M., L. T. Kok, A. B. Lamb, and C. S. Jubb. 2012. Laboratory rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A predator of the hemlock woolly adelgid (Hemiptera: Adelgidae). Psyche 2012: 9.

Siderhurst, L. A., H. P. Griscom, M. Hudy, and Z. J. Bortolot. 2010. Changes in light levels and stream temperatures with loss of eastern hemlock (*Tsuga canadensis*) at a southern Appalachian stream: Implications for brook trout. For. Ecol. Manage. 260: 1677-1688.

Solomon, M. E. 1949. The natural control of animal populations. J. Anim. Ecol. 18: 1-35.

Sumpter, K. L., T. J. McAvoy, C. C. Brewster, A. E. Mayfield, and S. M. Salom. 2018. Assessing an integrated biological and chemical control strategy for managing hemlock woolly adelgid in southern Appalachian forests. For. Ecol. Manage. 411: 12-19.

Tobin, P. C., R. M. Turcotte, L. M. Blackburn, J. A. Juracko, and B. T. Simpson. 2017. The big chill: Quantifying the effect of the 2014 North American cold wave on hemlock woolly adelgid populations in the central Appalachian Mountains. Popul. Ecol. 59: 251-258.

USDA. 2012. USDA Plant Hardiness Zone Map, https://planthardiness.ars.usda.gov/PHZMWeb/. United States Department of Agriculture.

Van Driesche, R., and T. S. Bellows. 1996. Biological Control, Chapman & Hall, New York.

Vieira, L. C., S. M. Salom, and L. T. Kok. 2012. Functional and numerical response of *Laricobius* spp. predators (Coleoptera: Derodontidae) on hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). Biol. Control 61: 47-54.

Virginia Tech. 2019. HWA Predator Database, http://hiro.ento.vt.edu/pdb/. Virginia Tech.

Wallace, M. S., and F. P. Hain. 2000. Field surveys and evaluation of native and established predators of the hemlock woolly adelgid (Homoptera: Adelgidae) in the southeastern United States. Environ. Entomol. 29: 638-644.

Zilahi-Balogh, G. M. G., C. D. Broeckling, L. T. Kok, and S. M. Salom. 2005. Comparison between a native and exotic adelgid as hosts for *Laricobius rubidus* (Coleoptera: Derodontidae). Biocontrol Sci. Technol. 15: 165-171.