ABSTRACT

POWERS, ZAIDEE LUCINA. Evaluating Techniques for Artificially Infesting Hemlocks (*Tsuga* spp.) with the Hemlock Woolly Adelgid (*Adelges tsugae*) for Genotype Resistance Screening. (Under the direction of Dr. Robert M. Jetton).

The search for natural resistance to the hemlock woolly adelgid (HWA, *Adelges tsugae*, Annand) in eastern (*Tsuga canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock requires research on the techniques used to test for tolerance/resistance in mass numbers of seedlings efficiently and effectively. The direct and suspended branch infestation techniques for detecting resistance to the hemlock woolly adelgid in hemlock (*Tsuga)* seedlings were analyzed to determine which method was most suitable for mass testing hemlock seedlings for resistance. Seedlings were infested with the progrediens and sistens generations in a completely randomized block design with the high direct infestation, low direct infestation, and a stationary suspended branch infestation. Following infestation, adelgid density on seedlings was evaluated in November. The progrediens infestations produced significantly greater adelgid densities on seedlings than the sistens infestations. When environmental conditions were favorable for adelgid growth and development, the suspended branch infestation technique produced significantly greater adelgid densities than the direct infestation technique on eastern hemlock seedlings. Densities achieved with the suspended branch technique were not significantly different from those achieved with the direct infestation technique on the Carolina seedlings. The suspended branch infestation with progrediens generation appears to be a more appropriate design than the high and low direct infestation with the progrediens generation, and all three infestation techniques with sistens generation, for testing mass numbers of seedlings for tolerance/resistance in a small scale experiment.

The suspended branch technique is the preferred technique for infestations but produces hot spots where adelgids are clustered below branches. An even dispersal of crawlers on seedlings below is necessary to fairly assess resistance. The density and distribution of emerging crawlers from stationary and rotating suspended branch techniques for the progrediens and sistens populations were evaluated using a randomized block design. To compare the effectiveness of the infestation techniques for tolerance/resistance testing, crawlers were collected on Tanglefoot® sticky sheets in March and May 2012. The progrediens generation did not produce significantly different adelgid densities (log) for either technique while the sistens generation produced significantly greater densities (log) of crawlers from the rotating branches than the stationary branches. The distribution of crawlers was significantly more dispersed/ less clustered for rotating branches than the stationary branches for the progrediens generation. The distribution of the sistens generation was not analyzed due to obstruction by fallen needles. The progrediens generation was determined to produce a larger density of adelgids, also noted in previous literature. In this study the progrediens branches had more eggs per ovisac than the sistens generation. It is recommended that tests for resistance consider the rotating suspended branch and use the progrediens generation.

Timing of adelgid dispersal has not been thoroughly tested in previous literature. The progrediens emergence timing was analyzed in a matched pair design where a small scale stationary suspended branch experiment was used to determine the number of days required for peak crawler emergence from infested branches, the number of days required for complete crawler emergence from the peak, and the influence of hydration on the density of emerging crawlers. Emerging crawlers were collected on Tanglefoot® sheets. The results indicated that 5 days were required for 1 peak in adelgid population emergence, population emergence began to decrease after the peak, and increase again by day 10. Crawler emergence began before day one and more days may be required to capture all crawlers before the peak. Hydrated branches produced significantly fewer dispersing crawlers than non-hydrated branches. Although the results suggest suspended branch infestations should be implemented for at least 5 days with non-hydrated infested branches for effective progrediens infestations, future research should consider the health of crawlers from non-hydrated branches before implementing in a mass testing procedure.

Evaluating Techniques for Artificially Infesting Hemlocks (*Tsuga* spp.) with the Hemlock Woolly Adelgid (*Adelges tsugae*) for Genotype Resistance Screening

by
Zaidee Lucina Powers

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Forestry and Environmental Resources
Entomology

Raleigh, North Carolina

2014

APPROVED BY:

Dr. Robert M. Jetton Committee Co-Chair	Dr. Albert (Bud) Mayfield
Dr. Fred Hain Committee Co-Chair	Dr. John Frampton

DEDICATION

To my parents and siblings for their constant love and support.

BIOGRAPHY

Zaidee Lucina Powers was born in upstate New York and lived in the country with her parents and four siblings. The forested property owned by her parents and grandparents gave her the opportunity to spend time outdoors and explore nature beginning at a young age. She further explored the outdoors on her family's annual trips to the Adirondack Mountains. Zaidee's interest in the sciences and outdoors became more focused while attending the SUNY College of Environmental Science and Forestry for a Bachelor's degree in Environmental Biology. Here she discovered a passion for entomology. Determined to work in the field of forest entomology she continued on to graduate school at North Carolina State University.

ACKNOWLEDGMENTS

Obtaining a Master's degree in Forestry and Entomology would not have been possible without support and encouragement from many people. First I thank my graduate advisor Robert Jetton for providing the opportunity to work on an exciting project with an important invasive forest pest. His dedication to the research and to my success as a graduate student is greatly appreciated. I am grateful for my committee: Robert Jetton, Albert Bud Mayfield, John Frampton, and Fred Hain. I worked closely with Robert Jetton and Bud Mayfield to set up my experiment and collect data, and received valuable advice regarding infestation techniques and experimental design. The support from Robert Jetton and John Frampton during the writing process is greatly appreciated. I am thankful to John Frampton for his valuable assistance with statistical analysis and experimental design. I appreciate the weekly lab meetings with Fred Hain and his lab discussing various research projects regarding the balsam woolly adelgid and hemlock woolly adelgid and receiving feedback on my research from Fred Hain, David Bednar, Ben Smith, Kelly Oten, Leslie Newton, Micah Gardner, Erin Mester, and Mary Talley.

There are a number of people outside my committee whose involvement is appreciated. Ben Smith (NCDA&CS/NCSU) provided technical assistance in the field and his help with the rotating suspended branch design, field work, and gopher are greatly appreciated. Andy Whittier (NCSU) dedicated many hours to travel and field work for the project. The experiment set up and data collection process would not have been as efficient and enjoyable without him. Frank Koch's (USDA Forest Service) spatial analysis expertise was greatly appreciated. The ArcGIS analysis process went smoothly due to his instructions and his willingness to meet and discuss the process and results in person. I thank Kaleb Rathbone and the NCDA&CS/NCSU Mountain Research Station in Waynesville, NC for providing space, equipment, and assistance. Thank you to the Coal Creek Company for site access and source of infested branches. Thank you to Weyerhaeuser Company for their donation of western hemlock seedlings.

A few people I met before graduate school played an important role in my life to get me to this point. I thank my undergraduate advisor Stephen A. Teale at the SUNY College of Environmental Science and Forestry for supporting my interest in forest entomology, providing various useful laboratory and field experiences, and aiding my search for graduate programs. I also thank the Thayer Family Trust Scholarship for their support throughout my undergraduate and graduate careers. My success is also due to the love, support, and encouragement from my parents Mom and Pop, siblings Joseph, Ariel, Leandra, and Quentin, and boyfriend Carlos Rosales.

This research project would not have been possible without funding from the USDA Forest Service Southern Research Station Cooperative Agreement No. 11-CA-11330129-064.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter II

Chapter III

<u>Chapter IV</u>

CHAPTER 1
A Review of the Literature Pertaining to Hemlock and the Hemlock Woolly Adelgid

Thesis Introduction

The hemlock woolly adelgid (HWA, *Adelges tsugae* Annand) is an invasive insect in North America that has caused significant decline in the ecologically important and ornamental eastern (*Tsuga canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock. The insect is native to Japan and was introduced to eastern North America on Southern Japanese hemlock nursery stock (Havill et al. 2006; Havill et al. 2007). It was first sighted in the 1950's in Richmond, Virginia and in following years has spread to 19 states on the east coast (Gouger 1971; USDA Forest Service 2012).

Eastern and Carolina hemlock are important ecological components of the forest. The removal of the species from the ecosystem dramatically changes overstory and understory canopy, animal inhabitants, and nutrient cycles (Jenkins et al., 1999; Ford et al., 2012; Knoepp et al., 2011; Rowell and Sobczak, 2008; Tingley et al., 2002; Ross et al., 2003; Ingwell et al. 2012). The adelgid causes significant decline in the tree by feeding on xylem ray parenchyma cells at the base of needles, depleting nutrients from storage cells and causing needles to grey and fall from the tree, bud mortality, reduced new growth, limb dieback, and whole tree mortality in as few as four years (Young et al. 1995; McClure et al. 2001). Two of the three adelgid generations, the progrediens (April-June) and sistens (June/July-March), survive and inhabit hemlock in North America (McClure 1987; McClure 1989), and disperse from tree to tree by wind, birds, deer, and humans (McClure 1990).

Integrated Pest Management (IPM) is used to control the pest in urban and forest settings, incorporating chemical (stem injections, trunk injections, foliar sprays), biological (native and non-native predators, fungi), host resistance, cultural treatments, and host gene conservation control mechanisms limited by land ownership, technology, funding, hemlock health, and HWA population level. Biological controls and resistance breeding are more feasible for managing the pest in the forest (McClure, 2007; Vose et. al., 2013).

Resistance research includes the breeding of native and tolerant non-native hemlock species as well as searching for natural resistance to the pest in native hemlock (Montgomery et al. 2009; Jetton et al. in press). Hemlock trees are tested for resistance by introducing the pest to the host and observing the host response. Infestations require consistent, reliable, and well distributed adelgid populations (Alfaro et al. 2008; Hodge and Dvorak 2007; Butin et al. 2007). The direct infestation is a technique that has been utilized in the past to successfully infest seedlings and mature trees by placing infested branches next to non-infested trees (Montgomery et al. 2009). The suspended branch technique is a newer method that is less labor intense than the direct infestation and involves placing infested branches above seedlings and allowing the crawler to fall onto the seedlings, resembling natural distribution (Newton et al. 2011; Jetton et al. in press).

The research presented focuses on improving the techniques used to test for resistance in seedlings. An effective and efficient technique is required for the mass testing of seedlings necessary to determine if tolerance/resistance is present in the native populations. Chapter II focuses on comparing two infestation techniques that have been used to successfully infest hemlock trees in previous studies, the direct infestation and suspended branch infestation. The density and population longevity of the adelgid was examined on infested eastern, Carolina, and western (*T. heterophylla* (Raf.) Sarg.) hemlock seedlings. Chapter III explores the suspended branch infestation and is focused on improving the distribution of crawlers falling from infested branches. Adelgid density and distribution were examined for stationary and rotating suspended branch techniques. Lastly, Chapter IV examines the emergence period of crawlers, a topic that has not been thoroughly studied in previous literature. The peak in population emergence, complete emergence from that peak, and the influence of hydrated branches on density of crawler emergence were observed. The remaining portion of Chapter I provides a history of the hemlock hosts and the adelgid pest, and explores the ecological and biological changes associated with the pest, management techniques, and conservation options.

Tsuga Characteristics

Distribution

The hemlock genus is composed of nine species. The five species that occur in Asia include the Northern Japanese hemlock, Chinese hemlock, Southern Japanese hemlock, Forrest's hemlock, and Himalayan hemlock (*Tsuga diversifolia* (Maxim.) Mast., *T. chinensis* (Franch.) E. Pritz., *T. sieboldii* Carr., *T. forrestii* Downie, and *T. dumosa* D. Don, respectively). The four species in North America include western hemlock, mountain hemlock, eastern hemlock, and Carolina hemlock (*T. heterophylla* (Raf.) Sarg., *T. mertensiana* (Bong.) Carr., *T. canadensis* (L.) Carr., *T. caroliniana* Engelm., respectively) (Farjon 1990). Hemlock trees grow in temperate zones. *Tsuga* Carr. are believed to have originally inhabited the four Northern hemispheres (Western North America, Eastern North America, Europe, and Asia). The groups in western North America diverged first in the late Eocene. The eastern North American group separated next during the Oligocene. In the Miocene, Carolina hemlock separated from Northern Japanese hemlock and Southern Japanese hemlock in Eurasia and the European trees became extinct. Changes in distribution were due to the shift to a colder and drier climate (late Tertiary) (LePage et al. 2003).

Between the late Cretaceous and the Plio-Pleistocene hemlock was present in North America and Eurasia. Hemlock first appeared in the fossil record in the Upper Cretaceous. Pollen near Opole, Silesia was discovered in rocks of the Upper Cretaceous. During the late Palaeocene and Eocene hemlock was present in England, Ireland, Spitsbergen, and Axel Heiberg Island, and the Western United States. At that time genes were moved between Western Europe and North America. Gene transfer was aided by the De Geer Route land bridge and the Thulian Route land bridge. There were a few major geographic and climate changes between the Eocene and Oligocene. Sea floor spreading caused the breakdown of the North Atlantic land bridge which aided in the movement of genes between North America and Europe. The Turgai Strait, a seaway that had previously prevented movement of hemlock westward, had dried. Overall the globe was cooling (LePage et al. 2003).

The location of hemlock in the Eocene and Oligocene was similar. The Eurasian species were not able to disperse to East Asia or North America. Climate in the late

Oligocene prevented movement of genes between Asia and Europe. During the Miocene genes were transferred between Asia and North America via the Beringian corridor. In the Pleistocene hemlock was widespread in Europe (LePage et al. 2003).

Currently the genus is present in the Pacific northwest of North America in the coastal mountains and islands. It is also located in the northern Rocky Mountains and Sierra Nevada Mountains. In the eastern part of the country the range is between Nova Scotia, Alabama, and Minnesota. The genus does not occur naturally in the middle of the North American continent or the subarctic north. The Asian species are present in Japan, China, and in some of the Himalayas. The mountains in Honshu, Shikoku, Kyushu, Yakushima, and Taiwan provide favorable habitat. The distribution of all members of the genus is discontinuous (Farjon 1990; Jetton et al. 2008).

The genus is divided into 9 species (Farjon 1990). The grouping is based on morphology, phytochemical characteristics, the nuclear ribosomal internal transcribed spacer region (ITS), chloroplast DNA (cDNA), and biogeography. Pollen morphology is an example of a characteristic that may be used to analyze phylogeny. The earliest pollen discovered is from the late Cretaceous (ca. 90mya) and was located in Poland. The pollen was bisaccate and resembled mountain hemlock. Additionally monosaccate pollen was discovered and resembled eastern hemlock and Northern Japanese hemlock. The phylogeny is checked with other characters as well. Software such as DIVa and AReA has been used to determine species ranges. The hypothesized distribution of *Tsuga* ultimately depends on the type of analysis (Havill et al. 2008).

The hemlock genus can be separated into three clades: 1) western hemlock and mountain hemlock in Western North America, 2) the species that occur in Asia and Carolina hemlock and 3) eastern hemlock. The bristlecone hemlock (*Nothotsuga longibracteata* (W.C.Cheng) Hu ex C.N.Page, formerly known as *T. longibracteacta* (W.C.Cheng) Hu ex C.N.Page) is currently considered an out group in the phylogeny of the hemlock genus. Mountain hemlock can be considered monotypic or a sister group to other *Tsuga* species and requires more analysis. Havill and others (2008) concluded that Carolina hemlock is not sister to eastern hemlock, but is nested in the Asian hemlock clade. *T. formosa* has previously been considered a variety of Chinese hemlock *(T. chinensis* (Tiawan)) and a sister to or nested in with Chinese hemlock on the China mainland. Havill considers *T. formosa* to be a species. Southern Japanese hemlock is closely related to Northern Japanese hemlock. Forrest's hemlock is not closely related to Himalayan hemlock and is nested in Chinese hemlock. The Himalayan hemlock is sister to the Asian clade and Carolina hemlock (Havill et. al. 2008).

Ecology

Hemlock is a long lived and slow growing tree. The genus is very shade tolerant and drought susceptible. The trees mature along streams and in areas where precipitation is constant during the growing season. Most species are in thick forests with high levels of humidity and moisture, often in subalpine and lowland forests (Evans et al. 1996; Farjon 1990).

The mature trees regulate growth under the canopy by controlling light, temperature and moisture. Hemlock is considered the dominant tree in mixed deciduous and conifer forests undergoing succession. The branches extend over the understory and block most light. Since the genus is shade tolerant, seedlings successfully mature under adult trees. Temperature is regulated in the understory and tends to be warmer in the winter months and cooler in the summer months. The ecosystem below the canopy is kept moist. Soils are usually acidic but can be neutral. The root system also aids in the tree's dominance (Evans et al. 1996; Farjon 1990).

In general, hemlock trees are monopodal (single stemmed) and straight with conical or pyramidal crowns. The characteristics of a tree depend on the environment. In North America the trees tend to exhibit these characters. The hemlocks in Asia often have multiple stems and domed or flat crowns. Seedlings exhibit drooping in the leader, terminal branches, and the stems have a whirling pattern (Farjon 1990).

Reproduction

The following information pertains to most hemlock species. The trees are monoecious. The female and male flowers occur on the same branch and mature between April and June. Female strobili are solitary and develop from terminal buds in the second year. Male strobili are solitary and develop from axillary buds in the second year. Both male and female strobili occur in high numbers on the outer part of the crown. The female structures are small and ovoid/ovoid-oblong before opening and ripen from a green/purple color to brown. Male structures are small, subglobular, and ripen from red color to yellow/red/purple (Farjon 1990).

When monosaccate pollen is present (ex: western hemlock) the cones are pendulous. The pollen contains spines that aid in attachment to the cone. After pollen has successfully attached, a pollen tube extends to the ovule. With bisaccate pollen (ex: mountain hemlock), the cones are upright. The pollen attaches to a pollen droplet and floats up to the ovule. After fertilization (six weeks), cones require 4 months to mature and seeds are distributed between mid-August and mid-September. Seeds are small, light brown, and have a membranous cup on one side (Farjon 1990; Jetton et al. 2008).

The eastern hemlock, Carolina hemlock, western hemlock, Chinese hemlock, and Southern Japanese hemlock are important species for research on the hemlock woolly adelgid (HWA). HWA was introduced to North America on Southern Japanese hemlock nursery stock. Eastern and Carolina hemlock are two native species in eastern North America that exhibit little to no resistance to HWA. Western hemlock is a North American species that appears to be more resistant to HWA (McClure 1987). Chinese hemlock has been considered for breeding a hybrid resistant hemlock that is most similar to the native hemlocks on the east coast of North America (Montgomery et. al. 2009). The role of these species in the progression of the HWA infestation will be discussed in the following sections.

Hosts: Eastern Hemlock (*Tsuga canadensis* (L.) Carr.), Carolina Hemlock (*T. caroliniana* Engelm.), and Western Hemlock (*T. heterophylla* Raf. Sarg.)

Eastern and Carolina hemlock have very little or no resistance to the hemlock woolly adelgid (HWA, *Adelges tsugae* Annand). HWA will likely decimate most eastern and

Carolina hemlock populations. A few trees (escapes or putatively resistant) may survive but the genetic diversity of the species will have been greatly reduced. Western hemlock is considered resistant to HWA on the west coast (McClure 1987; US Forest Service 2012).

Distribution and Ecology

Eastern hemlock is long living and a late successional climax species. It naturally occurs in areas that are cool, humid, and receive an annual precipitation of 700m-1500mm per year. Acceptable locations include land from eastern Canada to the north eastern United States and as far south as northern Georgia and Alabama. The range extends west to Minnesota. Some populations are scattered in Indiana and Ohio (Farjon 1990; Quimby et al. 1996).

The tree grows on rocky, acidic, loam, and silt loam soils with a pH of 3-4. The species may occur in a pure stand but is more often found mixed with deciduous trees and other conifers. In mixed stands eastern hemlock is associated with eastern white pine (*Pinus strobus* L.), red pine (*P. resinosa* Aiton), balsam fir (*Abies balsamea* (L.) Mill), red spruce (*Picea rubens* Sarg.), white spruce (*P. glauca* (Moench) Voss), tamarack (*Larix laricina* (Du Roi) K.Koch), birch species (*Betula* L.), sugar maple (*Acer saccharum* Marshall), northern red oak (*Quercus rubra* L.), white ash (*Fraxinus americana* L.), black ash (*F. nigra* Marshall), American beech (*Fagus grandifolia* Ehrh.), and *Populus* L. species (Farjon 1990; Quimby et al. 1996).

Carolina hemlock has a narrow range and is known to occur in Virginia, North Carolina, South Carolina, Tennessee, and Georgia. Populations are located at elevations of 600-1500m and some as low as 100m-600m (Farjon 1990; Jetton et al. 2008). The distribution is thought to be the result of Pleistocene glaciations. Carolina hemlock may have occupied a greater area that was narrowed and separated due to the glaciations (Jetton et al. 2008).

The populations grow in isolated groups and are often on ridges and rocky outcroppings. Carolina hemlock may also occur in cool meadows and along streams. The atmosphere is cool and humid with an annual precipitation of more than 1000mm. Precipitation is evenly distributed throughout the year (Farjon 1990).

The species grows in forests with hardwoods, in pure, small stands, or as a single tree. It is often on sandy-clay loam soils that are low in nutrients, very acidic, and drained. The soil content can vary greatly. The species is associated with eastern white pine, table mountain pine (*P. pungens* Engelm.), oaks (*Quercus* L.), red maple (*Acer rubrum*), mountain laurel (*Kalmia latifolia* L.), *Rhododendron L.,* and sometimes eastern hemlock. Southern populations are challenged by heat, fires, and presence of cliffs and rocky outcroppings. In the north, Carolina hemlock is limited by summer precipitation and hardwood forests (Jetton et al 2008; Farjon 1990).

The western hemlock is present on the Pacific coast of North America from Kenai Peninsula in Alaska to North Western California. It also occurs in the Cascade Range, Selkirk Mountains (B.C.), Idaho, and Northwestern Montana. The hemlock grows between sea level and 600m a.s.l. as well as up to 1800m in the Rocky Mountains. Suitable area in the Rocky Mountains is limited by dry summers. The climate in these areas is cool-maritime along the coast and cool-montane inland. In the coastal region the annual precipitation is (500-) 900-3800 mm. There is less precipitation inland (Farjon 1990).

Western hemlock occurs naturally in many soil types. The species is mostly found on soil with a pH of 3.5-5 and an acidic organic top layer (Farjon 1990). When moss covers the ground in some of these habitats the seeds are not able to grow through the moss. The seeds therefore germinate on dead trees that lay on the ground (Farjon 2010). The hemlock does not often grow in pure stands. The pure stands that do occur are present on the coast. It is an important component of maritime mesothermal coniferous forests (Farjon 1990).

Importance

Eastern hemlock is an ecologically important species. The hemlock provides a dense shade cover, produces an acidic “duff” layer, and regulates light, temperature, and moisture in the understory. The tree grows along streams and regulates the water temperature. Brook trout prefer conditions between 10°C and 20°C and depend on the hemlock for warm temperatures in the winter and cool temperatures in the summer. Roughed grouse, turkey, deer, snowshoe hare, and rabbit utilize the foliage for winter shelter. The tree also has a significant influence on invertebrates, amphibians, and reptiles. The acidic conditions influence soil moisture, stream flow, decomposition, and nutrient cycling (Evans et al. 1996; Quimby et al. 1996).

In Connecticut, 90% of birds depend on eastern hemlock for food, nesting, roosting, and shelter in the winter. The black-throated warbler is an example of a species that requires eastern hemlock sites. The solitary vireo, northern goshawk, and some plant species often prefer hemlock sites (Quimby et al. 1996). In addition to providing habitat, eastern hemlock regulates the presence of invasive plants. The removal of eastern hemlock from a forest stand may be followed by the successful establishment of Tree of Heaven (*Ailanthus altissima* (Mill.) Swingle.), Japanese barberry (*Berberis thunbergii* DC.), and Japanese stilt grass (*Microstegium vimineum* (Trin.) A. Camus) (Evans et al. 1996).

The eastern hemlock is not considered an important timber source. The wood is brittle, suffers from ring shake, uneven, usually cross-grained, and dry. It may be utilized for barns, sheds, and other structures. The tree is primarily used for pulp wood. Eastern hemlock is also a common landscaping tree with a variety of cultivars. It is valuable in landscaping as wildlife habitat (Krüssmann 1985; Quimby 1996). The eastern hemlock has more cultivars than western hemlock. The species is not preferred over western hemlock for planation forestry (Farjon 2010).

Eastern hemlock is aesthetically pleasing to humans. It provides shade along streams and in mountains which creates a comfortable environment during the summer. In the winter the understory is warmer while cool wind and noise is obstructed (Quimby et al. 1996).

Carolina hemlock is an ecologically important species. The hemlock has a dense crown and is long living (Farjon et al. 1990). The tree provides food for birds and mammals. The bark is eaten by beaver and sometimes porcupine and rabbit. Deer feed on the branches in winter and utilize the tree for shelter and bedding. The hemlock prevents soil erosion on rocky outcrops. The species is also often used by humans for landscaping. The aesthetic value and landscaping value are similar to eastern hemlock (Krüssmann 1985; Quimby et al. 1996). Carolina hemlock is not used as often for ornamental purposes as eastern hemlock because the seed is difficult to establish and the species is slow growing. It is not a lumber species because of the range in which it is found. The species is less common than other suitable lumber species and not widespread. The commercial use is therefore limited (Farjon 2010).

Western hemlock is an important species for timber production in the northwestern part of the United States and in western Canada. It is often used for pilings, poles, railway sleepers, construction, and wood pulp. The species is fast growing and was brought to Britain and northwestern Europe for forest plantations. Although the hemlock can survive when planted underneath the canopy of deciduous trees, it is not recommended in areas where native flora is protected. The tree produces heavy shade that will hinder growth of plants underneath the canopy. Western hemlock is also planted in arboreta and parks in the natural range as well as in the British Isles (Farjon 2010).

The hemlock provides some shelter and food for wildlife. Birds build nests in tree cavities. Elk and deer utilize the species for food. Snowshoe hair and rabbits also utilize the tree for food. The small mammals feed on seedlings (Moore 2002).

Pest: Hemlock Woolly Adelgid (*Adelges tsugae* Annand)

The hemlock woolly adelgid is an invasive insect in eastern North America and native to western North America. It was transported from Japan on Southern Japanese hemlock (*Tsuga sieboldii*) nursery stock (Havill et al. 2006). The insect was first sited in North America around the 1920's on the western coast in British Columbia. It first appeared on the east coast in the 1950s in Virginia and again in the 1960's in Pennsylvania (McClure, 1987). North American tree species susceptible to the hemlock woolly adelgid (HWA) include eastern (*Tsuga canadensis*) and Carolina hemlock (*Tsuga caroliniana*) (Havill et al. 2006; Havill et al. 2007).

Distribution

Members of the family Adelgidae occur on all species of hemlock and are adapted to live under different conditions. The adelgid located in China, Taiwan, and Japan are considered to be separate clades. The adelgid present in Japan and eastern North America are significantly similar and are of the same clade (Havill et al. 2006). The western North American populations occur naturally and the eastern North American populations were introduced from Japan (US Forest Service 2011). HWA is located in boreal and temperate areas of the northern hemisphere (Havill et al. 2007). The species is currently present in 19 states in the eastern United States (US Forest Service 2012).

Life Cycle

The life cycle of HWA in eastern North America differs from reproduction in the natural range of Asia. In the Asian range of HWA, the adelgid has a holocyclic life cycle in which both sexual and asexual reproduction takes place. The life cycle requires the presence of a primary and secondary host. *Picea* Mill. is the primary host and supports the sexual life stage: sexuales. Three asexual generations are produced on the secondary hosts: sexupara, progrediens, and sistens. The secondary host that occurs in North America is *Tsuga*. Acceptable spruce species are not present in North America. Only asexual forms of HWA can persist. The progrediens and sistens generations undergo parthenogenic reproduction and only two generations are produced per year (McClure 1989).

The progrediens population produces the sistens generation. Eggs are deposited in June. The sistens generation emerges from eggs in June/July and undergoes four instars before the adult stage. The second, third, fourth nymphal instars and the adult may be distinguished by thoracic sutures, winged bud notches, sclerotization, body size, body shape, thorax, and antennae. The wingless sistens generation is present on the tree June/July through March/April (McClure 1987; McClure 1989).

The first instar sistens start to feed at the base of needles in June/July. The nymphs, the immature form of the insect, stop feeding and aestivate during the summer. The aestivation period is similar to hibernation. The adelgid does not feed or move (McClure 1987; McClure 1989). Aestivation is regulated by temperature, photoperiod, and maternal conditions between the egg and second instar stages. Surpassing aestivation has been accomplished in the lab by conditioning adult progrediens to 12°C and 14°C. Photoperiod is not as effective in manipulating aestivation but a photoperiod of 12:12 (L:D) was almost successful (Salom et al. 2001).

During the fall and winter, sistens nymphs stop aestivation (usually around October) and begin to feed again. Over the winter the sistens generation matures. In February the adults start to deposit eggs and continue to produce eggs for 16 weeks. March through May the sistens produce a cottony, white, woolly ovisac that surrounds the egg and the adult. One ovisac houses 48.6 ± 5.8 eggs (McClure 1987; McClure 1989).

The sistens generation gives rise to the wingless progrediens and the winged sexupara. In eastern North America forty to fifty percent of the eggs will be progrediens and the remaining eggs will be sexupara. Both progrediens and sexupara eggs are clustered together and indistinguishable. The progrediens and sexupara start to emerge in April (McClure 1987; McClure 1989).

The progrediens are present April through June/July. The progrediens crawler stage of the life cycle is mobile for one to two days and may be transported to a new tree by wind. The crawlers require 4 weeks to mature to the 4^{th} instar. The adults produce cottony, white, woolly ovisacs around the adult and eggs June through July. Each ovisac contains 21.7 ± 3.6 eggs. These eggs hatch in June/July and give rise to the sistens generation (McClure 1987; McClure 1989).

Mature sexupara are winged and leave the hemlock to settle on spruce. Sexupara do not produce a woolly mass. Each adelgid produces 11.5 ± 3.6 eggs and they are deposited under the wings. Sexupara produce the sexual generation of the HWA life cycle: sexuales. The offspring of sexupara begin feeding in July. They live for a few days and do not survive past the first instar because of the lack of an acceptable host (McClure 1987; McClure 1989).

Feeding

HWA crawlers feed on xylem ray parenchyma cells at the base of the needle on the leaf cushion. Usually one crawler settles at the base of a needle on new growth. Future generations of crawlers are found at the base of the needle where a previous crawler had settled. Most adelgid species feed on cortical parenchyma cells and solutes from the phloem.

Unlike other adelgid species, HWA depletes nutrients only from storage cells (Young et al. 1995).

The adelgid feeds with a stylet bundle. The bundle is on the ventral surface of the body and is more than three times the length of the adelgid body (McClure et al. 2001). The stylet bundle has two outer mandibular stylets with deep grooves and two inner maxillary stylets that are inside the mandibular grooves. The maxillary stylets extend and retract. The feeding and salivary canals are one canal. When the insect molts the bundle is retracted (Young et al. 1995).

The bundle is inserted primarily in the intracellular and epidermal cells at the base of needles. Usually the insertion site is near the abscission layer, adaxial to the needle, near the vascular xylem. The stylet bundle moves inter and intracellularly. In the vascular tissue, the stylet bundle moves along the xylem vascular bundle between tracheids and ray parenchyma cells (Young et al. 1995).

The stylet stops at the xylem rays where nutrients are stored and transferred. Feeding removes stored nutrients, causing grey needle coloring and eventually needle loss (McClure et al. 2001). The crawler consumes nonstructural carbohydrates including starch and free sugar (Schwartzberg and Montgomery 2010). Trees exhibit varying levels of infestation, including bud mortality, needle loss, absence or reduction of new growth, and limb dieback progressing from the bottom of the tree to the crown. Tree mortality occurs in as little as 4 years (McClure et al. 2001).

HWA feeding is known to cause a hypersensitive response by the tree. The amount of hydrogen peroxide (H_2O_2) in the foliage increases after infestation and leads to cell death in the tree. The response likely takes place throughout the whole tree and is not concentrated at the site of feeding. Additionally, it has been observed that old growth needles are smaller than new growth needles after infestation (Radville et. al. 2011).

Transport

HWA crawlers and winged sexupara are transported by birds, wind, deer, and humans. McClure (1990) analyzed these four modes of transportation. McClure caught birds and dipped them in a detergent-water mixture. The water was filtered and HWA from the water were quantified. Of the sixteen species of birds caught at his sites in East Haddam, Connecticut, thirteen species carried HWA. The birds were diverse in their ecological roles and included ground dwelling species (McClure 1990). The size of the woolly masses has an influence on movement by birds (McClure 1987). Movement by birds can be a problem in residential areas where bird feeders are present year round (McClure 1990).

McClure analyzed wind distribution of HWA with sticky sheets at three different heights and collected HWA from heights of 5m, 10m and 15m above ground. Wind transport was found to be uniform vertically throughout the canopy. Wind played a role in moving a large amount of the adelgid population up to 300m out from the center of a stand. The winged generation was transported farther from the center of the stand than the non-winged generation (McClure 1990). The size of the woolly masses also influences movement by wind (McClure 1987).

Deer browse was examined by analysis of browsed and non-browsed branches. Movement of HWA far from the stand was partially due to deer browse. Transport by deer occurred when the animal fed on hemlock. There was a significant interaction between deer browsing and distance. HWA density was greatest at the center and edge of the stands examined by McClure (McClure 1990).

Human transport is possible through logging. The survival of HWA by logging dead and dying trees was determined in a laboratory experiment where McClure looked at adelgid survival without food. Transport by logging dead and dying trees was determined to be likely. Eggs and crawlers have been proven to remain viable without food for more than two weeks in a lab. When dead and dying hemlocks are removed from a stand the adelgid may be able to live on the material long enough to move to a more suitable environment (McClure 1990). The riparian corridors, trails and roads utilized by loggers and hikers aid in the transport of HWA (Koch et. al. 2006).

Population Dynamics

The absence of the sexual stage of the life cycle has reduced the potential damage the adelgid can inflict on susceptible hemlock populations. The damage caused by the asexual generations is still significant. One female produces up to 300 eggs in her lifetime and can easily lead to a large infestation (McClure et al. 2001).

Mark McClure (1991) explored the factors that influence HWA population density. Results were collected from hemlock in forest and plantation sites and illustrate how HWA populations respond to changes in tree health. Population density was proven to be influenced by environmental factors and conditions present after nymphs emerged (McClure 1991).

The initial infestation of HWA takes place on healthy hemlock trees. The adelgid feeds on new shoot growth in the first year of infestation. When infestation levels are relatively low (less than four adelgids per one 20 mm^2 branch) the new shoot growth is slowed or halted. The adelgid reaches a peak in population density in the first year. Discoloration and desiccation of needles, as well as branch dieback, is present in the lower crown (McClure 1991).

In the second year of infestation HWA has to feed on old growth. HWA fecundity and survival is significantly hindered when the adelgid consumes old growth material. The proportion of winged individuals produced increases significantly. The sexupara fly in search of a suitable spruce while the progrediens population density decreases on the hemlock. During the third year of infestation the hemlock is able to produce new shoots (11%-15% new shoot growth) because of the decrease in adelgid population. HWA population density peaks again and the adelgid begins to feed on the new growth. Hemlock mortality occurs in as few as four years. Only sexupara were produced in the fourth year of the McClure's study (McClure 1991).

Overall the sexupara population continues to increase as the infestation progresses. The shift in population density to sexupara is important for the decrease in overall population density. In the native range of HWA the adelgid was able to shift from one host species to another when a host species population began to decline and became detrimental to adelgid survival. Although HWA populations eventually crash on hemlock in eastern North America, the hemlock is not able to survive the infestation (McClure 1991).

Ecological Impacts

HWA infestations can change short-term and long-term physical characteristics of the stand including nutrient cycles, understory plant species, dominant tree species, and composition of animal inhabitants. HWA influences ecosystems differently in the north and south of eastern North America. The rate of hemlock decline and the influence of the decline on the ecosystem are dependent on site characteristics, climate, genetics, rate of HWA infestation spread, effects of biological control agents, as well as other factors (Ford et al. 2012). Studies on the ecological impacts of HWA sometimes differ in results due to differences in the factors listed above (Knoepp et al. 2011) and it is difficult to predict the long term effects of hemlock decline with the information available (Vose et al. 2013).

In the northeast 50% hemlock mortality can occur in 17 years (Lewis et al. 2008). The process is shorter in the south leading to 50% hemlock death in 7 years (Ford et al. 2012). Hemlock provides a large amount of shade and prevents growth of shade intolerant plants beneath the canopy. The death of hemlock trees initially leads a greater gap light index. Available sunlight is expected to change more dramatically as the hemlock loose branches and fall to the ground. The greater amount of sun that passes through the canopy can potentially cause the soil temperature to increase. In the south rhododendron may prevent soil temperature increase by inhibiting access of light to the understory. The ground also experiences a greater amount of leaf litter in infested stands than healthy stands for a few years after infestation due to the greater amount of needle fall from hemlock (Jenkins et al. 1999; Ford et al. 2012; Knoepp et al. 2011). The short term physical alterations to the stand are expected to change as other species replace the hemlock.

The gradual thinning of the canopy (Kizlinski et al. 2002) can result in greater seedling regeneration (Jenkins et al. 1999). In a hemlock-deciduous forests of the northeast, species commonly found in healthy stands include eastern hemlock, red maple (*Acer* *rubrum*), and white oak (*Q. alba* L.). Eastern hemlock mortality due to high infestation levels leads to short term changes in stand structure that include dominance by black birch (*Betula lenta* L.) as well as the presence of red maple , Canada mayflower (*Maianthemum canadense* Desf.), witch-hazel (*Hamamelis virginiana* L.), northern red oak (*Quercus rubra* L.), and chestnut oak (*Q. prinus* L.). Black birch could potentially become the established dominant tree species in forests without hemlock. More plant species richness is present in heavily infested stands than lightly infested stands. The species richness of shrubs is not significantly different between lightly and heavily infested areas (Ingwell et al. 2012; Jenkins et al. 1999).

In the northeast the forests experience a number of changes within a few years of infestation. The soil organic matter, total carbon, and total nitrogen in an ecosystem are not influenced by eastern hemlock mortality. Hemlock decline does lead to an increase in net nitrogen mineralization, nitrification, nitrogen turnover, and inorganic nitrogen availability. Infested areas also experience larger pools of ammonium-N and nitrate-N (Jenkins et al. 1999). Stream ecosystems are also affected by hemlock mortality. The increased light and nitrogen conditions can result in an increase in periphyton, a source of primary production (Rowell and Sobczak 2008).

Forests of the northeast may potentially shift from conifer to deciduous species. The decline of hemlock (Orwig and Foster 1998) will have a great influence on the long term effects of HWA on ecosystem processes (Jenkins et. al. 1999). A forest that is dominated by eastern hemlock and American beech experiences hemlock decline, the nitrogen cycle is not expected to change greatly because both species influence the cycle similarly (Finizi et al. 1998). A forest that was dominated by eastern hemlock and sugar maple with very basic soil is expected to have large changes in rates of carbon and nitrogen cycling after hemlock decline (van Breemen et al. 1997).

Southeast stands that experience hemlock decline undergo a large increase in the amount of rhododendron if rhododendron is already present. The amount of rhododendron is expected to continue to increase over time. Humans will not be able to completely remove the plant from the stand. Rhododendron inhibits growth below the canopy except where there are spaces between the patches. In these spaces some herbs and tree seedlings are able to develop (Ford et al. 2012). When rhododendron is not present, the stand is likely be dominated by maple, birch, beech (*Fagus* L.), and oak. Tree species that are present in hemlock forests increase in growth rate for a few years after hemlock decline. The trees take advantage of the light available. The ground layer species cover, richness, diversity, and tree seedling density varies between years. A few years after hemlock decline begins evergreen shrubs tend to increase growth. The number of species increases over time in the infested sites (Ford et al. 2012).

In the south soil carbon, nitrogen mineralization rate, and nitrogen concentration are not influence by hemlock decline within the first few years. The total nitrogen is initially greater in infested sites than healthy sites. The levels of the Oa and Oe soil horizon mass, carbon, nitrogen, and phosphorus are greater in infested stands than healthy stands. As the forest shifts to a completely hardwood site, the rate of the nutrient cycling is expected to increase and resemble the process in current hardwood sites (Knoepp et al. 2011). Rhododendron inhibits light availability to streams and may prevent changes in stream nutrient content. Within areas with hemlock decline in-stream respiration provided more net ecosystem production than gross primary production (GPP) (Northington et al. 2013).

Hemlock forests provide important nesting sites for a number of bird species. The composition of avian inhabitants is expected to change with the shift in forest structure. The new forests will potentially house a greater amount of eastern wood-pewee (*Contopus virens* Linnaeus), brown-headed cowbird (*Molothrus ater* Boddaert), tufted titmouse (*Baeolophus bicolor* Linnaeus), white-breasted nuthatch (*Sitta carolinensis* Latham), red-eyed vireo (*Vireo olivaceus* Linnaeus), and hooded warbler (*Wilsonia citrina* Boddaert). The species composition will vary from the current avian populations in healthy hemlock stands (black-throated green warbler (*Dendroica virens* Gmelin), Acadian flycatcher (*Empidonax virescens* Vieillot), blackburnian warbler (*Dendroica fusca* Müller), and hermit thrush (*Catharus guttatus* Pallas)). The black-throated green warbler, blackburnian warbler, and Acadian flycatcher are especially sensitive to hemlock removal in the north. The hermit thrush may benefit from hemlock decline (Tingley et al. 2002).

Fish are another animal that are dependent on the presence of hemlock. Two to three times more brook trout (*Salvelinus fontinalis* Mitchill) and brown trout (*Salmo trutta* Linnaeus) are present in streams with hemlock than hardwoods. Streams in hardwood forests support more insectivorous fish while streams in hemlock forests support more piscivorous fish. The shift from hemlock to hardwood forests will alter the functional feeding groups of fish as well as hinder biodiversity (Ross et al. 2003).

The diversity of above ground invertebrates that inhabit forests with hemlock in the northeast is greater in heavily infested stands than lightly infested stands. Invertebrates in highly infested stands include mainly acari and collembola, as well as some Coleopterans. Most of the invertebrates are predators. The arthropod species at the ground level do not differ significantly possibly due to delayed response or lack of dependence on hemlock leaf litter (Ingwell et al. 2012).

Future Distribution

Paradis et al. (2008) concluded that 91% mortality of the insect is required to prevent spread. The number of days of average daily minimum temperature, absolute daily minimum temperature, and average daily mean temperature are important environmental components that influence adelgid survival. Increased temperatures due to climate change are expected to aid spread of the adelgid. The gradual warming of the climate on the east coast will likely raise the winter temperatures, remove cold climate limits, and could potentially allow complete expansion of the northern edge of the infestation by the end of the century. In the next few decades temperatures are expected to increase 2-4°. Temperatures will likely increase by 5-8 ° by the end of the century. The climate change may also only allow spread to New York, leaving upper New York, Vermont, New Hampshire, and Northern Maine in an unfavorable isotherm (below -5°C) for HWA (Paradis et al. 2008).

The ability of HWA to survive depends on month, temperature, exposure time, and sample area. As temperature decreases, the ability of HWA to survive decreases as well.

HWA cannot survive below -30°C under laboratory conditions. HWA in January and February survive colder temperatures than individuals in March. Cold hardiness changes over time and is different for northern, central, and southern regions. HWA in the southern and central regions loose cold tolerance earlier in the year than HWA in the North. Cold tolerance is greater in northern regions (excluding pockets with variable temperatures) than central and southern regions. The difference in cold tolerance has not been proven to be a result of evolved tolerance in the North (Parker et al. 1999; Skinner et al. 2003). Survival may be estimated by accounting for minimum winter temperatures and latitude. In general, the majority of eastern hemlock and all of the Carolina hemlock occur in areas that will not limit HWA development with temperature (Trotter et al. 2009).

Management

A number of organizations in the United States collaborate to improve HWA management. The Hemlock Woolly Adelgid Initiative started in 2003 and was coordinated by the United States Department of Agriculture and Forest Service (USDA FS) and the Animal and Plant Health Inspection Service (APHIS), the National Association of State Foresters, and the National Plant Board. The program goal was to explore potential management procedures for HWA and methods of application. The focus in the program shifted in 2007 and the 2008-2012 goal is to improving research on management techniques, technology, and methods of informing the public (Onken and Reardon 2011).

The management of HWA in urban and forest settings requires the combination of strategies. The process for management begins with monitoring for the pest. Models illustrating the potential occurrence of the adelgid may then be developed. In order to reduce the effects of HWA on hemlock chemical controls, biological controls, cultural management, hemlock resistance, and hemlock gene conservation can be implemented. All of the control strategies are influenced by funding, land ownership and management, technology availability, hemlock health, and level of HWA infestation (Vose et al. 2013).

Integrated Pest Management (IPM) is implemented in urban and forest settings to reduce the spread of HWA. Removal of infested material in March and June, fertilizer after recovery from the pest, reduced tree stress (watering), and chemical applications are utilized in urban areas. Pest management in a forest is different from management in an urban area (McClure 2007).

The chemical applications include foliar sprays of horticultural soaps and oils and chemical insecticides, or systemic insecticides, applied by stem injections, soil injections, soil drenches, and slow release tablets (McClure 1992; McClure 1987; Cowles et al. 2006). Imidacloprid is a synthetic, systemic chemical that has been implemented in urban and forest settings and is best suited for single trees or small groups of trees (Webb et al. 2003; Vose et al. 2013). It is utilized for valuable trees as a stem injection and may also be applied as a soil drench and soil injection. Imidacloprid insecticides include Merit 75 WP and Advanced Tree and Shrub (Bayer) (Webb et al. 2003; Cowles et al. 2006; Vose et al. 2013). Imidacloprid is residual (Vose et al. 2013) and effective for long term management of HWA as a soil treatment (Joseph et al. 2011).

Adsorption of imidacloprid potentially protects microarthropods in some areas such as the Southern Appalachian Mountains by restricting chemical movement in soil organic matter (Knoepp et al. 2012). Soil treatments do not negatively affect aquatic macroinvertebrates either under some conditions in the Southern Appalachian Mountains (Churchel et al. 2011).

Dinotefuran is another chemical that is often used for management. The systemic chemical is applied as a trunk spray (Vose et al. 2013). Insecticides include Safari 2 G used as a broadcast application on soil, and Safari 20 SG used as a soil drench or injection (Durkin 2009). Dinotefuran is fast acting but does not protect the tree as long as imidacloprid (Joseph et al. 2011). Dinotefuran is not residual. Use of both imidacloprid and dinotefuran are highly regulated near water (Vose et al. 2013).

Insecticidal soaps and horticultural oils may be used in place of imidacloprid and dinotefuran. The soap and oil applications are more environmentally safe but do not protect the tree as long as the systemic chemical applications (Vose et al. 2013). Frank and Lebude (2011) illustrate the effectiveness of systemic insecticides in a report analyzing multiple types of chemical applications in nursery systems. Both horticultural oil and systemic insecticides successfully reduced first generation HWA. The level of second generation HWA on trees treated with horticultural oils was not significantly different from control trees while systemic insecticides such as imidacloprid and dinotefuran significantly reduced second generation HWA populations (Frank and Lebude 2011).

The alternative chemical controls, in addition to the systemic chemicals, can have an impact on non-target organisms. The broad spectrum horticultural oils as well as imidacloprid have negative effects on some insects in the canopy of the tree. Of the 293 species of phytophagous and transient insects in the canopy, 33 species are influenced by these chemicals. Changes occur in species richness, abundance, and composition (Dilling et al. 2009).

Chemicals cannot easily be implemented in a forest. The treatment is expensive and time consuming. Research on management methods in the forest focus on biological control and resistance breeding. There are no native biological control organisms in eastern North America that significantly reduce HWA populations (Wallace and Hain 2000). Non-native biological control predators may have a greater effect on the population (McClure et al. 2001). Parasitoids and pathogens are not currently major components of biological control (Onken and Reardon 2011).

Additional circumstances must be considered for biological control management that are not necessary for chemical or mechanical management procedures. The predators are mass-reared and require artificial diets. Laboratory conditions should include favorable temperature, humidity, lighting, and many other environmental factors for the predator. Pathogens should also be controlled in the lab (Cohen and Cheah 2011). Artificial diets may be utilized but do not allow for completion of the life cycle without incorporating HWA (Onken and Reardon 2011). Biological control rearing on HWA that surpasses HWA aestivation would allow for continuous rearing conditions for HWA predator controls and speed up the rearing process (Salom et al. 2001). Recent literature also indicates that HWA contains the components anthraquinone, chrysophanol and the anthrone precursor chrysarobin. The components were collected from HWA extracts. They could deter predation on the adelgid and should be considered when choosing biological control agents (Jones et al. 2012).

Laricobius Rosenhauer biological control agents include *Laricobius nigrinus* Fender, *L. rubidus* LeConte, and *L. osakensis* Montgomery and Shiyak (Story et al. 2012). *L. nigrinus* is native to western North America (Kohler et al. 2008). In addition to HWA on western hemlock, the beetle is known to occur with and potentially feed on the ragged spruce gall adelgid (*Pineus similis* Gillette) on western white pine (*P. monticola* Douglas ex D. Don), the larch cone adelgid (*A. lariciatus* Patch) on western larch (*Larix occidentalis* Nutt.), the cooley spruce gall adelgid (*A. cooleyi* Gillette) on Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and Engelmann spruce (*Picea engelmannii* Parry ex Engelm.) (Mausel 2011). *L. rubidus* is native to eastern North America. *L. rubidus* occurs on white pine (*Pinus strobus* L.) and feeds on the pine bark adelgid (*Pineus strobi* Hartig) and HWA (Clark and Brown 1960). *L. osakensis* is native to Japan (Montgomery et al. 2011). The predator has been observed to utilize the balsam woolly adelgid, pine bark adelgid, eastern spruce gall adelgid (*Aldelges abietis* Linnaeus), woolly alder aphid (*Paraprociphilus tessellates* Fitch), elongate hemlock scale (*Fiorina externa* Ferris), and pine needle scale (*Chionaspis pinifoliae* Fitch) as host material but is more likely to complete the life cycle on HWA (Lamb et al. 2011).

The three species are able to survive if released in the same area and do not negatively affect each other with regard to prey consumption, oviposition, and predation on each other. Although the species do not negatively influence each other in the field, laboratory experiments have noted that *L. rubidus* adults produce fewer eggs and consume fewer HWA ovisacs than the other two species (Story et al. 2012). *L. nigrinus* and *L. rubidus* can potentially hybridize and have a bias introgression towards *L. nigrinus* (Havill et al. 2012). Both *L. osakensis* and *L. nigrinus* have a type II functional response. *L. osakensis* is likely more effective than *L. nigrinus*. *L. osakensis* has a greater feeding rate response to fluctuations in the HWA population. The predator also has a greater population size response to changes in HWA numbers (Vieira et al. 2012).

L. nigrinus has been reared by Clemson University, the New Jersey Department of Agriculture, University of Georgia, University of Tennessee, and Virginia Tech. Releases have occurred in Maine, New Hampshire, Vermont, Massachusetts, Connecticut, Rhode Island, New York, Pennsylvania, New Jersey, Maryland, Virginia, West Virginia, North Carolina, South Carolina, Tennessee, and Georgia. *L. osakensis* has been reared by the University of Tennessee and Virginia Tech (Jubb 2011).

The lady beetle *Sasajiscymnus* (=*Pseudoscymnus*) *tsugae* Sasaji is a predator native to Japan. The coccinellid only feeds on aphids, scales, mealybugs, and adelgids. The adults are long living and highly fecund. *S. tsugae* is potentially a promising predator because it feeds on all parts of the HWA life cycle, including the second generation of progrediens and first instar aestivating nymphs (Cheah and McClure 2000). Only a small percentage of the beetles have been recovered from release sites, indicating the biocontrol may require more time to reach detectable levels (Hakeem et al. 2010). The lady beetle has been observed to co-exist with both *L. nigrinus* and *L. rubidus* in natural settings in eastern North America (Hakeem 2011). A number of labs have reared the lady beetle including Clemson University, North Carolina Department of Agriculture and Consumer Services, Northern Georgia College and State University, University of Tennessee, and Young Harris College. The predator has been released in Maine, New Hampshire, Vermont, Massachusetts, Connecticut, Rhode Island, New York, Pennsylvania, New Jersey, Maryland, Virginia, West Virginia, North Carolina, South Carolina, Tennessee, and Georgia (Jubb 2011).

Flies in the Chamaemyiidae (Diptera) are another potential predator group that feeds on all stages of the HWA life cycle (Ross et al. 2011). The *Leucopis* Meigen genus native to western North America has the potential to be a predator on HWA and has a wide range. The genus occurs on pine and spruce, and is known to survive on other insects including aphids and adelgids (McAlpine and Tanasijtshuk 1972). Chamaemyiids likely will co-exist well with *L. nigrinus* since they occur in the same native range (Kohler et al. 2008). An introduction with *Scymnus* species may be detrimental to *Leucopis* populations due to potential predation by the beetle. More research is required to determine if *Scymnus* is a threat to *Leucopis* populations (Mills 1990).

The lady beetle *Scymnus* (*Neopullus*) Sasaji from China is another biological control option. *S. sinuanodulus* Yu & Yao and *S. ningshanensis* Yu & Yao have been released in North America. The beetles have not been located at release sites since the year they were placed in the field, indicating that they may not have survived the introduction. The life cycle of these control agents may not be adequate for HWA management. The lady beetle adults are present for a few months before depositing eggs and during that time encounter predators and other environmental factors. *S. camptodromus* Yu & Liu is a lady beetle that may be better suited for HWA management because of true aestival diapause, ability to live in various habitats, presence during important life stages of HWA, and feeding throughout the year (Montgomery and Keena 2011). The beetle may also be more cold tolerant than the other species and therefore more useful in the northern range. The species is currently being evaluated for biological control release (Onken and Reardon 2011).

S. sinuanodulus has been reared by the University of Georgia and released in Vermont, New York, Pennsylvania, West Virginia, North Carolina, South Carolina, Tennessee, and Georgia. *S. camptodromus* has been reared by Virginia Tech and had not been released as of 2011 (Jubb 2011). The predator is expected to be released in 2013. *S. ningshanensis* has been released in North Carolina in 2010 but has not established in the area yet (Onken and Reardon 2011).

In addition to associated insect predators, fungi have the potential to influence HWA populations. Most entomopathogens can be mass produced, used in large quantities, and are effective soon after release. The fungi *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchnikoff) Sorokin utilized in the United States do not have an effect on HWA populations. *Lecanicillium* (Zimmerman) Viegas was a fungus that appeared to be very effective in laboratory test and did not hinder predator development. Research on the genus halted due to concerns for unknown environmental effects (Costa 2011).

Mycotol (*Lecanicillium muscarium* (Petch) Zare & W. Gams) has been used in other countries for insect control (white flies) and the environmental effects are well known, making it a good candidate for HWA application. The pathogen has the potential to influence populations of the sistens HWA generation. Further research is required to determine the influence of the fungus on HWA predators (Costa 2011). *L. muscarium* occurs in the eastern United States. The application of Mycotol in the United States has not yet been approved. Studies on the environmental application of the fungus have been observed in Tennessee (Onoken and Reardon 2011).

Most pathogens are applied in a spray to allow for adequate coverage of the plant and provide moisture needed for survival. Whey is incorporated in the spray formulation to provide nutrients for the fungus. The temperature in the HWA infested range may be a limiting factor for microbial development. Ambient temperatures in May and June (sistens generation) are better suited for fungal development while decreasing temperatures in late summer are not ideal (Costa 2011).

In some cases preemptive logging or salvaging may occur. Intensive logging of a site leads to quick removal of vegetation, as opposed to the gradual changes in vegetation due to HWA. In both cases black birch eventually becomes a dominant species. In some cases intensive logging could cause more changes to the environmental conditions than HWA. Logging leads to more shade intolerant species in the canopy. The procedure can also increase soil pH and nitrification, as well as decrease forest floor mass. The nitrogen lost from intense logging of healthy forests can potentially be greater than the loss from HWA infestation (Kizlinski et al. 2002)

When control procedures are not effective the manager can either decide to replace the hemlock with new hemlock or another species, utilize stand sanitation in which dead woody material is removed, or halt management. The management actions after the infestation has surpassed control efforts and has devastated an area are dependent on value of the timber, type of landscape, cost and funds available, and opportunity costs (example:

wildlife protection). Vose and others (2013) propose effective procedures for four different infestation situations (Vose et al. 2013).

Terpenoids

Previous research has examined the role of terpenoids in HWA host selection. Volatile terpenoids influence insect host selection. The volatiles can be deterrents and/or attractants. Volatiles that are important in plant-insect interactions are known as pheromones, kairomones, allomones, and synomones. In conifer species the terpenoid content is varied. Hemlock terpenoids are stored in the needles. Adelgids and aphids are responsive to a few monoterpenes. Lagalante and Montgomery (2003) initially hypothesized that α-pinene, β caryophyllene, and α-humulene are potential deterrents and isobornyl acetate is a potential attractant (Lagalante and Montgomery, 2003). Further study revealed that isobornyl acetate and α-humulene are likely involved in tree resistance to HWA (Montgomery and Legalante 2008).

The role of epicuticular lipids in host selection has not been analyzed as thoroughly as the volatile terpenoids with regard to HWA. More recent literature has explored the role of epicuticular lipids in short range host selection. Tolerant hemlock species have distinct epicuticular lipid chemical profiles that could influence tree defense against HWA at the feeding site. More research is needed to determine particular epicuticular lipids that alter HWA behavior (Oten 2011). Future research should be conducted on terpenoid content of needles as well as the leaf cushion (adelgid feeding site) at varying levels of adelgid population density (Montgomery and Legalante 2008).

Terpenoids have been examined in a number of hemlock species including eastern hemlock, Carolina hemlock, western hemlock (*T. heterophylla*), Asian species, and mountain hemlock (*T. mertensiana*). There are three groups of hemlock that have distinct terpenoid components: (1) eastern North American eastern and Carolina hemlock, (2) western hemlock and Asian species, and (3) mountain hemlock. The volatiles have been examined at low adelgid densities (Lagalante and Montgomery 2003).

It is hypothesized that eastern and Carolina hemlock may not have adequate defense against the HWA because of hemlock evolution in eastern North America. Eastern North

America was dominated by native leaf defoliators and did not have native adelgids and scale insects that fed on hemlock. Natural defense therefore evolved to prevent feeding by leaf defoliators. The ability to resist HWA infestations may no longer be present in the gene pool due to selective pressure or may be present at very low levels (Montgomery and Lagalante 2008). The search for resistant eastern and Carolina hemlock is difficult but necessary to improve HWA resistance research and conservation of eastern and Carolina hemlock.

There are a number of methods for extracting volatiles from needles. A method common throughout papers authored by Montgomery and Lagalante is Solid-Phase Microextraction (SPME). SPME does not incorporate solvents. Other forms of extraction include maceration, homogenization, steam distillation, Soxhlet extraction, and supercritical fluid extraction (Lagalante and Montgomery 2003).

Terpenoid content has been examined at multiple stages of tree development, including bud opening, shoot elongation, shoot maturation, and bud break. HWA avoids variable terpenoid levels by undergoing aestivation for part of the life cycle. A study conducted by Lagalante and others (2006) examined terpenoids in the needle and leaf cushion and compared terpenoids in new and old growth. New and old growth needles had similar chemistry. The leaf cushion new growth and old growth varied greatly and was unpredictable in the spring and summer. The leaf cushion terpenoids were more similar to the needles when mature. Germacrene D and isobornyl acetate varied in the new growth. Germacrene D was predicted to be a deterrent and isobornyl acetate is expected to be an attractant (Lagalante et al. 2006).

Volatiles have been compared between groups of eastern hemlock in various locations as well. Ingwell and others looked at terpenoids in potentially resistant trees, rooted propagules of the potentially resistant trees, and trees that are located outside of the HWA infestation. There was no significant difference in the terpenoid composition of these groups (Ingwell et al. 2009).

Genetic Conservation

Biological and chemical controls are used to manage HWA. These methods may not be successful in preventing hemlock decline. Conservation banks are an alternative to HWA management (Jetton et al. 2008). Genetic conservation is used for preservation as well as plant breeding purposes (Zobel and Talbert 2003). The conservation effort is an opportunity to preserve genetic variation in threatened species, including rare alleles. Population genetic variation is important for adapting to changing environmental conditions. Quick decline of a tree population can potentially remove the genetic material that would otherwise aid in resistance or tolerance to the negative environmental pressures (Schaberg et al. 2008). The genetic material may be threatened by a number of situations including the presence of insects and/or diseases, logging, clearing for agricultural or urban use, range limitations, and natural disasters. These situations are not dangerous unless regeneration is halted. In most circumstances the whole gene complex is considered for conservation. Many tree characteristics that allow adaption to pests and other environmental changes are controlled by multiple genes (Zobel and Talbert 2003).

Gene conservation can be *in situ* or *ex situ*. *In situ* conservation involves preserving a species in the native range. The tree species is conserved by allowing natural development of the area or by managing the stand. The natural development of the area is not preferred in some cases because natural succession can shift species composition of the stand and preferred species can be replaced. The quality of the land should be accounted for when selecting an area. The inexpensive, easily obtained locations are not always well suited for the species of concern. Neighboring plant composition should also be accounted for. In most cases it is preferred that nearby plants are not be able to breed with the tree (Zobel and Talbert 2003).

Ex situ conservation occurs outside the native range. The sites acceptable for *ex situ* conservation require similar habitat to the native range of the species. Temperature, rainfall, and soil conditions are a few important factors. In order to effectively establish a planting, participants should know how to store seeds, manage a nursery, and select appropriate sites for plantings (Jetton et al. 2008). Species reproduce though vegetative propagation, including grafting, rooting cuttings, and air layering. Applied improvement program clone banks will package, propagate, and outcross genes (Zobel and Talbert 2003).

Conservation may also take place through seed storage but the method is not suitable for all species. Pollen may be stored as well. Limitations include the potential for mutations in seed, and the presence of only half of the material in pollen. Tissue culture is a more modern method of *ex situ* conservation (Zobel and Talbert 2003).

The genetic material may then be used in the future to reintroduce tree species to the native range and/or develop resistant trees (Jetton et al. 2008). Overall, *ex situ* conservation is less difficult and less costly than *in situ* methods and will likely be utilized more often (Zobel and Talbert 2003). Conservation of hemlock may be *ex situ* or *in situ*. *Ex situ* conservation is preferred because HWA has infested and has the potential to infest the whole native range of eastern and Carolina hemlock (Jetton et al. 2008).

The process of establishing a site can be improved. Reproduction is manipulated to produce trees at a faster rate. Methods for quickening the cycle of coning and seed production include “top-working”, in which the part of a young tree is grafted to the top of an older tree, and plant-hormone (gibberellins) treatments (Jetton et al. 2008).

In most cases the morphology of a tree does not correlate with adaptability. Selection of genes should not depend solely on choosing the tallest, straightest, fast growing genotypes. Fifty tree samples are adequate for conservation. A sample number of 20 or less will likely restrict future breeding options (Zobel and Talbert 2003). The alleles of collected seed need to resemble the total population genetics so that genes will not be lost. For a tree with moderately diverse genetics (*T. caroliniana*) a sample size of 10-20 mother trees for 6-8 populations across the distribution is an adequate sample of alleles (Dvorak et al. 1999). If funds and resources allow, a collection of hundreds of samples from different geographic regions is the best option (Zobel and Talbert 2003).

Priority should be given to characteristics that are unique to each geographic region of the species. Species that have a wide range contain most of the adaptable genes for extreme conditions along the edge of the range. All of the genetic diversity cannot be collected. The most important alleles should be given priority in the collection process. Neutral alleles that do not currently appear to be important may be lost in the conservation effort due to genetic drift. Neutral alleles may have value in the future and should be considered (Zobel and Talbert 2003).

Genetic diversity occurs due to mutations, gene flow, natural selection, genetic drift, and human manipulation. The genetic diversity is influenced by three factors: the environment, the genetics of a species, and the interaction between the environment and genotypes. Genetic diversity (variability) is composed of additive (cumulative allele effects) and nonadditive (dominance and epistasis) factors. Traits controlled by additive factors such as wood specific gravity and bole straightness are more easily selected for in breeding programs than those controlled by nonadditive factors. Resistance to pests can be associated with additive and nonadditive factors. For some species, decent improvements to resistance are possible with the use of only additive components. Managing environmental conditions allows a breeder to explore more genetic diversity (Zobel and Talbert 2003).

Camcore (International Tree Conservation and Domestication) at North Carolina State University and the United States Department of Agriculture Forest Service (USDA FS) have combined efforts to conserve genetic material of eastern and Carolina hemlock. Since 2003 the organizations have been collecting seed from the geographic range of the species and placing the seed in conservation banks and cold storage (Jetton et al 2008; Jetton et al 2010). Fifty nine populations and four hundred and seven mother trees of eastern hemlock, as well as nineteen populations and one hundred thirty four families of Carolina hemlock, have been sampled (Dvorak et. al., 2012).Currently there is no management procedure that is effective at preventing decline of hemlock in forest stands due to HWA. The seed collected and planted by Camcore and the USDA FS will provide a source of genetic material from the native range of hemlock that may be used to conserve the species if the hemlock forests do not survive. It may also be used to repopulate hemlock forests once an effective control agent has been established (Tighe et al. 2005).

The Camcore and USDA FS *ex situ* conservation project has three sections. The first section took place in 2003. Carolina hemlock seed was gathered and placed in *ex situ* field sites (Jetton et al. 2010). The Carolina hemlock range contains a moderate amount of genetic diversity. Seedling banks are currently present in Chile, the Ozark Mountains of Arkansas, and Brazil. The locations have an environment that is most similar to the native range of Carolina hemlock and do not have HWA infestations. Samples have been collected from Hanging Rock, Caesar's Head, Cradle of Forestry, and Table Rock (Potter et al. 2010). A total of 134 mother trees and 19 populations have been sampled (Jetton et al. in press).

The second part of the Camcore and USDA FS *ex situ* conservation project began in 2005. Seed was gathered from the eastern hemlock native range and placed in *ex situ* field sites. The first two sections took place in the southern United States. For the third section of the project, eastern hemlock seed was collected from the northern and Midwest range (Jetton et al. 2008). Seed was first collected for the third section between 2005 and 2009 (Jetton et al. 2010).

Currently a greater number of collections have taken place in the southern United States than in the northern states. Most of Camcore's resources are in the south. It is more difficult to collect seed with fewer contacts in the north. Another challenge of locating healthy stands with a decent amount of healthy seed is the variation in HWA attack, seed production, and cone ripening (Jetton et al 2010).

As of 2008, most research on genetic diversity in eastern hemlock has been restricted to the northern populations. The trees do not vary greatly between populations with regard to genetics and moderate inbreeding occurs throughout the entire range. Northern and southern regions are not significantly different in the level of diversity (Potter et al. 2008). The lower genetic diversity in eastern hemlock may be due to a pest attack that had taken place 5,000 years ago. The trees in North America evolved along with chewing insects. Piercing insects (ex: HWA) were not common and defense against them is not a major component of the current hemlock genetics (Potter et al. 2008; Onken and Reardon 2011).

More research is needed for the southern range of eastern hemlock. The glacial refugia of eastern hemlock are in the south and expected to hold a large amount of genetic diversity. Eastern hemlock collections for *ex situ* conservation should focus on the southern Appalachians (ex: North Carolina, South Carolina, Tennessee) because they provide a great amount of genetic differentiation and a large amount of allelic richness. The Great Smoky Mountains should be explored as well (Potter et al. 2008, Lemieux et al. 2011). Sampling in New England, the Southern Appalachian Mountains, and disjunct populations should allow for the most genetic diversity to be captured (Potter et al. 2012).

Allozyme analysis has been used to test the glacial refugia theory and includes analysis of percent polymorphic loci, alleles per locus, and expected heterozygosity. The populations in the western part of the range exhibited little genetic diversity. Eastern populations had a greater genetic diversity. Location, heterozygosity, polymorphic loci, and alleles were statistically significantly related. Although sites in the eastern and western most part of the southern range contain less genetic diversity, samples should be collected there as well (Potter et al. 2008; Onken and Reardon 2011).

Camcore has established *in situ* conservation banks, including a site in Ashe County, North Carolina. The Carolina hemlock is chemically treated to prevent HWA infestation. The site serves as a learning tool for seed orchard establishment and breeding of hemlock. The conservation bank may be used to determine how seed from *ex situ* plantings will be reintroduced to the native range (Jetton et al. 2009).

***Tsuga* Resistance Screening**

Testing for tree resistance to insects involves introducing the pest organism to the host and observing physiological and chemical responses of the tree. Artificial infestation consists of placing the insect on the tree at a point during the insect and tree life cycle when the insect would naturally infest the host. An infestation technique that mimics natural dispersal of the pest is preferred. Artificial infestations have been used for insect pests and fungal diseases to test for resistance (Alfaro et al. 2008; Hodge and Dvorak 2007).

Infestation Techniques for Hemlock Woolly Adelgid

Montgomery and others (2009) have conducted research on the potential resistance in multiple *Tsuga* species to HWA. Seedlings were inoculated with HWA by placing infested branches on each seedling and restricting crawler movement by enclosing the seedling and infested branch with a mesh bag until the crawler’s settled (July). Each seedling was infested with the same number of adelgids. Branches were removed and sampled twice throughout the study. HWA density was determined by counting the number of live HWA on the previous year's growth. Seedling height, spread at widest diameter, and bud break were also reported (Montgomery et al 2009). A similar method of artificial infestation is incorporated in my analysis of infestation methods for hemlock woolly adelgid on hemlock.

High populations of HWA were found on eastern hemlock, Carolina hemlock, and Southern Japanese hemlock. The Southern Japanese hemlock was susceptible but tolerant of the insect. Chinese hemlock (*T. chinensis*) had low populations of adelgids and exhibited resistance. The hybrids of Chinese hemlock with Carolina hemlock and Southern Japanese hemlock had intermediate HWA infestations. The resistance was either antixenosis (non preference) or antibiosis (reduced adelgid survival). The hybrids could be useful in replacing decimated hemlock populations. The process would be efficient with the use of Chinese hemlock mother trees (Montgomery et al 2009).

Carolina hemlock is more closely related to the Asian hemlock species and is a better candidate for resistance breeding. In order to produce a cross that is similar to the Carolina hemlock, the Carolina and Chinese hemlock have been backcrossed. The goal is to backcross until the tree only contains resistance genes from the Chinese hemlock. The Chinese hemlock's reaction to abiotic and/or biotic stress can be expressed in more than 100 hemlock sequence contigs. The database of expressed sequenced tags (EST's) developed by Smith and others (2010) will aid in determining resistance/ susceptibility levels in hemlock. There is currently little documentation of gene sequencing in hemlock. Greater research in hemlock gene sequencing may aid in screening for resistance (Smith et al. 2010).

An infestation is successful when the number of settled adelgids is high enough to influence tree survival. Elizabeth Butin and others (2007) observed how infestation methods influence settlement rate of HWA on 16 mature eastern hemlock trees. Differences in infestation level were recorded for the location of infested branches, number of infested branches, method used to attach the infested branches to the tree, and the use of a mesh sleeve. Butin examined the influence of temperature as well (Butin et al. 2007).

The infestation techniques examined include 1) one infested branch attached to the top of an uninfested branch with loose gardening wire and covered with a mesh sleeve, 2) one infested branch attached underneath an uninfested branch with loose gardening wire and covered with a mesh sleeve, 3) three infested branches attached on top of an uninfested branch with loose gardening wire and covered with a mesh sleeve, 4) one infested branch attached tightly on the top of an uninfested branch with flagging tape and covered with a mesh sleeve, 5) one infested branch attached on the top of an uninfested branch with loose gardening wire and not covered with a mesh sleeve, and 6) one infested branch attached on the top of an uninfested branch with loose gardening wire covered with a mesh sleeve where the branches were gathered from one tree in Forest Park. The technique for temperature was examined similarly to number 6. For the temperature observations the foliage was collected on a different date and placed in a refrigerator during the winter months. All infested branches were hydrated with aquapics (Butin et al. 2007).

The best method of infesting mature trees was to use loose gardening wire to attach infested material to uninfested material and cover the branches with a mesh sleeve. The recommended time for infestation was during the sistens generation in spring. The crawlers collected earlier in the year infested the material at higher densities than crawlers later in the year (Butin et al. 2007).

The rate of crawler settlement did not differ for infested branches attached to the tree on the top and underside of the uninfested branches. There was no difference in HWA density at 1 and 3 infested branches. Adelgids on the 3 branch infestations may have settled on the infested material instead of the uninfested material. The crawler settlement was 56% less for infested branches without mesh bags and for tight attachment with the flagging tape. The mesh bag may have been beneficial to the infestation process because of a unique microclimate which aided HWA infestations, restricted dispersal of crawlers, and provided protection from natural predators. The flagging tape could have caused mortality of HWA during attachment (Butin et al. 2007).

The technique of directly attaching an infested branch to a tree or seedling has been utilized in the past to successfully infest hemlock seedlings and is incorporated in my analysis of HWA infestation techniques. Recently there has been some research on a suspended branch technique, an infestation process similar to the natural dispersal of HWA. Jetton et al. (in press) conducted a preliminary study to determine if a suspended branch technique could be implemented in resistance testing of large numbers of seedlings. The number of settled crawlers, density, and distribution of crawlers emerging from the suspended branches were observed. The materials and methods utilized in the experiment are similar to the materials and methods of this thesis project (Jetton et al. in press).

A high infestation technique with 48 infested branches (approximately 513,000 crawlers in March and 33,000 crawlers in May) suspended over eastern and Carolina hemlock seedlings was compared to a low infestation technique where 24 infested branches (approximately 289,000 crawlers in March and 17,000 crawlers in May) were suspended over eastern and Carolina seedlings. The March high infestation had a significantly greater number of adelgids dispersing from the branches than the low inoculation. The high inoculation did not significantly differ in the number of adelgids that settled on Carolina and eastern hemlock seedlings in the low infestation. The May high infestation had a significantly greater number of crawlers dispersing from the branches than the low inoculation. The number of settled crawlers in the high and low May infestations was much lower than number settled in the March infestations. The suspended branch was more effective in March and should be applied for resistance testing programs in March. The reduced number of emerging and settled crawlers in May could be due to greater "competition" for food due to decline in the tree from which the infested branch collected (Jetton et al. in press).

The suspended branch technique at the high and low infestations met and exceeded damage threshold densities. The distribution of emerging crawlers was observed to have "hot spots" where adelgids were clustered. Fewer crawlers were present near the edge of the collection sheet. Uniform distribution is important for resistance testing. A method with a more uniform distribution would be an acceptable method for mass testing (Jetton et al. in press).

Infestation Techniques for Balsam Woolly Adelgid

The balsam woolly adelgid (BWA, *Adelges piceae* Ratz.) is a pest in North America. Leslie Newton and others (2011) conducted research on screening methods for BWA resistance in Fraser fir (*Abies fraseri* (Pursh) Poir.). Genetic differences in host resistance were observed while noting the level of effectiveness of each technique and how affordable the materials were. Newton compared a technique where cut infested bark was placed next to the uninfested seedling, a technique in which the cut infested bark was attached to the uninfested seedling, and suspended bolt technique where 1.2 m long infested bolts were hung above the seedlings (Newton et al. 2011). The suspended bolt technique is similar to the HWA suspended branch technique.

The cut bark technique is time consuming but has been successfully used in the past to infest seedlings. BWA infests the bole of the tree, unlike HWA which settles at the base of needles. Newton et al. (2011) utilized bark and tree bole infested material. The use of infested bolts is potentially as effective and may be easier to apply. Newton et al. measured density and distribution of crawler emergence from the bolt to ensure that the infestation was widely dispersed. Paper grids were placed below 1.2 m and 1.5 m infested bolts, sprayed the grids with Tanglefoot ®, and analyzed the BWA that fell on the grids (Newton et al. 2011).

The suspended bolt and cut bark attached to the seedling produced higher infestations than the cut bark placed next to the seedling. For all three treatments there was a significant interaction by age of the tree (2 and 7 years) and season (summer and fall). The cut bark and suspended bolt infestations were both effective (Newton et al. 2011).

The paper grids had a concentration of crawlers directly below the bolt. Crawlers were also dispersed below on either side of the bolt. Newton concluded that a highly infested, 10cm diameter bolt, is able to successfully inoculate material within a 30cm diameter. The bolt produced “hot spots” where the adelgid distribution was concentrated. Resistance testing requires even dispersal of crawlers on the seedlings below so that each seedling is equally infested. The “hot spots” could be more evenly distributed by altering the bolt placement. The suspended bolt is most suitable for seedling inoculations. The technique requires less effort and is more time efficient than the cut bark technique. The time required for the infestation process was reduced by a few hours. The suspended bolt also provided constant supply of BWA and a greater number of BWA for the infestation than did the bark pieces (Newton et al. 2011).

REFERENCES

Alfaro, R I, King J N, Brown R G, Buddingh S M. 2008. Screening of Sitka spruce genotypes for resistance to the white pine weevil using artificial infestations. *Forest Ecology and Management* 255: 1749-1758.

Annand, P N. 1924. A New Species of Adelges (Hemiptera, Phylloxeridae). *The Pan-Pacific Entomologist* 1.2: 79-82.

Butin, E., Preisser E, and Elkinton J. 2007. Factors Affecting Settlement Rate of the Hemlock Woolly Adelgid, Adelges Tsugae, On Eastern Hemlock, Tsuga Canadensis. *Agricultural and Forest Entomology* 9.3: 215-219.

Churchel, M A, Hanula J L, Berisford C W' Vose J M, Dalusky M J. 2011. Impact of Imidacloprid for Control of Hemlock Woolly Adelgid on Nearby Aquatic Macroinvertebrate Assemblages. *Southern Journal of Applied Forestry* 35.1: 26-32.

Cheah, C A S J and McClure M S. 2000. Seasonal synchrony of life cycles between the exotic predator, Pseudoscymnus tsugae (Coleoptera: Coccinellidae) and its prey, the hemlock woolly adelgid Adelges tsugae (Homoptera: Adelgidae).*Agricultural and Forest Entomology* 2.4: 241-251.

Clark, R.C. and Brown N R. 1960. Studies of predators of the balsam woolly aphid, Adelges picae (Ratzburg) (Homoptera: Adelgidae), Laricobius rubidus LeConte (Coleoptera: Derodontidae), a predator of Pineus strobi (Hartig.) (Homoptera: Adelgidae).*The Canadian Entomologist* 92.3: 237-240.

Cohen, A C and Cheah C. 2011.Chapter 14: Development of Artificial Diets for Predators of Hemlock Woolly Adelgids. In: B. Onken and Richard Reardon (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid*. USDA Forest Service FHTET-2011-04. 148-157.

Costa, Scott D. 2011. Chapter 9: Insect-Killing Fungi for HWA Management: Current Status. In: B. Onken and Richard Reardon (Editors). *Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service,* FHTET-2011-04. 107-115.

Cowles, R S, Montgomery M E, and Cheah C A S J. 2006. Activity and residues of imidacloprid applied to soil and tree trunks to control hemlock woolly adelgid (Hemiptera: Adelgidae) in forests. *Journal of Economic Entomology* 99.4: 1258-1267.

Dilling, C, Lambdin P, Grant J, and Rhea R. 2009. Community Response of Insects Associated with Eastern Hemlock to Imidacloprid and Horticultural Oil Treatments. *Environmental Entomology* 38.1: 53-66.

Durkin, P R. 2009. Dinotefuran Human Health and Ecological Risk Assessment Final Report. *USDA Forest Service*. Forest Service, <http://www.fs.fed.us/foresthealth/pesticide/pdfs/0521803b_Dinotefuran.pdf >.

Dvorak, W S, Hodge G R, López J L, Espinoza J A, Woodbridge W C, Jump R M, Jetton R M, Whittier W A, McGee R, Gutiérrez E A. 2012. “2012 Camcore Annual Report International Tree Breeding and Conservation”. Rep. Camcore, North Carolina State University. 33. <http://www.camcore.org/publications/documents/AnnReport%202012%20Online.pdf>.

Dvorak, W S, Hamrick, J, Hodge, G R.1999. Assessing the sampling efficiency of ex situ gene conservation in natural pine populations in Central America. *Forest Genetics* 6.1: 21-28.

Evans, R A, Johnson E, Shreiner J, Ambler A, Battles J, Cleavitt N, Fahey T, Sciascia J, and Pehek E. 1996. Potential Impacts of Hemlock Woolly Adelgid (Adelges Tsugae) on

Eastern Hemlock (Tsuga Canadensis) Ecosystems. *Proceedings of the first hemlock woolly adelgid review. Charlottesville, Virginia, USA.12 October 1995:* 42-57.

Farjon, A. 1990. *Pinaceae. Drawings and Descriptions of the Genera Abies, Cedrus, Pseudolarix, Keteleeria, Nothotsuga, Tsuga, Cathaya, Pseudotsuga, Larix and Picea*.Königstein: Koeltz Scientific Books.

Farjon, A. 2010. *A Handbook of the World's Conifers Vol. 2*. Leiden; Boston: Brill. 1008-1009, 1015-017.

Finzi, A C, van Breemen N, Canham C D. 1998. Canopy Tree-Soil Interactions Within Temperate Forests: Species Effects On Soil Carbon And Nitrogen. *Ecological Applications* 8.2: 440-446.

Ford, C R,   Elliott K J, Clinton B D, Kloeppel B D, and Vose J M. 2012. Forest dynamics following eastern hemlock mortality in the southern Appalachians. *Oikos* 121.4: 523-536.

Frank, S D and Lebude A. 2011. Season-Long Insecticide Efficacy for Hemlock Woolly Adelgid, Adelges tsugae (Hemiptera: Adelgidae), Management in Nurseries."*Florida Entomologist* 94.2: 290-295.

Gouger, R J. 1971. Control of *Adelges tsugae* on hemlock in Pennsylvania. Sci. Tree Topics 3: 1-9.

Hakeem, A, Grant J F, Lambdin P L, Buckley D, Hale F A, Rhea J R, Wiggins G J, Taylor G. 2010. Recovery of Sasajiscymnus tsugae, released against hemlock woolly adelgid, Adelges tsugae, in the southern Appalachians. *Biocontrol Science and Technology* 20.10: 1069-1074.

Hakeem, A, Grant J F, Wiggins G J, Lambdin P L, Rhea J R. 2011.Establishment and coexistence of two predators, Laricobius nigrinus and Sasajiscymnus tsugae, introduced against hemlock woolly adelgid on eastern hemlock. *Biocontrol Science and Technology* 21. 6: 687-691.

Havill, NP and Foottit R G. 2007. Biology and Evolution of Adelgidae. *Annual Review of Entomology* 52: 325-349.

Havill, NP, Campbell C S, Vining T F, LePage B, Bayer R J, Donoghue M J. 2008. Phylogeny and Biogeography of *Tsuga* (Pinaceae) Inferred from Nuclear Ribosomal Its and Chloroplast DNA Sequence Data. *Systemic Botany* 33.3: 478-489.

Havill, NP, Montgomery M E, Guoyue Y; Shiyake S, Caccone A. 2006. Mitochondrial DNA from Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Suggests Cryptic Speciation and Pinpoints the Source of the Introduction to Eastern North America. *Annals of the Entomological Society of America* 99.2: 195-203.

Ingwell, L, Brady J, Fitzpatrick M, Maynard B, Casagrande R, Preisser E. 2009. IntraspecificVariation in Tsuga Canadensis Foliar Chemistry. *Northeastern Naturalist* 16.4: 585-594.

Ingwell, L, Miller-Pierce M, Trotter R T III, and Preisser E L. 2012. Vegetation and Invertebrate Community Response to Eastern Hemlock Decline in Southern New England. *Northeastern Naturalist* 19.4: 541-58.

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

Jetton, R M, Dvorak W S, Whittier W A. 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. *Forest Ecology and Management* 255: 3212-3221.

Jetton, R M, Whittier A W, Dvorak W S, Rhea J R. 2010.Status of Gene Conservation for Eastern and Carolina Hemlock in the Eastern United States. *Fifth Symposium on Hemlock Woolly Adelgid in the Eastern United States.* USDA Forest Service.

Jetton, RM, Whittier A W, Dvorak W S, and Potter K M. 2008. Status of *Ex Situ* Conservation Efforts for Carolina and Eastern hemlock in the Southeastern United States. *Fourth Symposium on Hemlock Woolly Adelgid in the Eastern United States*. USDA Forest Service.

Jetton, R M., Mayfield, A E; Powers, Z L. Development of a Rain Down Technique to Artificially Infest Hemlocks with the Hemlock Woolly Adelgid (Hemiptera: Adelgidae). In press.

Jetton, R M, Dvorak W S, Whittier W A. 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. *Forest Ecology and Management* 255: 3212-3221.

Jetton, R, Dvorak W, Whittier A, Potter K, Rhea R. 2009. Genetics and Conservation of Hemlock Species Threatened by the Hemlock Woolly Adelgid. *USDA Proceedings 20th U.S. Department of Agriculture Interagency Research Forum on Invasive Species 2009*. USDA Forest Service, 2009.

Jones, A C, Mullins D E, Jones T H, and Salom S M. 2012 Potential Feeding Deterrents Found in Hemlock Woolly Adelgid, Adelges Tsugae. *Naturwissenschaften* 99.7: 583-86.

Joseph, SV, Braman K S, Quick J, Hanula J L. 2011. The Range and Response of Neonicotinoids on Hemlock Woolly Adelgid, Adelges tsugae (Hemiptera: Adelgidae). *Journal of Environmental Horticulture* 29.4:197–204.

Jubb, C S. 2011. Chapter 11: Rearing Labs and Distribution of Predators for Release. In: Onken and Richard Reardon (Editors).*Implementation and Status of Biological Control of Hemlock Woolly Adelgid.* USDA Forest Service. FHTET-2011-04. 125-132.

Kizlinski, ML, Orwig D A, Cobb R C, Foster D R. 2002. Direct and indirect ecosystem consequences of an invasive pest on forests dominated by eastern hemlock. *Journal of Biogeography* 29.10-11: 1489-1503.

Knoepp, J D, Vose J M, Michael J L, Reynolds B C. 2012.Imidacloprid Movement in Soils and Impacts on Soil Microarthropods in Southern Appalachian Eastern Hemlock Stands. *Journal of Environmental Quality* 41: 469–478.

Knoepp, J D, Vose J M, Clinton B D, Hunter M D. 2011. Hemlock Infestation and Mortality: Impacts on Nutrient Pools and Cycling in Appalachian Forests. *Soil Science Society of America Journal* 75.5: 1935-1945.

Koch, F. H., Cheshire H M, Devine H A. 2006. Landscape-Scale Prediction of Hemlock Woolly Adelgid, Adelges tsugae (Homoptera: Adelgidae), Infestation in the Southern Appalachian Mountains. *Environmental Entomology* 35.5: 1313-1323.

Kohler, G R, Stiefel V L, Wallin K F, Ross D W. 2008. Predators associated with the hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific Northwest. *Environmental Entomology* 37.2: 494-504.

Krüssmann, G, Warda H D, Daniels G S. 1985. *Manual of Cultivated Conifers 2nd edition.* Gerd Krüssmann ; Editor, Hans-Dieter Warda ; Translator, Michael E. Epp ; Technical Editor, Gilbert S. Daniels.. Portland, Or. : Timber Press.

Lagalante, A F, and Montgomery M E. 2003. Analysis of Terpenoids from Hemlock (Tsuga) Species by Solid-Phase Microextraction/ Gas Chromatography/ Ion-Trap Spectrometry. *Journal of Agriculture and Food Chemistry* 51.8: 2115-2120.

Lagalante, AF, Lewis N, Montgomery M E, Shields K S. 2006. Temporal and Spatial Variation of Terpenoids in Eastern Hemlock (Tsuga Canadensis) in Relation to Feeding by Adelges Tsugae. *Journal of Chemical Ecology* 32: 2389-2403.

Lamb, A, Montgomery M E, Viera L C, Shiyake S, Salom S. 2011. Chapter 7: Laricobius osakensis, a Hemlock Woolly Adelgid Predator from Japan. In: Onken B and Reardon R (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid*. USDA Forest Service. FHTET-2011-04. 90-96.

Lemieux, MJ, Beaulieu J, and Bousque J. 2011. Chloroplast DNA polymorphisms in eastern hemlock: range-wide genogeographic analyses and implications for gene conservation. *Canadian Journal of Forest Research* 41.5: 1047-1059.

Lepage, B A. 2003. A New Species of Tsuga (Pinaceae) from the Middle Eocene of Axel Heiberg Island, Canada, and an Assessment of the Evolution and Biogeographical History of the Genus. *Botanical Journal of the Linnean Society* 141.3: 257-96.

Lewis J D, Licitra J, Tuininga A R, Sirulnik A, Turner G D, Johnson J. 2008.Oak seedling growth and ectomycorrhizal colonization are less in eastern hemlock stands infested with hemlock woolly adelgid than in adjacent oak stands. *Tree Physiology* 28: 629–636.

Mausel, D L, Davis G A, Lamb A S, Zilahi-Balogh G M G, Kok L T, Salom S M. 2011. Chapter 6: Laricobius nigrinus Fender (Coleoptera: Derodontidae). In: Onken B Reardon R (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid*. USDA Forest Service. FHTET-2011-04. 77-89.

McAlpine, J F and Tanasijtshuk V N. 1972. Identity of *Leucopis Agenticollis* and Description of a New Species (Diptera: chamaemyiidae). *The Canadian Entomologist* 104:12 : 1865-1875.

McClure, M S. 1987. Biology and Control of Hemlock Woolly Adelgid. *Bulletin of the Connecticut Agricultural Station 851*: 1-9. Web.

McClure, M S. 1991. Density-Dependent Feedback and Population Cycles in Adelges Tsugae (Homoptera: Adelgidae) on Tsuga Canadensis. *Environmental Entomology* 20.1: 258-264.

McClure, M S. 1992. Effects of Implanted and Injected Pesticides and Fertilizers on the Survival of *Adelges tsugae* (Homoptera: Adelgidae) and on the Growth of *Tsuga Canadensis*. *Journal of Economic Entomology* 85.2: 468-472.

McClure, M S. 1989. Evidence of a Polymorphic Life Cycle in the Hemlock Woolly Adelgid, Adelges Tsugae (Homoptera: Adelgidae). *Annals of the Entomological Society of America* 82.1: 50-54.

McClure, M S. 2007. Hemlock woolly adelgid, Adelges tsugae (Annand). *The Connecticut Agricultural Experiment Station*

McClure, M S. 1990. Role of Wind, Birds, Deer, and Humans in the Dispersal of Hemlock Woolly Adelgid (Homoptera: Adelgidae). *Environmental Entomology* 19.1: 36-43.

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

Mills, N J. 1990. Biological control of forest pests in Africa. *Bulletin of Entomological Research* 80.1: 31-36.

Montgomery, M E, Shiyake S, Havill N P, Leschen R A B. 2011. A New Species of Laricobius (Coleoptera: Derodontidae) From Japan With Phylogeny and a Key for Native and Introduced Congeners in North America. *Annals of the Entomological Society of America* 104.3: 389-401.

Montgomery, M E and Lagalante A F. 2008. The Role of Volatile Terpenoids in the Relationship of the Hemlock Woolly Adelgid and its Host Plants." *Fourth Symposium on Hemlock Woolly Adelgid in the Eastern United States*: 118-123.

Montgomery, M E and Keena M A. 2011. Chapter 5: Scymnus (Neopullus) Lady Beetles from China. In: Onken B and Reardon R (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid*. USDA Forest Service. FHTET-2011-04. 53-76.

Montgomery, M E, Bentz S E, and Olsen R T. 2009. Evaluation of Hemlock (Tsuga) Species and Hybrids for Resistance to Adelges Tsugae (Hemiptera: Adelgidae) Using Artificial Infestation. *Journal of Economic Entomology* 102.3: 1247-1254.

Moore, L M. 2002. Western Hemlock Tsuga Heterophylla (Raf.) Sarg. *Plant Guide*. USDA Natural Resources Conservation Service.

Newton L, Frampton J, Monahan J, Goldfarb B, Hain, F. 2011. Two novel techniques to screen Abies seedlings for resistance to the balsam woolly adelgid, Adelges piceae. *Journal of Insect Science* 11:158: 1-16.

Northington, R M, Webster J R, Benfield E F, Cheever B M, Niederlehner B R. 2013. Ecosystem Function in Appalachian Headwater Streams during an Active Invasion by the Hemlock Woolly Adelgid. *PLoS ONE* 8.4: 1-8.

Onken B and Reardon R. 2011. “Preface”. In: Onken B and Reardon.R (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid.* USDA Forest Service. FHTET-2011-04.

Onken B. and Reardon R. 2011. Chapter 22: An Overview and Outlook for Biological Control of Hemlock Woolly Adelgid. In: Onken B and Reardon R (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid*. USDA Forest Service, FHTET-2011-04. 222-228.

Orwig, D A and David R. 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. Foster. *Journal of the Torrey Botanical Society* 125.1 : 60-73.

Oten K. 2011. *Host-plant selection by the hemlock woolly adelgid, Adelges tsugae Annand: sensory systems and feeding behavior in relation to physical and chemical host-plant characteristics*. Ph.D. Thesis, Department of Entomology, North Carolina State University, Raleigh, NC, USA.

Paradis A, Elkinton J, Hayhoe K, Buonaccorsi J. 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. *Mitigation and Adaptation Strategies for Global Change* 13.5-6: 541-54.

Parker B L, Skinner M, Gouli S, Ashikaga T, and Teillon H B. 1999. Low Lethal Temperature for Hemlock Woolly Adelgid (Homoptera : Adelgidae). *Environmental Entomology* 28.6:1085-1091.

Potter, K M, Dvorak W S, Crane B S, Hipkins V D, Jetton R M, Whittier W A, Rea R. 2008. Allozyme variation and recent evolutionary history of eastern hemlock (*Tsuga canadensis*) in the southeastern United States. *New Forests* 35.2: 131-145.

Potter, KM, Jetton R M, Dvorak W S, Frampton J, and Rhea R. 2010. *Ex situ* Seed Collection Represents Genetic Variation Present in Natural Stands of Carolina Hemlock. *Fifth Symposium on Hemlock Woolly Adelgid in the Eastern United States*: 181-189.

Potter, K M, Jetton R M, Dvorak W S, Hipkins V D, Rhea R, Whittier W A. 2012. Widespread inbreeding and unexpected geographic patterns of genetic variation in eastern hemlock (Tsuga canadensis), an imperiled North American conifer. *Conservation Genetics* 13: 475–498.

Quimby, J W. 1996. Value and Importance of Hemlock Ecosystems in the Eastern United States. *Proceedings of the first hemlock woolly adelgid review. Charlottesville, Virginia, USA.12 October 1995.* 1-8.

Radville, L, Chaves A, Preisser E L. 2011. Variation in Plant Defense against Invasive Herbivores: Evidence for a Hypersensitive Response in Eastern Hemlocks (*Tsuga canadensis*). *Journal of Chemical Ecology* 37.6: 592-597.

Ross, D W, Gaimari, S D, Kohler C R, Wallin K F, Grubin, S M. 2011. Chapter 8: Chamaemyiid predators of the hemlock woolly adelgid from the Pacific Northwest. In: Onken B and Reardon R (Editors).*Implementation and Status of Biological Control of Hemlock Woolly Adelgid*. USDA Forest Service, FHTET-2011-04. 97-106.

Ross, R M, Bennett R M, Snyder C D, Young J A, Smith D R, Lemarie D P. 2003. Influence of eastern hemlock (Tsuga canadensis L.) on fish community structure and function in headwater streams of the Delaware River basin. *Ecology of Freshwater Fish* 12.1: 60-65.

Rowell, T J and Sobczak W V. 2008. Will Stream Periphyton Respond to Increases in Light Following Forecasted Regional Hemlock Mortality? *Journal of Freshwater Ecology* 23.1: 33-40.

Salom, S M, Sharov A A, Mays W T, and Neal J W Jr.. 2001. Evaluation of Aestival Diapause in Hemlock Woolly Adelgid (Homoptera: Adelgidae). 30.5: 887-882.

Schaberga, P G, DeHayesb D H, Hawleyc G J, Nijensohn S E. 2008.Anthropogenic alterations of genetic diversity within tree populations: Implications for forest ecosystem resilience. *Forest Ecology and Management* 256.5: 855-862.

Schwartzberg, L, and Montgomery M. 2010. Relationships of Nonstructural Carbohydrates and the Hemlock Woolly Adelgid. *Fifth Symposium on Hemlock Woolly Adelgid in the Eastern United States*: 198-202.

Skinner, M, Parker B L, Gouli S, Ashikaga T. 2003. Regional Responses of Hemlock Woolly Adelgid (Homoptera: Adelgidae) to Low Temperatures. *Environmental Entomology* 32.3: 523-28.

Smith, K, Mwaniki A, Morse A M, Davis J M, Peter G F, Nelson C D. 2010. Gene Discovery in Carolina Hemlock (*Tsuga caroliniana)* Targeted for Response to Hemlock Woolly Adelgid. *Fifth Symposium on Hemlock Woolly Adelgid in the Eastern United States:* 100-103.

Story, H M, Vieira L C, Salom S M, Kok L T. 2012. Assessing Performance and Competition Among Three *Laricobius* (Coleoptera: Derodontidae) Species, Predators of Hemlock Woolly Adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). *Environmental Entomology* 41.4: 896-904.

Tighe, M E, Dvorak W S, Whittier W A, Romero J L, Rhea J R. 2005. The ex situ conservation of Carolina hemlock." *Third Symposium on Hemlock Woolly Adelgid in the Eastern United States*: 180-190.

Tingley, MW, Orwig D A, Field R, Motzkin G. 2002. Avian response to removal of a forest dominant: consequences of hemlock woolly adelgid infestations. *Journal of Biogeography* 29.10-11: 1505-1516.

Trotter, R T, and Shields K S. 2009. Variation in Winter Survival of the Invasive Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Across the Eastern United States. *Environmental Entomology* 38.3: 577-87.

USDA Forest Service 2011. Counties with established *A. tsugae* populations 2012.

Distribution map available online at http://na.fs.fed.us/fhp/hwa/maps/2012.pdf.

van Breemen, N, Finizi A C, Canham C D. 1997. Canopy tree-soil interactions within temperate forests: effect of soil elemental composition and texture on species distributions. *Canadian Journal of Forest Research* 27: 1110-1116.

Vieira, L C, Salom S M, Kok L T. 2012. Functional and numerical response of Laricobius spp. predators (Coleoptera: Derodontidae) on hemlock woolly adelgid, Adelges tsugae (Hemiptera: Adelgidae). *Biological Control* 61.1: 47-54.

Vose J M, Wear D N, Mayfield A E III. 2013. Hemlock woolly adelgid in the southern Appalachians: Control strategies, ecological impacts, and potential management responses. *Forest Ecology and Management* 291: 209-219.

Webb R E, Frank J R, Raupp M J. 2003. Eastern Hemlock Recovery from Hemlock Woolly Adelgid Damage following Imidacloprid Therapy. *Journal of Arboriculture* 29.5: 298-302.

Young R F, Shields K S, Berlyn G P. 1995. Hemlock woolly adelgid (Homoptera: Adelgidae): stylet bundle insertion and feeding sites. *Annals of the Entomological Society of America* 88: 827-835.

Zobel B and Talbert J. 2003. *Applied Forest Tree Improvement*. New Jersey: The Blackburn Press, 50-52, 67, 70, 459-478.

CHAPTER II
Evaluation of Infestation Techniques for Host Resistance Screening to the Hemlock Woolly Adelgid

ABSTRACT

The hemlock woolly adelgid (HWA, *Adelges tsugae* Annand) is an invasive pest from Japan that causes significant decline in eastern (*Tsuga. canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock in eastern North America. The purpose of this experiment was to compare the effectiveness of two artificial methods for infesting hemlock seedlings with the adelgid for resistance screening. Adelgid density and population longevity were observed for two direct infestation treatments and one suspended branch infestation treatment on eastern, Carolina, and western (*T. heterophylla* (Raf.) Sarg.) hemlock. Infestations took place over two years (2012 and 2013) using the progrediens (March) and sistens (May) generations. The March 2012 high direct infestations resulted in significantly greater crawler densities than low direct and suspended branch infestations for all three host species, averaging 1.89 ± 0.23, 1.08 ± 0.18, and 0.57 ± 0.13 crawlers per 10 cm branch, respectively. The May 2012 high direct, low direct, and suspended branch infestations were less than March infestations and were not significantly different from each other with average values of 0.25 ±0.08, 0.17 ± 0.07, and 0.37 ± 0.10 crawlers per 10 cm branch, respectively. The progrediens infestations in 2013 on eastern and Carolina hemlock took place in more favorable environmental conditions for the adelgid with the result that the suspended branch infestation on eastern hemlock produced significantly greater infestations than the direct infestations on eastern hemlock, with an average of 17.72 ± 1.02 crawlers per 10 cm branch.

The high direct and suspended branch infestations produced greater crawler densities on Carolina hemlock with an average of 8.64 ± 0.73 and 8.31 ± 0.74 crawlers per 10 cm branch, respectively. The suspended branch infestation with the progrediens generation is recommended for resistance testing. In addition to adelgid settlement, population longevity under more favorable conditions and tree health should be incorporated in the analysis for determining effectiveness of infestation techniques. Tree health factors may include height, stem caliper at soil surface, and percent new growth.

INTRODUCTION

The hemlock woolly adelgid (HWA, *Adelges tsugae* Annand) is an introduced pest from Japan transported to eastern North America on southern Japanese hemlock (*Tsuga sieboldii* Carr.) nursery stock (Havill et al. 2006). HWA infestations were first observed in Virginia in the 1950s (McClure 1987) and as of 2013 have spread to 19 states on the east coast (USDA Forest Service 2012).

HWA has caused significant decline in eastern (*T. canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock populations along the east coast (Havill et al. 2006; Havill et al. 2007). The populations in eastern North America have two generations per year, progrediens generation (April-June) and sistens generation (June/July-March) (McClure 1987; McClure 1989). The adelgid feeds in the xylem ray parenchyma cells at the base of needles which depletes nutrients from storage cells and causes needles to grey and fall from the tree, bud mortality, reduced new growth, limb dieback, and ultimately whole tree mortality in as few as four years (Young et al. 1995; McClure et al. 2001).

Both eastern and Carolina hemlock are ecologically important tree species. The hemlocks regulate ecosystem conditions and provide food and shelter for a number of animals. Neither species is a significant source of timber but both have been used by humans for ornamental purposes (Evans et al. 1996; Krüssmann 1985; Quimby 1996; Tingley et al. 2002; Ross et al. 2003; Ingwell et al. 2012). The loss of hemlock from forests is expected to lead to significant changes in ecosystems including changes in understory and dominant plant species, nutrient cycles, and animal inhabitants (Ford et al. 2012). Hemlock ecosystems will likely be replaced by hardwood species in the north and south or with rhododendron in the south (Jenkins et al. 1999; Ford et al. 2012; Knoepp et al. 2011; Northington et al. 2013; Ingwell et al. 2012; Finzi et al. 1998; van Breemen et al. 1997).

Control mechanisms for the pest include chemical controls, biological controls, mechanical controls, using resistant planting stock, and a combination of these factors. Chemical applications are ideal for urban environments and include horticultural soaps and oils, and systemic insecticides (imidacloprid, dinotefuran) in the form of single tree injections, drenches, or foliar sprays (McClure 1987; Webb et al. 2003; Vose et al. 2013). Chemical controls are not effective at a landscape level due to time and money constraints (McClure 2007). Biological controls have the potential to be more effective in the forest setting and currently include releases of HWA predators (*Laricobius nigrinus* Fender, *Sasajiscymnus tsugae* (Sasaji and McClure), and *Scymnus* (Kugelann) species) that can reduce the HWA populations (Story et al. 2012; Kohler et al. 2008; Clark and Brown 1960; Montgomery et al. 2011; Cheah and McClure 2000; Montgomery and Keena 2011;

McAlpine and Tanasijtshuk 1972; Jubb 2011). Preemptive logging is a management method that does not require the introduction of chemicals but can lead to abrupt microenvironmental changes, decrease shade cover, and alter tree species composition (Kizlinski et al. 2002).

The use of trees selected or bred for tolerance or resistance is another method that could be applied at the forest setting. Some hemlock species exhibit tolerance or resistance to HWA feeding, including Southern Japanese hemlock, Chinese hemlock (*T. chinensis* (Franch.) E. Pritz.), hybrids of Chinese and Carolina hemlock, western hemlock (*T. heterophylla* (Raf.) Sarg.), and potentially Carolina hemlock (Montgomery et al. 2009; Havill et al. 2006; Oten 2011). There may be genes for tolerance to HWA in eastern and Carolina hemlock whose effects are not readily observed in the forest due to low populations of tolerant trees. Significant stand decline may not be prevented when very few tolerant trees are present. Rare genetic material can limit the adaptability of the forest to insect attack if the gene frequency has not been altered through natural conditions or silvicultural actions. (Schaberg et al. 2008). Conservation banks are utilized to store genetic material and breed plants (Zobel and Talbert 2003). Present conservation of eastern and Carolina hemlock takes place at *in situ* and *ex situ* conservation banks. The stored genetic material can be reintroduced to the native range and/or used to develop tolerant/resistant trees (Jetton et al. 2008).

Plants are tested for resistance by introducing the pest to the host during the lifecycle when it naturally infests the host and noting physiological and chemical responses of the plant. The infestation should mimic natural dispersal of the pest and is successful when host plant survival is hindered (Alfaro et al. 2008; Hodge and Dvorak 2007; Butin et al. 2007). Hemlock trees have been tested for susceptibility to the adelgid with a few different techniques. Direct infestation involves placing a branch infested with HWA next to a non-infested seedling and enclosing the branch with a mesh cloth or similar material. Adelgid crawlers move from the branch onto the seedling (Montgomery et al. 2009). A good method for direct infestation is to loosely attach infested branches to the seedling. The mesh sleeve may not be necessary for successful infestations (Butin et al. 2007).

Suspended branch infestation is a method in which branches are placed above seedlings and crawlers fall off onto the seedlings below. This method simulates the natural distribution of adelgids onto seedlings and is potentially less labor intensive than direct infestation. Suspending branches above multiple seedlings likely requires less effort than infesting each tree individually with infested branches. Suspended branch and direct infestations have been compared for balsam woolly adelgid (BWA, *Adelges piceae* Ratz.), an adelgid pest in North America that settles on the bark of fir (*Abies* Mill.) (Balch 1952). Newton et al. (2011) examined direct infestation where cut pieces of bark were placed on or near seedlings and suspended log infestation where an infested bolt was placed above seedlings. The suspended bolt was recommended for resistance testing because the method produced larger infestations, was less time consuming, and less labor intense than the direct infestations (Newton et al. 2011). Suspended branch infestation has been used to produce infestations above the damage threshold for HWA, was most effective following March infestations, and is a good candidate for resistance testing (Jetton et al. 2013).

Direct and suspended branch infestation techniques have not been directly compared for HWA. In order to identify hemlock woolly adelgid resistance in eastern and Carolina hemlock, mass numbers of seedlings need to be tested. This will require artificial infestation techniques that are both efficient in terms of time and cost and result in reliable seedling infestations. The objective of this project is to determine the best technique for infesting hemlock seedlings with HWA to test for resistance. The infestation techniques examined are direct infestation and suspended branch infestation.

MATERIALS AND METHODS

2012 Experiment

Location and Experimental Design

Source Material-Infested Branches

Suspended branch and direct infestations were conducted for two experiments in 2012 to compare the techniques. The March experiment used eggs of the progrediens generation and the May experiment used eggs of the sistens generation. Infested branches were gathered with pole pruners from infested eastern hemlocks in McDowell County near Marion, North Carolina (35.606825,-82.100354) and near Petros, Tennessee (36.09395, -84.409075) in March, and from Petros, Tennessee only in May. Branches 40 and 20 cm long were cut for suspended branch treatments and direct infestation treatments, respectively. The average number of ovisacs per branch and eggs per ovisac were estimated from 15 branch samples collected from the progrediens and sistens infested material. The 40 and 20 cm cut branches were stored in 11L buckets with water or Falcon 25 mL centrifuge tubes with water, respectively.

Source Material-Seedlings

The experiments were conducted on eastern, Carolina, and western hemlock seedlings. Eastern hemlock from Pikes Peak Nursery in Pennsylvania, western hemlock from Weyerhaeuser Company in Oregon, and Carolina hemlock from Foggy Mountain Nursery in North Carolina were potted in 5.7 L pots with Fafard 3B mix medium with Multicoat 15-16-17 fertilizer incorporated in January and stored in a greenhouse with a maximum temperature of 21°C until March. In March the seedlings for the progrediens infestation were used for the experiment and the seedlings for the sistens infestation were moved outdoors near the greenhouse. The 2-4 year-old eastern, Carolina, and western hemlock seedlings had an average caliper at soil surface and average height of 9.85 ± 0.33 cm and 48.60 ± 1.10 cm, 9.89 ± 0.33 cm and 74.06 ± 1.96, and 7.95 ± 0.16 cm and 46.30 ± 1.15 cm, respectively.

Progrediens Infestation (March 2012)

Research was conducted at the North Carolina Department of Agriculture & Consumer Services Mountain Research Station in Waynesville, NC (35.487319°N, -82.966883°W). Seedlings were placed in a 1 m^3 cube with a frame of 2 cm diameter schedule 40 PVC pipe. The frame was wrapped around four sides with clear 6 mil plastic sheeting to deter HWA movement among plots. The sheeting was secured to the frame with paper binder clips. The bottom of the cube was not covered. Cubes were placed in a building that was not climate controlled but provided shelter from rain and direct sunlight.

Each cube was one research plot. The plots were organized in a randomized block design with four blocks and four research plots per block. Each plot had three eastern hemlock, three Carolina hemlock, and three western hemlock seedlings. The seedlings were arranged in a Latin square design and were placed in a three by three pattern in which the vertical and horizontal rows had one eastern, one western, and one Carolina hemlock.

HWA infested branches were introduced March 7, 2012. The two infestation techniques compared in the study were suspended branch and direct infestation. Suspended branch infestations were introduced to one plot in each block. A section of approximately 1 m^2 poultry wire was placed over the top of the cube and secured with plastic cable ties. Forty infested branches 40 cm in length, including the main branch and side branches, were then set on top of the chicken wire in four rows of ten branches with the bottom side of the branches facing down. There were approximately 19 woolly masses per cm and an average of 40.50 ± 1.64 eggs per ovisac. Thus approximately 1,231,200 progrediens eggs per 1 m^2 area were placed above nine seedlings. The branches were covered with a second sheet of 1 m^2 poultry wire that was secured with plastic cable ties. Progrediens crawlers fell off the branches onto the seedlings below.

Direct infestations were introduced to two plots in each block. The infested branches 20 cm in length were placed next to seedlings either in the soil or attached to the branches with a plastic cable tie. Approximately 15 cm of branch, including the main branch and side branches, was exposed to the seedlings. The infested branches were attached to the seedling if the seedling branches were too high to provide sufficient contact with the infested branch in the soil (greater than about 15 cm). Three infested branches were used for high direct infestations and one infested branch was used for low direct infestations. There were approximately 19 woolly masses per cm of infested branch and an average of 40.50 ± 1.64 progrediens eggs per ovisac. The high direct infestations provided approximately 34,628 progrediens eggs. The low direct infestations provided approximately 11,543 progrediens eggs. Within each block, one plot received high direct infestations and one plot received low direct infestations.

Direct infestation branches were hydrated with a method similar to the water tube method used by Butin and others (2007). Infested branches were placed in Falcon 25 mL centrifuge tubes filled with water. The openings were covered with Parafilm® to prevent water evaporation and the stems of the branches were pushed through the Parafilm®. About 2- 5 cm of branch was inserted into the water and 15 cm of branch was exposed to the seedlings. In addition to the three infested plots, one plot in each block was a non-infested control used to monitor the movement of HWA among plots. Within each block, plots were randomly assigned to the treatments.

Infested branches were removed from plots after 9 days on March 16, 2012. Seedlings were then moved outside and placed in direct sunlight on top of a black ground cover weed barrier. For each outdoor plot, four T posts were placed in the corners of a 1 m^2 square. Landscape cloth was wrapped twice around the sides of the posts. The cloth was used to deter movement of HWA among plots. The seedlings were arranged again in a Latin square design for each plot.

Sistens Infestation (May 2012)

The sistens infestation was conducted at the same location as the progrediens infestation. The seedling arrangements, experimental design, cubes, and infestation methods were the same as the progrediens infestation. The control plot for the March infestation was reused as the control plot for May. Infested branches were introduced May 11, 2012 and removed after 14 days on May 25, 2012. The branches had an average of approximately 14 woolly masses per cm branch and an average of 6.57 ± 0.49 sistens eggs per ovisac. Approximately 147,168 sistens eggs per 1 m^2 were suspended above seedlings. High direct infestation seedlings were infested with approximately 4,139 sistens eggs each. Low direct infestation seedlings were infested with approximately 1,380 sistens eggs per seedling. Seedlings were moved outdoors to a different set of plots with the same design as the March 2012 infested plots for a total of seven research plots per block for the study.

Data Collection

Branch samples were collected from infested seedlings on November 2, 2012. For each seedling, four side branches and one terminal branch were removed. The 10 cm samples were stored in a walk-in cooler at the North Carolina State University Camcore lab at 6°C until they were processed. The number of HWA on the branches and the life stage were recorded while observing through a dissecting microscope. The number of live sistens nymphs was analyzed. Sistens nymphs that were alive when the branches were cut had wool.

Seedlings were evaluated for woolly masses on March 7, 2013 to determine the progress of the infestation. Visual counts of full seedlings indicated that no new woolly masses were present. The sistens population had not survived. A few old woolly masses were present and may have developed before death of the adelgid population on the seedlings. Wool was considered healthy if it was not compressed.

Statistical Analysis

For each seedling, an average density of settled live nymphs was calculated based on counts from 5 branches. These data were analyzed using a generalized linear fixed-effect model. The null hypothesis that block, month, hemlock species and/or treatment did not have a significant influence on the average nymph density was tested. The month by treatment interaction was significant and the results were sliced by month. Assuming a Poisson distribution and employing a log link function, data were analyzed using the GLMMIX procedure of SAS Enterprise Guide 5.1 (SAS EG) with the following model:

$\log(Y_{hijkl}) = \mu + \alpha_h + \beta_i + \upsilon_j + \tau_k + (\beta\upsilon)_{ij} + (\beta\tau)_{ik} + (\upsilon\tau)_{jk} + \varepsilon_{hijkl}$

where,

Y_{hijkl} = hemlock woolly adelgid average live nymph count per 10cm branch;

μ = base level;

α_h = *h*th block effect (h=1 to 4);

β_i = *i*th month effect (i=1 to 2);

υ_j = *j*th hemlock species effect (j=1 to 3);

τ_k = *k*th treatment effect (k=1 to 3);

$(\beta\upsilon)_{ij}$ = interaction of the *i*th month and the *j*th hemlock species;

$(\beta\tau)_{ik}$ = interaction of the *i*th month and the *k*th treatment;

$(\upsilon\tau)_{jk}$ = interaction of the *j*th hemlock species and the *k*th treatment;

ε_{hijkl} = unaccounted for variation.

The Tukey-Kramer HSD method was employed to test if differences among pairwise least squares means for month, hemlock species, and treatments were significant at α=0.05. All reported means and standard errors had been transformed back to the original scale by applying the inverse link function.

The control treatment was used to determine if there was movement of HWA among plots. The same set of control trees were placed with infested seedings for the March and the May infestations but were not included in the above analysis. The control had an average of 0.094 ± 0.22 sistens crawlers per 10 cm branch.

2013 Experiment

Location and Experimental Design

Source Material-Infested Branches

Suspended branch infestations and direct infestations were conducted for one experiment in 2013 to compare the infestation techniques. The March experiment used eggs from the progrediens generation. The collection and processing of infested branches was the same as the 2012 experiment. Infested branches were collected from Asheville, NC at the Forest Service Southern Research Station (35.611966,-82.562181) in March.

Source Material-Seedlings

The experiment was conducted on eastern and Carolina hemlock. The seedling sources and potting procedure were the same as the 2012 experiment. The trees were stored on an outdoor porch at the Bent Creek Experimental Forest Southern Research Station (35.612441,-82.563268) until the infestations in March.

Progrediens Infestation (March 2013)

Infestations were conducted at the Forest Service Bent Creek Experimental Forest (35.612529,-82.563332). Seedlings were placed in the cubes utilized for the progrediens and sistens 2012 infestations. The trees were stored inside a climate controlled building at room temperature.

Each cube was one research plot. The plots were placed in a randomized block design with four blocks and four research plots per block. The plots contained four eastern hemlock and four Carolina hemlock. The seedlings were randomly arranged within each plot.

Infested branches were introduced March 26, 2013. Branches used to infest seedlings had an initial infestation with approximately 9 ovisacs/cm branch and an average of 77.69 ± 4.48 progrediens eggs per ovisac. Suspended branch infestations and direct infestations were compared in the study. Suspended branch infestations were introduced to one plot per block. The 1 m^2 area was infested with approximately 1,118,736progrediens eggs. Direct infestations were introduced to two plots per block. One plot received high direct infestations and one plot received low direct infestations. Each seedling in the high direct treatments was infested with approximately 31,464 progrediens eggs. Each seedling in the low direct treatment was infested with approximately 10,488 progrediens eggs. The methods for suspended branch infestation, high direct infestation, and low direct infestation were the same as the methods used for both progrediens and sistens 2012 infestations. In addition to the three infested plots, one plot in each block was a control. The plot was used to monitor the movement of HWA between plots and was not purposefully infested with HWA. Plots were randomly assigned to treatments.

Infested branches were removed from plots after 10 days on April 5, 2012. Seedlings were then moved to a new outdoor location. The outdoor plot design was the same as the outdoor plots for the 2012 experiment. Seedlings were placed under a deciduous tree canopy instead of direct sunlight. The actual seedling location in each plot was not consistent with the location in the building.

Data Collection

The initial level of infestation on the branches was documented using the same method as the 2012 experiment. The seedling infestation level for the sistens generation was recorded from branch samples collected June 10, 2013. The 10 cm branches were not removed from the trees. Visual counts of the ovisacs were taken for four lateral branches and one terminal branch.

Statistical Analysis

All statistical methods were similar to those described for the 2012 data except the following model was employed: $\log(Y_{hijk}) = \mu + \alpha_h + \beta_i + \upsilon_j + (\beta\upsilon)_{ij} + \varepsilon_{hijk}$

where,

Y_{hijk} =	hemlock woolly adelgid average live nymph count per 10cm branch;
μ =	base level;
α_h =	*h*th block effect (h=1 to 4);
β_i =	*i*th hemlock species effect (i=1 to 2);
υ_j =	*j*th treatment effect (i=1 to 3)
$(\beta\upsilon)_{ij}$ =	interaction of the *i*th hemlock species and the *j*th treatment;
ε_{hik} =	unaccounted for variation;

The Tukey-Kramer HSD method was employed to test if differences among pairwise least squares means for hemlock species and treatments were significant at α=0.05. Significant interactions were sliced for further analysis. All reported means and standard errors have been transformed back to the original scale by applying the inverse link function.

The control treatment was used to determine if there was movement of HWA among plots. The control trees were not included in the above analysis. The control seedlings had an average of 0.01± 0.05 crawlers per 10 cm branch.

RESULTS

2012 Experiment

The density of nymphs that broke aestivation by November on the hemlock seedlings differed significantly with block, month, and the treatment by month interaction at $\alpha = 0.05$ (Table 1). Treatment, species, and the interactions of treatment by species and month by species were not significant. Although not significant, adelgid density was highest for high direct infestation followed by low direct and suspended branch infestation (Figure 1), and was four times higher for March compared to May infestation (Figure 2). Although not significant at $\alpha = 0.05$, adelgid density was highest for Carolina hemlock, followed by eastern and western hemlock (Figure 3). Adelgid density was greater for all three species with progrediens high direct and low direct infestations, followed by the progrediens suspended branch infestations and all three treatments for sistens infestations (Figure 4).

March high direct infestations resulted in significantly greater adelgid densities than March low direct and suspended branch infestations (Figure 5). All treatments in May produced lower adelgid densities than in March and were not significantly different from each other (Figure 5). The average number of sistens nymphs was 1.89 ± 0.23, 1.08 ± 0.18, and 0.57 ± 0.13 per 10 cm following March high direct , low direct, and suspended branch infestations, respectively. May treatments had an average of 0.25 ±0.08, 0.17 ± 0.07, and 0.37 ± 0.10 adelgids per 10 cm for high direct, low direct, and suspended branch infestations, respectively.

2013 Experiment

The density of progrediens nymphs/adults settled on hemlock seedlings was significantly different for block, treatment, species, and the treatment by species interaction (Table2). Adelgid density was highest for suspended branch infestations followed by high direct and low direct infestations (Figure 6), and was greater on eastern hemlock than Carolina hemlock (Figure 7). On eastern hemlock, adelgid density was highest for suspended branch infestation on eastern hemlock seedlings with a mean of 17.72 ± 1.02 adelgids per 10 cm, differing significantly from infestation levels on seedlings treated with high direct (12.12 ± 0.87 adelgids per 10 cm) and low direct (9.57 ± 0.80 adelgids per 10 cm) infestations (Figure 8). On Carolina hemlock, the infestation density of progrediens nymphs/adults on seedlings in the high direct infestation treatment was not significantly different from the suspended branch treatment with an average density of 8.31 ± 0.74 adelgids per 10 cm (Figure 9). The low direct infestation was not significantly different from the suspended branch infestations and lower than the high direct infestations with a density of 6.31 ± 0.61 adelgids per 10 cm (Figure 9).

DISCUSSION

The 2012 results indicated that all three seedling species had the greatest adelgid density after infestation with the progrediens high direct technique. After the 2013 infestations, the suspended branch eastern hemlock had a greater adelgid density than direct infestations (high and low). High direct and suspended branch infestations on Carolina hemlock were most successful in producing infestations. The infestation techniques in 2012 were examined under less ideal environmental conditions than infestation techniques in 2013. The influence of environment on adelgid health was apparent in the lower adelgid densities collected in 2012 (average of 0 to 8.6 adelgids per 10cm branch) than 2013 (average of 0 to 34.4 adelgids per 10 cm branch). The 2012 seedlings were located on black ground cloth in direct sunlight during the summer. The 2013 seedlings were placed under a deciduous tree canopy in the spring and received more shade than the 2012 seedlings. The adelgid aestivates during the summer to avoid extreme conditions but cannot move to more favorable habitat if direct sunlight and/or temperature are too harsh (McClure 1987; McClure 1989; Tauber 1986). Research conducted by Mayfield and Jetton (2012) suggests adelgid densities on hemlock seedlings are positively associated with increasing levels of shade. In their study, the sistens and progrediens generations on hemlock seedlings had significantly fewer adelgids in direct sunlight than in shaded plots and populations exposed to direct sunlight were nearly zero. The Mayfield and Jetton study took place near the 2012 experiment with the same black ground cloth and potted seedling source and age (Mayfield and Jetton 2012). The infestations were conducted at the same time as the 2012 experiment infestations and the shade study results directly relate to the influence of direct sunlight in the 2012 experiment. The Mayfield and Jetton (2012) study and this study indicate that future infestations should not take place in direct sunlight if large adelgid populations are desired. Additionally, researchers searching for natural tolerant/resistant genotypes should be aware that low populations of adelgid on forest hemlock trees in direct sunlight may be due to environmental conditions rather than tolerance/resistance of the tree alone.

The settled adelgids from the 2012 experiment did not survive the winter, indicating a low fitness level. Some live nymphs were present at the end of the summer and were collected in November. In addition to extreme summer conditions, winter temperatures are considered to have detrimental effects on sistens survival in healthy populations. The winter of 2012-2013 did not have the extreme temperatures required to damage hemolymph and cause mortality in healthy populations (Paradis et al. 2008; Gouli et al. 2000; Parker et al. 1999). Although adelgid winter mortality is not a major threat to populations in the southern portion of its range (estimated 0.8-0.9 survival rate) (Trotter and Shields 2009), the unhealthy population in the 2012 experiment may have experienced mortality from less extreme winter temperatures (minimum temperature of -2.6°C) (State Climate Office North Carolina, 2013).

The removal of sample branches in November may have hindered adelgid winter survival as well. The subtraction of live sistens nymphs on seedlings further decreased the population on the tree. The removal of seedling foliage and new growth from the young seedlings could have reduced adelgid fecundity and survival (McClure 1991). There may have been greater population survival if branches remained on the tree. Further research should consider the influence of data collection methods on adelgid survival.

Adequate infestations have been produced with the direct and suspended branch techniques in previous studies. A similar study by Jetton et al. (in press) utilized 24 and 48 branches to infest seedlings with the suspended branch technique. The lowest infestation on Carolina hemlock in the Jetton study was 46.8 crawlers per 10 cm. Montgomery et al. (2009) utilized a slightly different direct infestation technique to produce infestations exceeding 4 adelgids per 1 cm branch on susceptible and tolerant hemlock species. The average number of settled nymphs from high and low direct infestations utilized in the study by Butin et al. (2007) was approximately a maximum of 0.82 and 1.2 per cm branch, respectively, and was more similar to the high and low direct infestations in the 2012 study.

The adelgid density for experiments in 2012 and 2013 were not significantly different for some high and low direct infestations. May 2012 high and low direct infestations were statistically similar for all species (Figure 5) and March 2013 eastern hemlock high and low direct infestations were statistically similar (Figure 8). Similar studies utilizing high and low direct infestations to infest mature trees and seedlings concluded that the low direct infestation is as effective as the high direct infestation (Butin et al 2007; Jetton et al. 2008). The Butin et al. (2007) study took place in late April on mature eastern hemlock and noted that the similar levels of infestation on the tree may be due to the production of more HWA than the tree could support from the high infestations, and/or the crawlers on the high direct infestation settled on the branches rather than the tree. The results from 2012 may be influenced by environmental conditions.

The infestations in 2012 and 2013 produced the highest HWA seedling infestations in March. March 2012 infested material had an average of 40.50 ±1.64 eggs per ovisac and March 2013 had an average of 77.69 ± 4.48 eggs per ovisac. May 2012 had an average of 6.57 ± 0.49 eggs per ovisac. March 2012 infestations had approximately 6 times the number of eggs per ovisac than May 2012. The result is supported by previous literature. The month of March is better suited for infestations due to the initial density of the progrediens population on infested trees. March material is known to have more eggs per ovisac than the May. Northern populations have been observed with 48.6 ± 5.8 eggs per ovisac in March and 21.7 ± 3.6 eggs per ovisac in May (McClure 1989). The total number of eggs in a given length of branch has also been observed to be greater in March than May. Southern populations have an average of 216.6 ± 93.3 and 647.5 ± 109.6 ovisacs per 40 cm branch and an average of 45.7 ± 5.8 and 2.8 ± 0.6 eggs per ovisac for March and May, respectively (Jetton et al. in press). The difference between eggs per ovisac values in March and May 2012 was not as great as the 16 fold difference in the experiment by Jetton et al. (in press). The results support the use of infestations in March for large seedling infestations.

The 2013 infestation level on eastern hemlock was significantly greater than the infestation level on Carolina hemlock. Research conducted by Jetton et al. (2008) produced similar results with seedlings of different ages. Research by Oten (2011) also indicated that eastern hemlock is potentially more susceptible to HWA infestations than Carolina hemlock. Carolina hemlock is more closely related to resistant Asian hemlock (Havill et al. 2008) and it is reasonable, phylogenetically, to argue that the hemlock has potentially greater tolerance to HWA. Future studies should consider breeding a more tolerant Carolina hemlock along with hybridizing Chinese and Carolina hemlock.

Fecundity for populations in the 2012 experiment was not obtained due to the decline in adelgid populations throughout the winter. Jetton et al. (2008) noted that Carolina hemlock had much greater HWA fecundity than eastern and western hemlock. Although there is evidence of some tolerance in the Carolina hemlock, the species is a susceptible host for HWA populations and does experience decline and mortality due to infestations.

CONCLUSION

The comparison of suspended branch and direct infestations suggest that March suspended branch infestations are most appropriate for infesting mass numbers of seedlings to test for tolerance and/or resistance to HWA. The 2012 experiment indicated that March suspended branch infestations were not the best option for infestations under unfavorable conditions. The environmental conditions that negatively affected the densities in 2012 likely happened after the treatments were applied, and likely affected all treatments equally. The 2013 experiment resulted in significantly greater crawler densities for March suspended branch infestations on eastern hemlock than direct infestations (high and low), and March high direct and suspended branch infestations on Carolina hemlock than low direct infestations, under favorable environmental conditions. Experimental conditions including light intensity, temperature, adelgid distribution, data collection method, and stage in the adelgid life cycle should be considered in order to produce healthy adelgid populations.

A number of organizations in the United States work to improve HWA management on the east coast, including the United States Department of Agriculture Forest Service (USDA FS), the Animal and Plant Health Inspection Service (APHIS), the National Association of State Foresters, and the National Plant Board. The organizations have collaborated to explore potential management procedures and methods of application, and improve research on management techniques, technology, and methods of informing the public (Onken and Reardon, 2011). Results from this experiment expand current knowledge on HWA infestation technology by determining an efficient and reliable method to test seedlings for tolerance and/or resistance to HWA. Eastern and Carolina hemlock are ecologically important tree species that may require replacement in the future. Mass screening of hemlock species will be important for the advancement of tolerance/ resistance selection and breeding. Mass breeding for resistant genomes, deployment, and restoration by organizations such as Camcore (International Tree Conservation and Domestication, North Carolina State University) will benefit from efficient and effective adelgid infestation testing procedures.

ACKNOWLEDGMENTS

We thank Kaleb Rathbone and the NCDA&CS/NCSU Mountain Research Station in Waynesville, NC for providing space, equipment, and assistance; Ben Smith (NCSU-ASTF) and Andy Whittier (NCSU-Camcore) for technical assistance; the Coal Creek Company for site access and source of infested branches; and Weyerhaeuser Company for the donation of Western hemlock seedlings. This work is funded by USDA Forest Service Southern Research Station Cooperative Agreement No. 11-CA-11330129-064.

REFERENCES

Alfaro R I, King J N, Brown R G, Buddingh S M. 2008. Screening of Sitka spruce genotypes for resistance to the white pine weevil using artificial infestations. *Forest Ecology and Management* 255: 1749-1758.

Balch R E. 1952. Studies of the balsam woolly aphid, *Adelges piceae* (Ratz.) and its effect on balsam fir, *Abies balsamea* (L.) Mill. *Canadian Department of Agriculture Publication* 867.

Butin E, Preisser E, Elkinton J. 2007. Factors affecting settlement rate of the hemlock woolly adelgid, *Adelges tsugae*, on eastern hemlock, *Tsuga canadensis*. *Agricultural and Forest Entomology* 9: 215-219.

Cheah C A S J and McClure M S. 2000. Seasonal synchrony of life cycles between the exotic predator, *Pseudoscymnus tsugae* (Coleoptera: Coccinellidae) and its prey, the hemlock woolly adelgid *Adelges tsugae* (Homoptera: Adelgidae). *Agricultural and Forest Entomology* 2.4: 241-251.

Clark R C and Brown N R. 1960. Studies of predators of the balsam woolly aphid, *Adelges picae* (Ratzburg) (Homoptera: Adelgidae), *Laricobius rubidus* LeConte (Coleoptera: Derodontidae), a predator of *Pineus strobi* (Hartig.) (Homoptera: Adelgidae). *The Canadian Entomologist* 92.3: 237-240.

Evans R A, Johnson E, Shreiner J, Ambler A, Battles J, Cleavitt N, Fahey T, Sciascia J, Pehek E. 1996. Potential Impacts of Hemlock Woolly Adelgid (Adelges Tsugae) on Eastern Hemlock (Tsuga Canadensis) Ecosystems. *Proceedings of the First Hemlock Woolly Adelgid Review*. *Charlottesville, Virginia, USA.12 October 1995*. (pp 42-57). USDA Forest Service. FHTET.

Finzi, A C, van Breemen N, Canham C D. 1998. Canopy Tree-Soil Interactions Within Temperate Forests: Species Effects On Soil Carbon And Nitrogen. *Ecological Applications* 8.2: 440-446.

Ford C R, Elliott K J, Clinton B D, Kloeppel B D, Vose J M. 2012. Forest dynamics following eastern hemlock mortality in the southern Appalachians. *Oikos* 121.4: 523-536.

Gouli V, Parker B L, Skinner M. 2000. Haemocytes of the hemlock woolly adelgid *Adelges tsugae* Annand (Hom., Adelgidae) and changes after exposure to low temperatures. *Journal of Applied Entomology* 124: 201–206.

Havill N P and Foottit R G. 2007. Biology and Evolution of Adelgidae. *Annual Review of Entomology* 52: 325-349.

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

Hodge G R, Dvorak W S. 2007. Variation in pitch canker resistance among provenances of *Pinus patula* and *Pinus tecunumanii* from Mexico and Central America. *New Forests* 33: 193-206.

Ingwell L L, Miller-Pierce M, Trotter R T III, Preisser E L. 2012. Vegetation and Invertebrate Community Response to Eastern Hemlock Decline in Southern New England. *Northeastern Naturalist* 19.4: 541-58.

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

Jetton, R M, Hain F P, Dvorak W S, Frampton J. 2008. Infestation Rate of Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Among Three North American Hemlock (*Tsuga*) Species Following Artificial Inoculation. *Journal of Entomological Science* 43.4: 438-442.

Jetton R M, Whittier A W, Dvorak W S, Potter K M. 2008. Status of Ex Situ Conservation Efforts for Carolina and Eastern hemlock in the Southeastern United States. *Fourth Symposium on Hemlock Woolly Adelgid in the Eastern United States.* USDA Forest Service.

Jetton R M, Mayfield A E, and Powers Z L. Development of a Rain Down Technique to Artificially Infest Hemlocks with the Hemlock Woolly Adelgid (Hemiptera: Adelgidae). In press.

Jubb C S. 2011. Chapter 11: Rearing Labs and Distribution of Predators for Release. In: Onken B and Reardon R (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid.* USDA Forest Service. FHTET-2011-04.

Kizlinski M L, Orwig D A, Cobb R C, Foster D R. 2002. Direct and indirect ecosystem consequences of an invasive pest on forests dominated by eastern hemlock. *Journal of Biogeography* 29.10-11: 1489-1503.

Knoepp J D, Vose J M, Clinton B D, Hunter M D. 2011. Hemlock Infestation and Mortality: Impacts on Nutrient Pools and Cycling in Appalachian Forests. *Soil Science Society of America Journal* 75.5: 1935-1945.

Kohler GR, Stiefel VL, Wallin KF, Ross DW. 2008. Predators associated with the hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific Northwest. *Environmental Entomology* 37: 494-504

Krüssmann G, Warda H D, Daniels G S. 1985. *Manual of Cultivated Conifers.* Warda H D (Editor), Translator, Michael E (Translator), Technical Editor, Daniels G S (Technical Editor). 2nd ed. Portland, Or. : Timber Press. Print.

Mayfield A E III and Jetton R M. 2013. A shady situation: Evaluating the effect of shade on hemlock woolly adelgid densities on potted hemlock seedlings. Poster presented at the 55th Southern Forest Insect Work Conference, 23-26 July 2013, New Orleans, LA.

McAlpine J F and Tanasijtshuk V N. 1972. Identity of Leucopis Agenticollis and Description of a New Species (Diptera: chamaemyiidae). *The Canadian Entomologist* 104:12: 1865-1875.

McClure M S. 1987. Biology and Control of Hemlock Woolly Adelgid. *Bulletin of the Connecticut Agricultural Station* 851: 1-9.

McClure M S. 1989. Evidence of a polymorphic life cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). *Annals of the Entomological Society of America* 82: 50-54.

McClure M S. Hemlock woolly adelgid, Adelges tsugae (Annand). 2007. *The Connecticut Agricultural Experiment Station.*

McClure M S, Salom M S, Shields K S. 2001. *Hemlock Woolly Adelgid*. USDA Forest Service. 414 FHTET-2001-03.

Montgomery M E and Keena M A. 2011. Chapter 5: Scymnus (Neopullus) Lady Beetles from China. In: Onken B, Reardon R (Editors). *Implementation and Status of Biological Control of Hemlock Woolly Adelgid*. USDA Forest Service. FHTET-2011-04.

Montgomery M E, Bentz S E, Olsen R T. 2009. Evaluation of hemlock (*Tsuga*) species and hybrids for resistance to *Adelges tsugae* (Hemiptera: Adelgidae) using artificial infestation. *Journal of Economic Entomology* 102: 1247-1254.

Montgomery M E, Shiyake S, Havill N P, Leschen R A B. 2011. A New Species of Laricobius (Coleoptera: Derodontidae) From Japan With Phylogeny and a Key for Native and Introduced Congeners in North America. *Annals of the Entomological Society of America* 104.3: 389-401.

Newton L, Frampton J, Monahan J, Goldfarb B, Hain F. 2011. Two novel techniques to screen *Abies* seedlings for resistance to the balsam woolly adelgid, *Adelges piceae*. *Journal of Insect Science* 11:158. Available online: http://www.insectscience.org/11.158

Northington RM, Webster J R, Benfield E F, Cheever B M, Niederlehner B R. 2013. Ecosystem Function in Appalachian Headwater Streams during an Active Invasion by the Hemlock Woolly Adelgid. *PLoS ONE* 8.4:1-8.

Oten K. 2011. *Host-plant selection by the hemlock woolly adelgid, Adelges tsugae Annand: sensory systems and feeding behavior in relation to physical and chemical host-plant characteristics*. Ph.D. Thesis, Department of Entomology, North Carolina State University, Raleigh, NC, USA.

Paradis A, Elkinton J, Hayhoe K, Buonaccorsi J. 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. *Mitigation and Adaptation Strategies for Global Change* 13.5-6: 541-54.

Parker B L, Skinner M, Gouli S, Ashikaga T, and Teillon H B. 1999. Low Lethal Temperature for Hemlock Woolly Adelgid (Homoptera : Adelgidae). *Environmental Entomology* 28.6: 1085-1091.

Quimby J W. Value and Importance of Hemlock Ecosystems in the Eastern United States. 1996. *Proceedings of the first hemlock woolly adelgid review. Charlottesville, Virginia, USA.12 October 1995.* pg 1-8.

Ross R M, Bennett R M, Snyder C D, Young J A, Smith D R, Lemarie D P. 2003. Influence of eastern hemlock (Tsuga canadensis L.) on fish community structure and function in headwater streams of the Delaware River basin. *Ecology of Freshwater Fish* 12.1: 60-65.

Schaberg P G, DeHayesb D H, Hawleyc G j, Nijensohn S E. 2008. Anthropogenic alterations of genetic diversity within tree populations: Implications for forest ecosystem resilience. *Forest Ecology and Management* 256.5: 855-862.

State Climate Office of North Carolina. 2013. Cronos database » mountain research station (wayn). Retrieved from NC CRONOS Database website: http://www.nc-climate.ncsu.edu/cronos/index.php?station=WAYN

Story H M, Vieira L C, Salom S M, Kok L T. 2012. Assessing Performance and Competition Among Three Laricobius (Coleoptera: Derodontidae) Species, Predators of Hemlock Woolly Adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). *Environmental Entomology* 41.4: 896-904.

Tauber M J, Tauber C A, Masaki S. *Seasonal adaptations of insects*. New York: Oxford University Press, 1986.

Tingley M W, Orwig D A, Field R, Motzkin G. 2002. Avian response to removal of a forest dominant: consequences of hemlock woolly adelgid infestations. *Journal of Biogeography* 29.10-11: 1505-1516.

Trotter R T and Shields K S. Variation in Winter Survival of the Invasive Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Across the Eastern United States. *Environmental Entomology* 38.3: 577-87.

USDA Forest Service 2011. Counties with established *A. tsugae* populations 2012. Distribution map available online at http://na.fs.fed.us/fhp/hwa/maps/2012.pdf.

van Breemen N, Finzi A C, Canham C D. 1997. Canopy tree – soil interactions within temperate forests: effect of soil elemental composition and texture on species distributions. *Canadian Journal of Forest Research* 27: 1110–1116.

Vose J M, Wear D N, Mayfield A E III. 2013. Hemlock woolly adelgid in the southern Appalachians: Control strategies, ecological impacts, and potential management responses. *Forest Ecology and Management* 291: 209-219.

Webb R E, Frank J R, Raupp M J. 2003. Eastern Hemlock Recovery from Hemlock Woolly Adelgid Damage following Imidacloprid Therapy. *Journal of Arboriculture* 29.5: 298-302.

Young R F, Shields K S, Berlyn G P. 1995. Hemlock woolly adelgid (Homoptera: Adelgidae): stylet bundle insertion and feeding sites. *Annals of the Entomological Society of America* 88: 827-835.

Zobel B and Talbert J. 2003. *Applied Forest Tree Improvement*. New Jersey: The Blackburn Press, 50-52, 67, 70, 459-478.

Table 1. Results of analysis of variance for settled HWA nymphs on hemlock seedlings exposed to the progrediens and sistens generation in March and May 2012.

Effect	**DF**	**F**	***P***
Block	3	6.52	0.0003
Treatment	2	2.00	0.1387
Month	1	41.73	<0.0001
Species	2	2.45	0.0885
Treatment*Month	2	5.86	0.0034
Treatment*Species	4	0.97	0.4244
Month*Species	2	1.50	0.2252

Table 2. Results of analysis of variance for settled HWA nymphs on hemlock seedlings exposed to the progrediens generation in March 2013.

Effect	**DF**	**F**	***P***
Block	3	3.52	0.0184
Treatment	2	14.49	<0.0001
Species	1	57.29	<0.0001
Treatment*Species	2	4.17	0.0187

Average Density of Hemlock Woolly Adelgid per 10 cm Branch

for Three Methods of Artificial Infestation

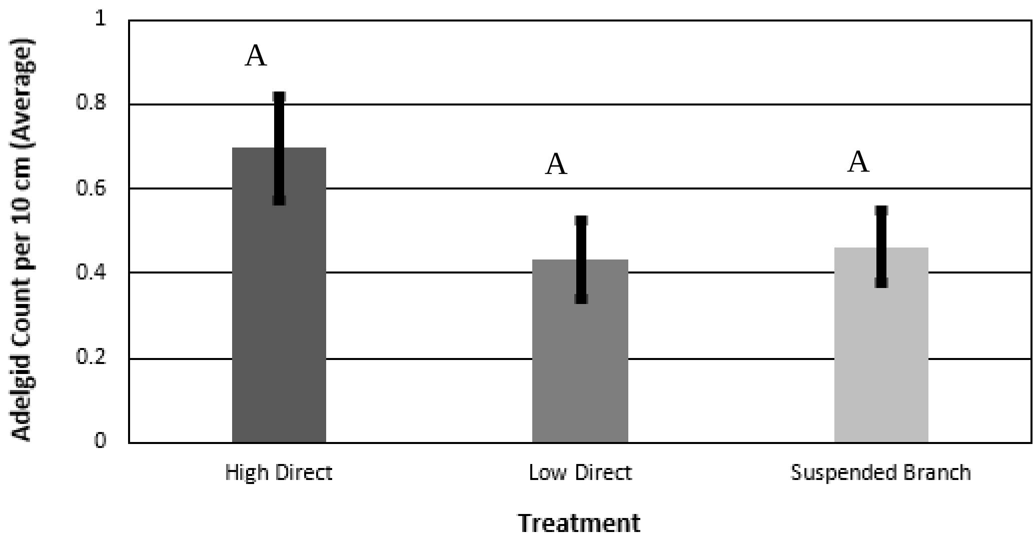


Figure 1. Least squares means (± SE) density of settled HWA nymphs per 10 cm branch on seedlings exposed to the progrediens and sistens generation with high direct, low direct, and suspended branch infestation treatments. Means are averaged across the March and May 2012 infestations. Bars with the same letters are not significantly different. There is no significant difference among treatments.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch for

March and May 2012 Infestations

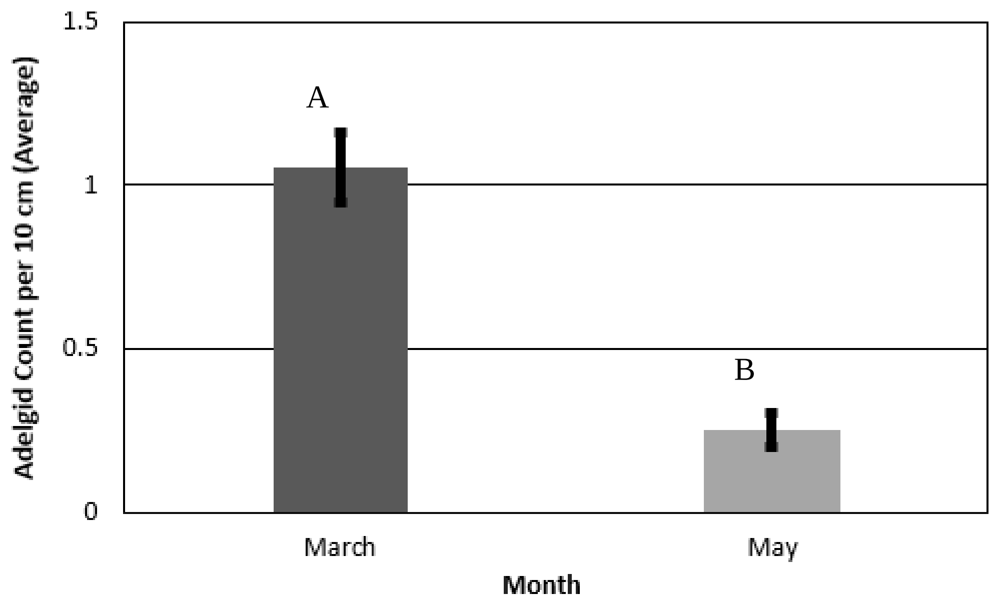


Figure 2. Least squares means (± SE) density of settled HWA nymphs per 10 cm branch on seedlings exposed to the progrediens and sistens generation in March and May 2012. Bars with the same letters are not significantly different.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch for Carolina, Eastern, and Western Hemlock

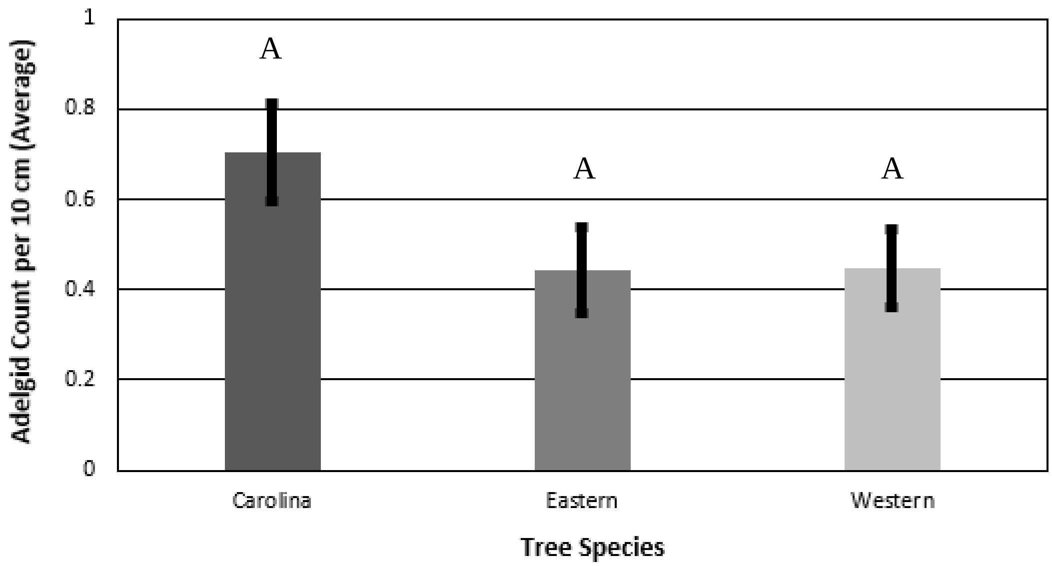


Figure 3. Least squares means (± SE) density of settled HWA nymphs per 10 cm branch for Carolina, eastern, and western hemlock exposed to the progrediens and sistens generations averaged across March and May 2012 infestations. Bars with the same letters are not significantly different. There is no significant difference among tree species.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch on Carolina, Eastern, and Western Hemlock for Three Methods of Artificial Infestation in March and May 2012

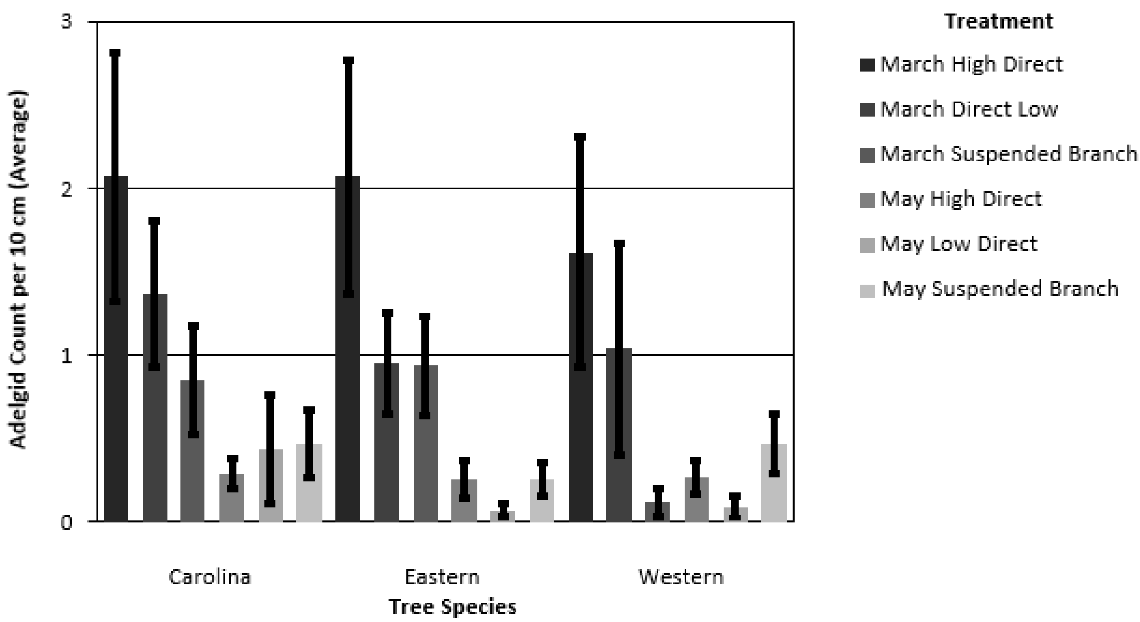


Figure 4. Average (SE) density of settled HWA nymphs per 10 cm branch on Carolina, eastern, and western hemlock exposed to the progrediens (March) and sistens (May) generation in 2012 with high direct, low direct, and suspended branch infestation treatments.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch for Three Artificial Infestation in March and May 2012

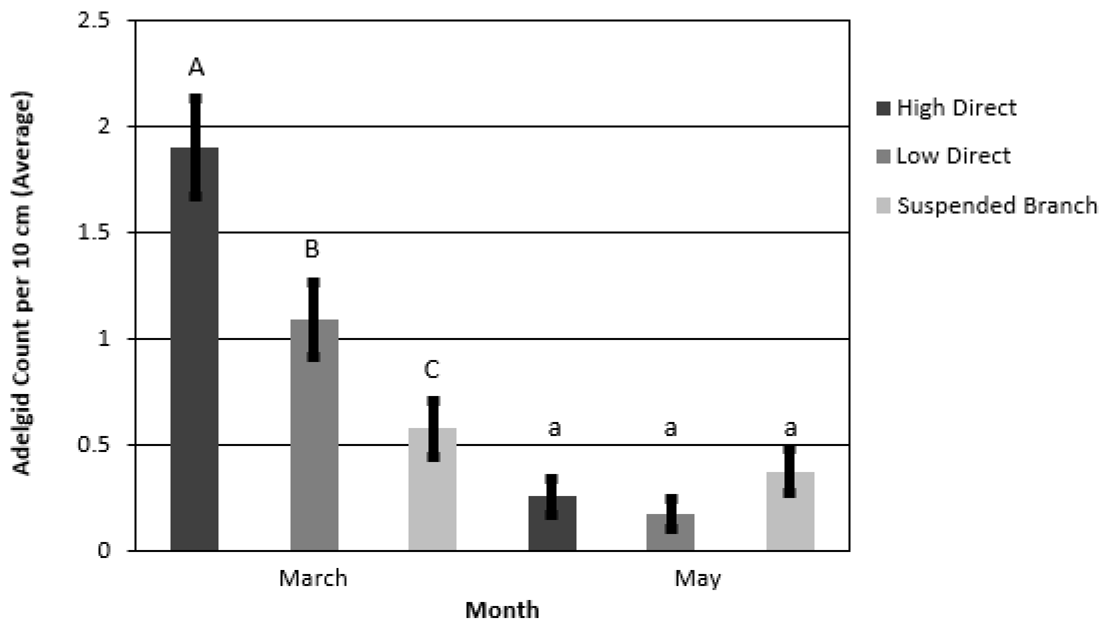


Figure 5. Least squares means average (SE) density of settled HWA nymphs per 10 cm branch on hemlock seedlings exposed to the progrediens (March) and sistens (May) generation 2012 with high direct, low direct, and suspended branch infestation treatments. Letters indicate significance levels calculated with Tukey-Kramer grouping when $\alpha = 0.05$. The month by treatment interaction was significant and the results were sliced by month. Significance levels for March are indicated by upper case letters and significance levels for May are indicated by lower case letters. Treatments with different letters are significantly different from each other.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch for Three Methods of Artificial Infestation in March 2013

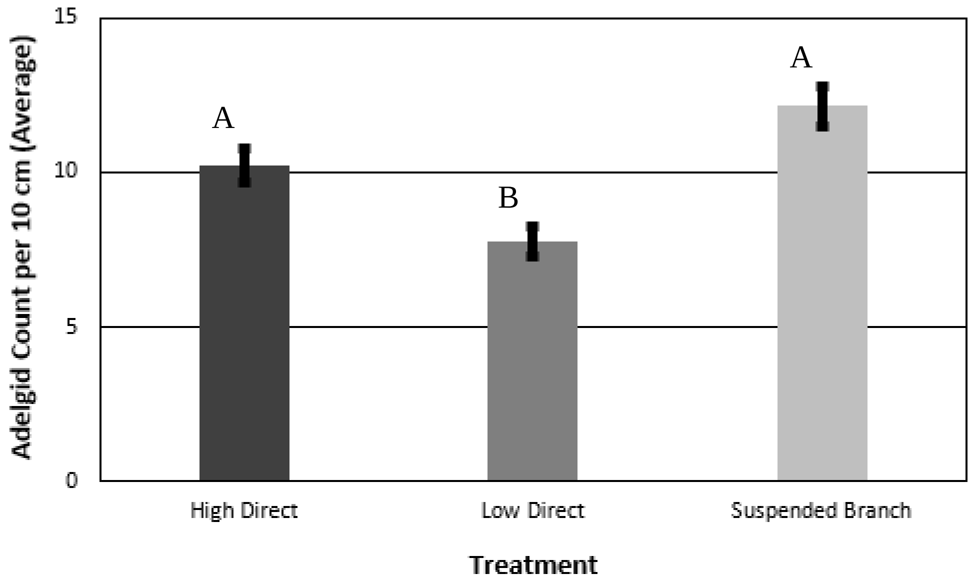


Figure 6. Least squares means (± SE) density of HWA nymphs per 10 cm branch on hemlock seedlings exposed to the progrediens generation in March 2013 with high direct high, low direct, and suspended branch infestation treatments. Bars with the same letter are not significantly different.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch on Eastern and Carolina Hemlock Seedlings Infested March 2013

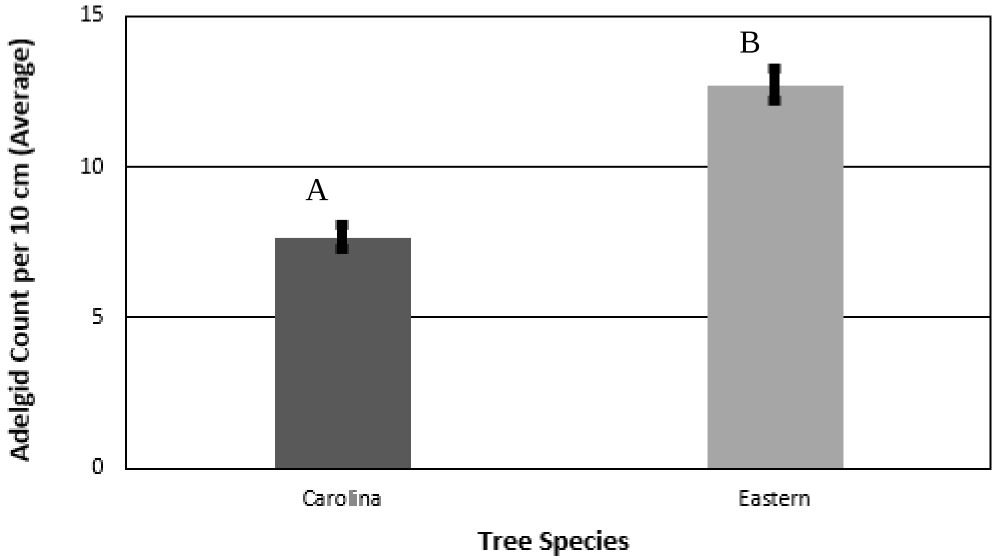


Figure 7. Least squares means (± SE) density of settled HWA nymphs per 10 cm branch for Carolina and eastern hemlock seedlings exposed to the progrediens generation in March 2013. Bars with the same letter are not significantly different. The density of settled adelgids was significantly different for tree species.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch on Eastern Hemlock Seedlings Infested with Three Methods of Artificial Infestation March 2013

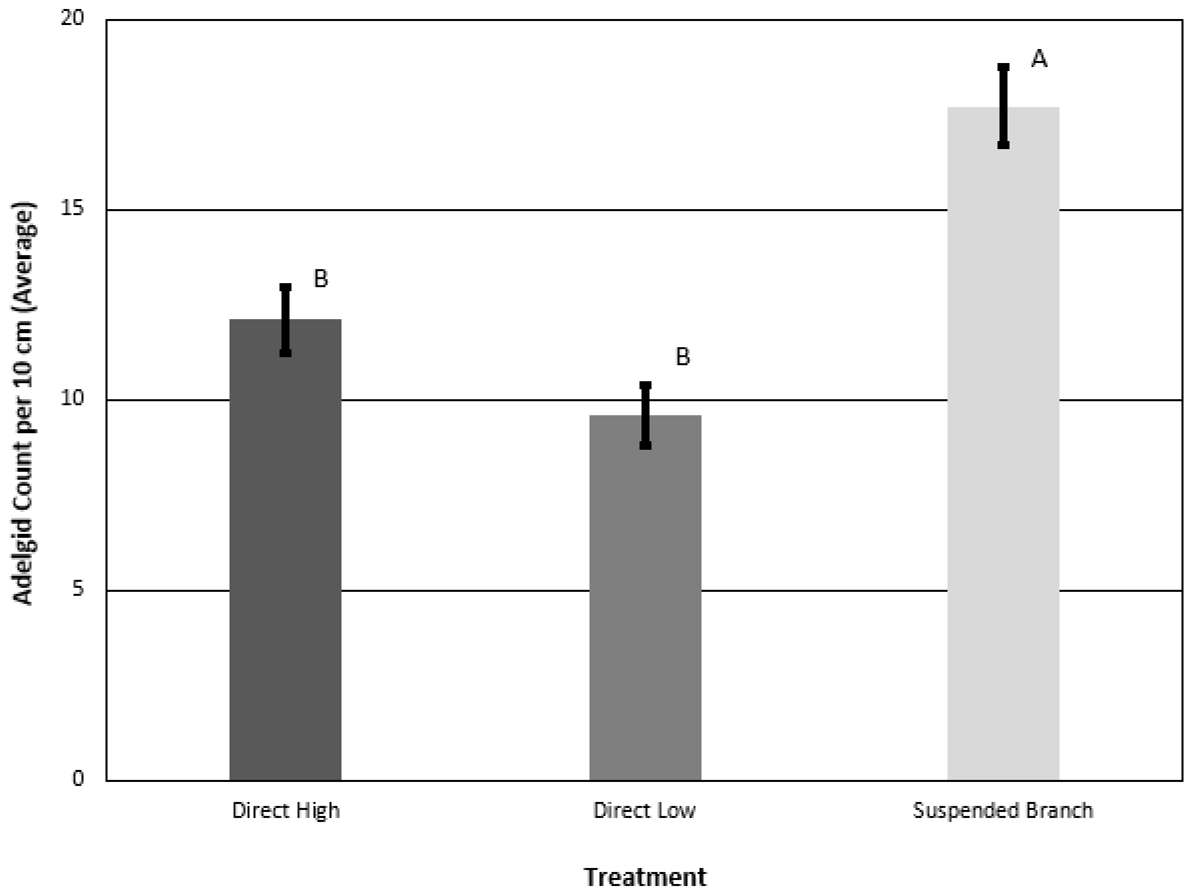


Figure 8. Least squares means average (SE) density of HWA nymphs per 10 cm branch on eastern hemlock exposed to the progrediens generation in March 2013 with high direct, low direct, and suspended branch infestation treatments. Letters indicate significance levels calculated with Tukey-Kramer Grouping (α = 0.05). Bars with the same letter are not significantly different.

Average Density of Hemlock Woolly Adelgid per 10 cm Branch on Carolina Hemlock Seedlings Infested with Three Methods of Artificial Infestation March 2013

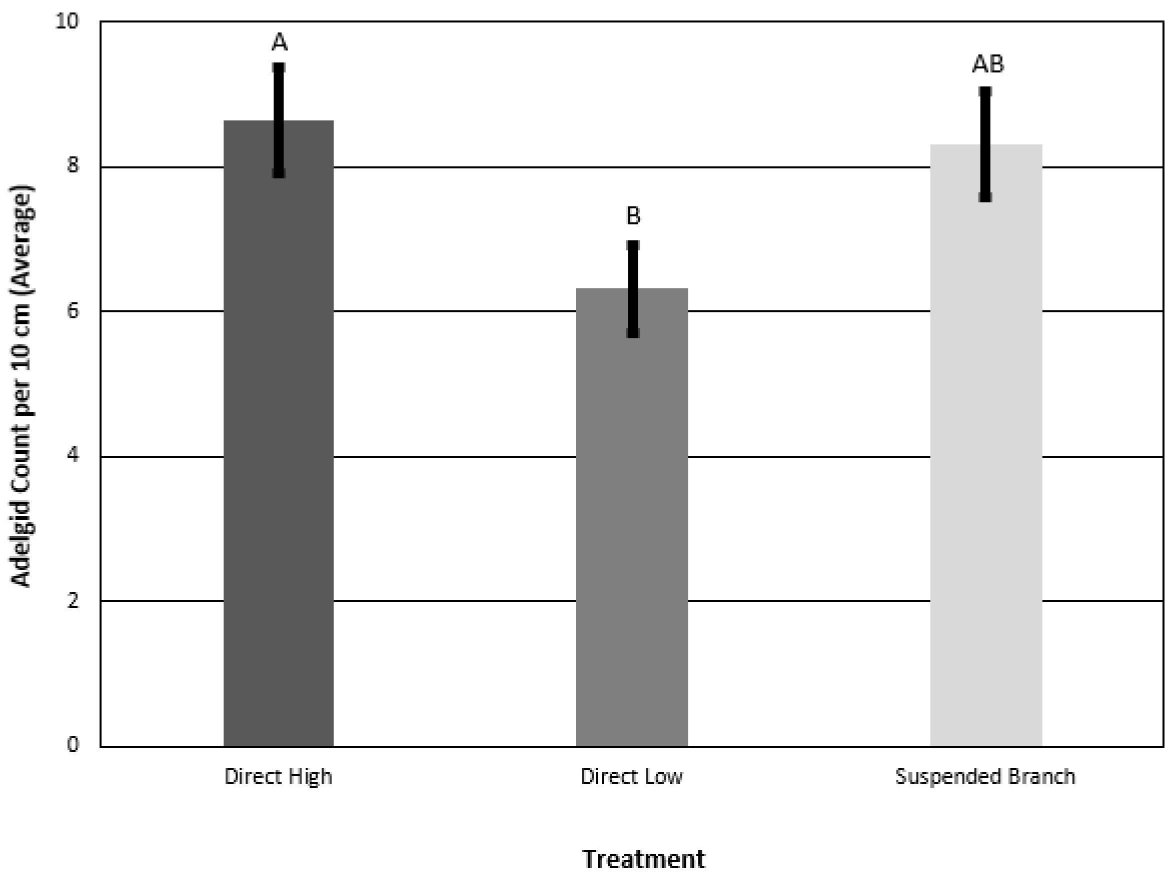


Figure 9. Least squares means average (SE) density of HWA nymphs per 10 cm branch on Carolina hemlock exposed to the progrediens generation in March 2013 with high direct, low direct, and suspended branch infestation treatments. Letters indicate significance levels calculated with Tukey-Kramer Grouping ($\alpha = 0.05$). Bars with the same letter are not significantly different.

CHAPTER III

Comparison of the Stationary and the Rotating Suspended Branch Infestation Techniques for Screening Hemlocks Resistant to the Hemlock Woolly Adelgid

ABSTRACT

The invasive hemlock woolly adelgid (HWA, *Adelges tsugae* Annand) has caused significant decline in eastern (*Tsuga canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock populations in eastern North America. The purpose of this study was to compare the effectiveness of stationary and rotating suspended branch infestations for tolerance/resistance screening of hemlock seedlings. Crawler density and distribution were observed on Tanglefoot® coated paper placed below suspended infested branches. The progrediens (March) and sistens (May) generations were observed. The density of progrediens crawlers that dispersed from branches per cm^2 was not significantly different between rotating (4.25± 0.34, non-transformed data) and stationary (6.71 ± 0.88, non-transformed data) suspended branches. The density of sistens crawlers per 2 cm^2 was significantly greater for rotating (14.41± 0.24, non-transformed data) than stationary (14.76 ± 0.42, non-transformed data) suspended branches. Adelgid distribution was analyzed for progrediens crawlers only. With respect to number of single or clustered adelgid points, crawler distribution was significantly clustered for stationary suspended branches and significantly dispersed for the rotating suspended branches. Distribution analysis that accounted for the number of adelgids at each point indicated that both stationary and rotating suspended branch crawler density was clustered, and stationary suspended branches produced more clustering at all distances between points than rotating suspended branches.

These results indicated the rotating suspended branch technique should be considered for resistance testing of HWA on hemlock seedlings. Rotating branches decreased the amount of adelgid clustering below branches and produced crawler densities greater than or equal to crawler densities from stationary suspended branches.

INTRODUCTION

Eastern (*Tsuga canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock populations in North America are threatened by the hemlock woolly adelgid (HWA, *Adelges tsugae* Annand). The invasive pest, introduced on southern Japanese hemlock (*T. sieboldii* Carr.) nursery stock, as of 2013 has spread to 19 states on the east coast in the last 60 years (Havill et al. 2006; Havill et al. 2007; McClure 1987; USDA Forest Service 2012). The decline in hemlock populations caused by HWA produces significant changes in hemlock ecosystems including shifts in over story and understory canopy, animal inhabitants, and nutrient cycles (Jenkins et al., 1999; Ford et al., 2012; Knoepp et al., 2011; Rowell and Sobczak, 2008; Tingley et al., 2002; Ross et al., 2003; Ingwell et al. 2012).

Integrated Pest Management (IPM) is utilized in urban and forest ecosystems to manage the pest. IPM includes a combination of chemical, biological, host resistance, cultural treatments, and host gene conservation control mechanisms that are limited by land ownership, technology, funding, hemlock health, and HWA population level. Insecticides are an important part of IPM and are useful when other controls are not effective, such as locations greatly impacted by the adelgid. Chemical controls are not feasible in the forest due to high rates of hemlock mortality, regulation limits, and budget limits. Biological controls are applied in the forest setting and will be most effective with the combination of multiple natural enemies. Resistance breeding also has the potential to successfully limit hemlock decline in the forest (McClure, 2007; Vose et. al., 2013). Resistance research includes breeding of native Carolina hemlock with non-native hemlock species such as Chinese hemlock (*T. chinensis* (Franch.) Pritzel ex Diels.) and Southern Japanese hemlock (Montgomery et al. 2009), as well as locating putative natural tolerance/resistance to the adelgid in native populations (Jetton et al. in press).

Hemlock trees are tested for resistance to the adelgid by introducing the pest to the host and observing the host response. The adelgid is introduced during the life cycle when it naturally disperses onto the host. A technique that resembles natural distribution of the pest is preferred (Alfaro et al. 2008; Hodge and Dvorak, 2007). An alternative to direct infestation utilized in previous literature (Montgomery et al. 2009; Butin et al. 2007; Newton et al. 2011) is the suspended branch infestation. Infested branches are placed above seedlings and the adelgid crawlers fall off onto the seedlings below, mimicking natural dispersal of the crawlers. Suspended branches have been utilized in stationary infestations where hotspots of clustered adelgids occurred below the branches (Jetton et al. in press; Newton et al. 2011). Even dispersal of the pest is important for resistance testing (Jenkins et al. 1982). Rotating suspended branches may produce a more dispersed distribution of crawlers.

Insect pest research has incorporated image analysis tools to reduce meticulous and time consuming work, prevent destructive analysis, as well as reduce human error (Mains et al. 2008; Teale et al. 2009). Research projects have included beech scale density assessment with wax mass area (Teale et al. 2009), adult mosquito counts in traps (Kesavaraju and Dickson 2012), ant nest structure analysis (Halley et al. 2005), detecting pupae in wheat (Toews et al. 2006), and vector control through fecundity assessment (Mains et al. 2008). The use of Image J, a public domain image processing program used to calculate area, distance, angle, etc. and edit images, is restricted by color and behavior of the insect. For example, the analysis of egg counts for some *Aedes* species is possible due to the ovipositioning behavior on moist solid surfaces instead of water. The dark egg color allows for the contrast with background material that is required for analysis (Mains et al. 2008). Image J is appropriate for HWA crawler counts because the adelgid can be collected on a solid surface and the dark crawlers on white paper provide a good contrast.

The objective of this study was to improve the technique for infesting seedlings with suspended branches. The density and distribution of adelgids that dispersed from stationary and rotating suspended branches were compared. Data were collected with Image J as well as direct observation under a dissecting microscope.

MATERIALS AND METHODS

Location and Experimental Design

Source Material-Infested Branches

Stationary and rotating suspended branch methods were compared in 2012. Progrediens generation eggs were used for the March infestation and sistens generation eggs were used for May. Infested branches were gathered with pole pruners from eastern hemlocks in McDowell County near Marion, North Carolina (35.606825,-82.100354) and near Petros, Tennessee (36.09395, -84.409075) in March. Branches were collected from Petros, Tennessee only in May. Branches 40 cm long were cut for stationary and rotating suspended branch treatments. The average number of ovisacs per branch and eggs per ovisac were estimated with 15, 40 cm branch samples each from progrediens and sistens infested material, including the main stem and side branches. The cut ends of the 40 cm branches used for the infestation experiment were placed in 11 L buckets with water until used.

Progrediens and Sistens Introductions (March and May 2012)

Research took place at the North Carolina Department of Agriculture & Consumer Services Mountain Research Station in Waynesville, NC (35.487319°N, -82.966883°W). Branches were placed above 1 m^3 cubes with a 2 cm diameter frame of schedule 40 PVC pipe. The four sides of the cube were enclosed with clear 6 mil plastic sheeting to deter movement of HWA between plots. Sheeting was secured with binder clips. The bottom of the cube was not enclosed and the top of the cube was covered with a m^2 section of poultry wire secured with plastic cable ties. The experiment took place in a building that protected against rain and direct sunlight but was not climate controlled.

Cubes were positioned into three blocks with two research plots per block randomly arranged. The progrediens generation was introduced March 7, 2012 and branches were removed March 16, 2012. One plot per block had infested stationary suspended branches where 40 cm branches, including the main branch and side branches, were placed on the poultry wire in four rows of ten with the abaxial side down. The branches were covered with a second 1 m^2 section of poultry wire secured with plastic cable ties (Figure 1) and a sheet of paper 1 m^2 with a 10 cm^2 grid was sprayed with Tanglefoot ® and placed under the cube. Each branch had approximately 19 ovisacs per cm and an average of 40.50 ± 1.64 eggs per ovisac. Approximately 1,231,200 progrediens eggs were placed above the 1 m^2 Tanglefoot ® paper below (Figure 2).One plot per block had infested rotating suspended branches positioned and secured similar to the stationary suspended branches and utilized the same Tanglefoot® paper and adelgid infestation level. For each plot the top corners of the cube were attached with a wire to a rotating motor that ranged between 2.25 rpm and 2.5 rpm. The motor was suspended from a wooden frame and branches were 105 cm to 110 cm above the ground (Figure 1).

The sistens experiment took place at the same location as the progrediens experiment. The experimental design, cubes, and infestation methods were the same as the progrediens generation. Infested branches were introduced May 11, 2012 and removed May 25, 2012. Each branch had approximately 14 ovisacs per cm and an average of 6.57 ± 0.49 eggs per ovisac. Approximately147,168 sistens eggs were placed above the 1 m^2 Tanglefoot ® paper below.

Data Collection

Progrediens infested branches were removed after 9 days on March 16, 2012 and the Tanglefoot ® paper was collected. The paper was cut into 20 cm^2 sections (twenty five squares per plot). Density and distribution data was collected from the Tanglefoot ® paper with Image J. The paper was cleared of needles and debris, scanned with a Zeutschel OS 12000 Bookcopy overhead scanner at the Natural Resources Library at North Carolina State

University, and saved in Tagged Image File Format (TIFF). A ruler was scanned, saved as a TIFF file, and was used to convert size measurements in Image J from pixels to cm. Within Image J, additional objects that were not crawlers were erased. The photos were enhanced 0.4%, converted to binary, and the Analyze Particles option was used to examine the 20 cm^2 sections.

The area of each single adelgid and adelgid cluster was determined. In Image J, the size range was set to 0-∞ cm^2 and the circularity range was set to 0-1. The Image J output included an image with adelgid areas identified by number, as well as a results table with the number, label, area (cm^2), and x, y coordinates of each area. Grid lines and crawlers that fell on the grid lines were not included in the results.

The number of HWA per cm^2 on the sheets was estimated using two regression equations, one for each suspended branch technique. The area measurements in the output from Image J were utilized to develop the equations. To determine the equation for the stationary suspended branch technique a subsample was collected from the data. All the areas for the stationary suspended branch technique were placed in one Excel file and ordered from least to greatest area value. The areas were accompanied by the x, y coordinate and point number. The data was then separated into 20 intervals. Each interval had the same number of data points. From each interval 2 samples were randomly collected using the random number generator RANDBETWEEN in Excel.

Next, the number of HWA in the given area was determined for the 2 samples per interval. The x, y coordinate and point number were used to locate the area. The number of

HWA on the original sheet in the area was determined visually with a dissecting microscope. A total of 40 samples were collected. There were many small adelgid areas (areas with one or two crawlers) and no large adelgid areas (areas with more than 8 crawlers) in the sample. The procedure did not account for the larger areas because the vast majority of observations were small areas.

A second sample was therefore collected from the stationary suspended branch data set where the values above the largest area in the first sample were selected. The larger areas were separated into 20 intervals. Each interval had the same number of data points and 2 samples were collected from each interval for a total of 40 samples. The procedure for counting the number of crawlers in each area was the same as the first 40 samples. A total of 80 samples were collected from the stationary suspended branch sheets and used to determine the regression equation. Samples that were not crawlers or had a combination of crawlers and other material (example: dirt) were indicated as missing values in the data set. The same procedure was used to sample the rotating suspended branch sheets.

Sistens infested branches were removed after 14 days on May 25, 2012 and the Tanglefoot ® paper was collected with the same method as the progrediens collections. The analysis differed from the progrediens analysis. Image J was not the best option because sheets were covered with needles. All needles would have had to be removed, a time consuming method that would have removed crawlers as well. The crawler density was determined visually with a dissection microscope. A subsample of five 2 cm^2 sections per 10 cm^2 grid on the m^2 sheet was used to determine the density of crawlers per 2 cm^2. The distribution of crawlers for the area could not be sufficiently analyzed due to needle coverage. The average crawler densities from the subsample were mapped using ArcGlobe 10.1 (Figure 13).

Statistical Analysis

Progrediens Infestation (March 2012)

For each suspended branch technique, regression equations to predict crawler density per area were developed based on 80 subsamples. These data were analyzed using linear regression (full model fitted). The null hypothesis that the linear regression model does not fit the model better than the baseline model ($\beta_1 = 0$) was tested. Data were analyzed with the REG procedure of SAS Enterprise Guide 5.1 (SAS EG 5.1).

For each suspended branch technique, crawler density was calculated based on predicted values of the number of crawlers per cm^2 from the regression equations. These data were analyzed using a two sample t-test. The null hypothesis that suspended branch treatment had no significant influence on the predicted density values of progrediens per cm^2 was tested. Variances for the two infestation methods were significantly different ($F=2.93$, $P<0.0001$) according to a folded F test. Satterthwaite's approximation was used to calculate standard errors used in the t-tests. The log transformed data were analyzed with the TTEST procedure ($\alpha = 0.05$) of SAS EG 5.1.

For each suspended branch technique, crawler distribution was calculated based on approximate crawler counts from the regression equations for all samples collected with Image J. This data was analyzed using Average Nearest Neighbor. The null hypothesis that crawlers were randomly distributed (complete spatial randomness, CSR) was tested. The Euclidean distance was utilized to analyze the data with ArcGlobe 10.1.

Average Nearest Neighbor utilized the x, y coordinates of single and clustered adelgids to calculate a nearest neighbor index based on the average distance between each point and it's nearest neighboring point. The analysis did not account for the number of crawlers at each coordinate. The data was also analyzed with Ripley's-K. The null hypothesis that crawlers were randomly distributed at given distances between points was tested. Data were analyzed with isotropic corrections in R-3.0.1.

Sistens Infestation (May 2012)

For each suspended branch technique, crawler density was calculated based on subsample counts from Tanglefoot® sheets. These data were analyzed using a two sample t-test. The null hypothesis that there is no significant difference in mean crawler density between rotating and stationary treatments was tested. Variances for the two infestation methods were significantly different ($F=2.66$, $P<0.0001$) according to a folded F test. Satterthwaite's approximation was used to calculate standard errors used in the t-tests. The log transformed data were analyzed with the TTEST procedure ($\alpha = 0.05$) of SAS EG 5.1.

RESULTS

The linear regression for the progrediens in March resulted in a stationary suspended branch regression equation of $y = 985.25934\,x - 0.08187$ that was significantly different from the base line and indicated that an area of 1 cm^2 contained approximately 985 crawlers (Table 1, Figure 3). The rotating suspended branch regression equation of $y = 683.74464\,x$ +

0.72521 was significantly different from the baseline and indicated that an area of 1 cm² contained approximately 684 crawlers (Table 1, Figure 4). A large portion of variability was explained by both models and the equations are only suitable for estimating values within the range they were sampled. The rounded total number of crawlers on each March sheet, based on regression equation calculations, were 142,797 (sheet 1A stationary), 180,966 (sheet 2A stationary), 230,776 (sheet 3A stationary), 138,922 (sheet 1B rotating), 220,681 (sheet 2B rotating), and 132,173 (sheet 3B rotating).

The average progrediens crawler density per cm² in March was not significantly different between the rotating (4.25± 0.34, non-transformed data) and stationary (6.71 ± 0.88, non-transformed data) suspended branches ($t = 0.11$, $P = 0.92$, transformed data). The average sistens crawler density per 2 cm² in May was significantly different between the rotating (14.41± 0.24, non-transformed data) and stationary (14.76 ± 0.42, non-transformed data) suspended branch infestations ($t = 8.55$, $P < 0.0001$, transformed data). The log transformed results were different from the two sample t-test on the original data.

Progrediens and sexupara eggs are deposited at the same time in the same cluster. Approximately 40 % to 50 % of the eggs would become sexupara and indistinguishable from the progrediens generation during the collection process (McClure 1987; McCLure 1989). The number of progrediens eggs was approximately 4 times as great as the sistens eggs but resulted in lower densities of dispersing nymphs. The Average Nearest Neighbor analyses of progrediens stationary suspended branch sheets had Average Nearest Neighbor ratios of 0.95, 0.97, and 0.96 for sheets 1A, 2A, and 3A, respectively. The occurrence of points had an aggregated distribution. The Z-scores were -34.16, -20.20, and -26.10 for sheets 1A, 2A, and 3A, respectively, indicating that the null hypothesis was rejected for all sheets. The P values for all three sheets were 0.00 and indicated that it is unlikely the observed spatial pattern is due to random processes, and the null hypothesis was rejected (Figure 5, Figure 6, Figure 7). The Average Nearest Neighbor analysis of progrediens rotating suspended branch sheets had Average Nearest Neighbor ratios of 1.04, 1.04, and 1.01 for sheets 1B, 2B, and 3B, respectively. The occurrence of points had a dispersed distribution. The Z-scores were 31.03, 38.89, and 10.69 for sheets 1B, 2B, and 3B, respectively, indicating that the null hypothesis was rejected for all sheets. The P values for all three sheets were 0.00 and indicated that it is unlikely the observed spatial pattern is due to random processes, and the null hypothesis is rejected (Figure 8, Figure 9, Figure 10).

The Average Nearest Neighbor is a simple and elegant test but does not account for multiple crawlers at one location. The results therefore reflect the clustering or dispersion of points with single or grouped crawlers. The multi-distance spatial cluster Ripley's K analysis was used to determine if individual crawlers were clustered at different distances, accounting for the number of crawlers at each point. The graphs for both treatments (Figure 11, Figure 12) illustrate that the observed K is greater than the expected K and the high confidence interval, indicating that the distribution of crawlers was significantly more clustered than random in both treatments at all distances (scale of analysis). The observed K for rotating and stationary suspended branches was close to neutral at short distances and the gap between observed K and expected K increased at larger distances, indicating more clustering at longer distances. The rotating suspended branches had a smaller gap between observed K and expected K than the stationary suspended branches and therefore produced less clustering at all distances. The distribution of sistens crawlers in May on all three rotating suspended branch Tanglefoot® sheets approximated a ring/circular pattern (Figure 13). None of the three stationary suspended branch Tanglefoot® sheets had a similar adelgid pattern (Figure 13).

DISCUSSION

The regression equations may be used to estimate the number of HWA in a given area as long as the values are within the range used to produce the equations (stationary: 0.000162 cm^2 - 0.04 cm^2; rotating: 0.000162 cm^2 - 0.018 cm^2) . Area measurements can be limited by overlapping individual crawlers (Mains et al. 2008). Although there was some overlap of crawlers on the Tanglefoot® papers, the equations have high R-square values of 0.86 (Figure 3) and 0.78 (Figure 4) and are a good representation of the number of adelgids per cm^2.

The progrediens density was not significantly different between the stationary and rotating suspended branch sheets. The sistens density was significantly different between the two techniques, with greater densities for the rotating than the stationary suspended branches. The progrediens and sistens infested branches were collected, stored, and placed above sticky sheets with the same procedure for both months. The significance of the results could have differed for March and May due to differences in sampling technique. A subsample of 80 points were collected for each treatment from a total count of adelgid area collected in March with Image J while 20% of each sheet was subsampled visually with a dissecting microscope for May. The results may have also been influenced by unintended disruption of branches (ex: when replacing motor batteries) and/or adelgid mortality while securing plastic cable ties (Butin et al. 2007). Differences due to the movement of branches could be avoided by rotating the seedlings instead of the branches.

An even distribution of the pest onto the host is necessary for effective resistance testing (Smith et al. 1970; Newton et al. 2011). Equal distribution of crawlers over all trees ensures that each tree receives the same HWA population pressure. Uneven distribution may result in false resistance or false susceptibility. A susceptible seedling that does not receive crawlers due to high clustering during the infestation may exhibit a false resistance when the health does not decline. A low/moderately resistant seedling that receives a hot spot due to high clustering during the infestation may exhibit a false susceptibility when seedling health declines compared to the other seedlings.

The Average Nearest Neighbor output indicated that rotating suspended branches produced a significantly dispersed pattern and stationary suspended branches produced a significantly clustered pattern of progrediens crawlers. The data were further explored with Ripley’s K where results of the progrediens generation indicated that clustering was less apparent in rotating suspended branches than stationary suspended branches. Rotating suspended branch plots had no significant clustering or a small degree of clustering at shorter distances. As the distance increased the amount of clustering increased slightly (Figure 11). The stationary suspended branches had a larger degree of clustering at short distances than the rotating suspended branches, and the clustering increased greatly as distance increased

(Figure 12). The results are supported by a previous study where infestations of the leaf miner *Liriomyza munda* on plants produced a more even oviposition pattern on plants with a rotating infestation technique. The technique reduced the pattern produced by stationary infestations but it was not removed completely (Smith et al. 1970).

The sistens crawlers produced a similar pattern to the progrediens distribution. The rotating suspended branch Tanglefoot® sheets had a ring/circular pattern of adelgids while one of the stationary suspended branch Tanglefoot® sheets exhibited random hot spots. The distribution of crawlers was not statistically analyzed due to high needle content on sticky sheets from the sampling method and time of year. Branches were not hydrated during the experiment, drying them out and causing some needle loss. The branches were then moved during the Tanglefoot® sheet collection process and additional needles fell onto the sheets. With regard to time of year, the branches collected in May had experienced both progrediens and sistens generations while branches in March were exposed to only one generation. May branches were less healthy and more prone to needle loss. Overlapping needles obstructed the view of crawlers. The removal of all needles in order to view crawlers in Image J would have been a time consuming process that eliminated crawlers as well as needles from the analysis. The crawler counts from these papers would then be inaccurate. It may not be necessary to statistically analyze the distribution of sistens crawlers in future research due to the greater fecundity of HWA in March than in May, discussed in the following paragraph. March appears to be better suited for seedling infestations to test for resistance.

HWA infestations are known to have greater fecundity in March than May (McClure 1989). The March populations are recommended for producing infestations (Butin et al. 2007; Jetton et al. in press) above the damage threshold (McClure 1991). The branches in this experiment contained 1,242,000 eggs per m^2 in March and 151,000 eggs per m^2 in May, exemplifying the greater fecundity in March. Additionally, the March crawler density is significantly similar for both infestation techniques, indicating that either technique would be appropriate in March. Since the rotating suspended branches produced a better distributed/ less clustered pattern than the stationary suspended braches, infestations in March should incorporate the rotating suspended branch technique (Figure 11, Figure 12).

The total number of crawlers emerging from the 1,242,000 eggs in March ranged from 142,797 crawlers to 230,776 crawlers on the stationary suspended branch sheets and 132,173 crawlers to 220,681 on the rotating suspended branch sheets. Less than half of the initial population dispersed onto the Tanglefoot® sheets below during the 10 day emergence period. The density of settled crawlers on hemlock trees was not observed in the study. The next step is to compare the settled crawlers on seedlings for both techniques to determine if adequate infestation levels occur. Crawlers are transported by wind, deer, birds, and humans (McClure 1990). Their settlement and survival should not be influenced by movement of branches or the fall from the branches onto the seedlings.

Future studies should focus on improving the rotating suspended branch technique. The crawler distribution was more dispersed for the rotating suspended branches than the stationary suspended branch technique, but the corners of the 1 m^2 Tanglefoot® sheet had a smaller density of crawlers than the middle of the sheet. Seedlings in an infestation study would receive fewer crawlers if they were placed in the corners than in the middle and center of the sheet. Seedlings placed within in a circle rather than a square, a technique used in previous studies (Smith et al. 1970), may provide a more even infestation. The distribution should be observed with a circular Tanglefoot ® sheet. An alternative to rotating the infested branches is to rotate the seedlings (Smith et al. 1970). The rotation of seedlings would avoid disturbance to the branches and prevent some of the difficulties that occurred with data collection and processing.

The use of Image J is recommended for future studies. A large portion of variability is explained by the regression models developed (Figure 3, Figure 4). The program is free and requires an inexpensive scanner. Images of crawlers can be saved and used in the future for manual counts if necessary. The program reduces human error and time required for counts.

The process for analyzing Tanglefoot® sheets in Image J can be improved. The sheets collected dirt and dust from the floor. The dust pieces may be identified as crawlers in Image J if they are the same size and circularity as the crawlers. Future experiments should take place where the floor has been thoroughly cleaned of debris and there is no unnecessary air movement in the room such as wind blowing under the door or air vents directed toward the sheets. Raising the sheets above ground may also decrease the amount of debris.

The processing of the Tanglefoot ® sheets is slowed by needle removal. Branches lost needles during the 10 day period due to the absence of water. When the cages are moved to remove the sheets underneath, needles easily fall from the poultry wire above. If the cages did not have to be moved the sheets would not collect as many needles and less time would be required to analyze the sheets. A wooden square frame 1.1 m x 1.1 m x 10 cm with a solid bottom and open top could be built with a 1 m^2 side opening where the sheet could slide in and out without disturbing the branches. The processing with Image J is also slowed by the removal of gridlines. There should be a minimal amount of grid lines on the sheet.

Previous studies have utilized hydrated branches instead of non-hydrated branches (Butin et al. 2007). Hydrated branches retain needles for a longer period of time than non-hydrated branches and would quicken the data collection and processing. Chapter IV examines the topic and demonstrated that the number of crawlers dispersing from hydrated branches was fewer than non-hydrated branches, with approximately twice the number of crawlers dispersing from non-hydrated branches. The crawlers on the non-hydrated branches may have received more pressure to search for new material due to the reduced health of the branches. The health of crawlers on infested and non-infested branches is one aspect of the system that was not examined in Chapter IV and should be observed in future studies because resistance/tolerance testing requires healthy and reliable populations.

A concern with documenting crawler area instead of crawler count is the change in area that takes place when pressure is applied to crawlers during the collection. With applied pressure the adelgid is flattened and the area measured by Image J increases. During the collection process clear sheets were placed on the Tanglefoot® side of the paper and some pressure was imposed to secure the sheets. The 150 papers were then stored in a binder where papers in the back of the binder received additional pressure due to the weight of the sheets in the front of the binder. In the future researchers should be aware of pressure on the crawlers during collection and storage.

CONCLUSION

The results suggest that infestations on hemlock seedlings should take place in March with rotating suspended branches. March branches had a greater number of eggs per ovisac. The rotating suspended branch sheets had a similar density to the stationary suspended branch sheets and a more dispersed/less clustered crawler pattern. May could be a valid alternative, with significantly greater crawler densities from rotating suspended branches than stationary suspended branches. The rotating suspended branch technique should be further explored regarding the design of the infestation and adelgid settlement on seedlings before implementing in mass testing programs for tolerance/resistance in hemlock. The advancement in infestation technology will aid mass selection for resistant phenotypes, deployment, and restoration.

ACKNOWLEDGMENTS

We thank Kaleb Rathbone and the NCDA&CS/NCSU Mountain Research Station in Waynesville, NC for providing space, equipment, and assistance; Ben Smith (NCSU-ASTF) and Andy Whittier (NCSU-Camcore) for technical assistance; Frank Koch (USDA FS) for distributional analysis assistance; and the Coal Creek Company for site access and source of infested branches. This work is funded by USDA Forest Service Southern Research Station Cooperative Agreement No. 11-CA-11330129-064.e

REFERENCES

Alfaro R I, King J N, Brown R G, Buddingh S M. 2008. Screening of Sitka spruce genotypes for resistance to the white pine weevil using artificial infestations. *Forest Ecology and Management* 255: 1749-1758.

Butin E, Preisser E, Elkinton J. 2007. Factors affecting settlement rate of the hemlock woolly adelgid, *Adelges tsugae*, on eastern hemlock, *Tsuga canadensis*. *Agricultural and Forest Entomology* 9: 215-219.

Ford, C R, Elliott K J, Clinton B D, Kloeppel B D, and Vose J M. 2012. Forest dynamics following eastern hemlock mortality in the southern Appalachians. Oikos 121.4: 523-536.

Halley J D, Burd M, Wells P. 2005. Excavation and architecture of Argentine ant nests. *Insect Soc.* 52:350–356.

Havill N P and Foottit R G. 2007. Biology and Evolution of Adelgidae. *Annual Review of Entomology* 52: 325-349.

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

Hodge G R, Dvorak W S. 2007. Variation in pitch canker resistance among provenances of *Pinus patula* and *Pinus tecunumanii* from Mexico and Central America. *New Forests* 33: 193-206.

Ingwell, L, Miller-Pierce M, Trotter R T III, and Preisser E L. 2012. Vegetation and Invertebrate Community Response to Eastern Hemlock Decline in Southern New England. *Northeastern Naturalist* 19.4 : 541-58.

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

Jenkins, J N, Parrott W L, McCarty Jr. J C, White W H. 1982. Breeding cotton for resistance to tobacco budworm: Techniques for uniform field infestation. *Crop Science* 22: 400-404.

Jetton R M, Mayfield A E, Powers Z L. Development of a Rain Down Technique to Artificially Infest Hemlocks with the Hemlock Woolly Adelgid (Hemiptera: Adelgidae). In press.

Kesavaraju B and Dickson S. 2012. New Technique to Count Mosquito Adults: Using ImageJ Software to Estimate Number of Mosquito Adults in a Trap. *Journal of the American Mosquito Control Association* 28.4:330-333.

Knoepp, J D, Vose J M, Clinton B D, Hunter M D. 2011. Hemlock Infestation and Mortality: Impacts on Nutrient Pools and Cycling in Appalachian Forests. *Soil Science Society of America Journal* 75.5: 1935-1945.

Mains, J W, Mercer D R, Dobson S L. 2008. Digital Image Analysis to Estimate Numbers of Aedes Eggs Oviposited in Containers. *American Mosquito Control Association* 24.4: 496–501.

McClure M S. 1987. Biology and Control of Hemlock Woolly Adelgid. *Bulletin of the Connecticut Agricultural Station* 851: 1-9.

McClure, M S. 1989. Evidence of a Polymorphic Life Cycle in the Hemlock Woolly Adelgid, Adelges Tsugae (Homoptera: Adelgidae). *Annals of the Entomological Society of America* 82.1: 50-54.

McClure, M S. 1990. Role of Wind, Birds, Deer, and Humans in the Dispersal of Hemlock Woolly Adelgid (Homoptera: Adelgidae). *Environmental Entomology* 19.1:36-43.

McClure, M S. 1991. Density-Dependent Feedback and Population Cycles in *Adelges Tsugae* (Homoptera: Adelgidae) on *Tsuga Canadensis*. *Environmental Entomology* 20.1: 258-264.

McClure, M S. 2007. Hemlock woolly adelgid, Adelges tsugae (Annand). *The Connecticut Agricultural Experiment Station*.

Montgomery M E, Bentz S E, Olsen R T. 2009. Evaluation of hemlock (*Tsuga*) species and hybrids for resistance to *Adelges tsugae* (Hemiptera: Adelgidae) using artificial infestation. *Journal of Economic Entomology* 102: 1247-1254.

Newton L, Frampton J, Monahan J, Goldfarb B, Hain F. 2011. Two novel techniques to screen *Abies* seedlings for resistance to the balsam woolly adelgid, *Adelges piceae*. *Journal of Insect Science* 11:158. Available online: http://www.insectscience.org/11.158.

Ross, R M, Bennett R M, Snyder C D, Young J A, Smith D R, Lemarie D P. 2003. Influence of eastern hemlock (Tsuga canadensis L.) on fish community structure and function in headwater streams of the Delaware River basin. *Ecology of Freshwater Fish* 12.1: 60-65.

Rowell, T J and Sobczak W V. 2008. Will Stream Periphyton Respond to Increases in Light Following Forecasted Regional Hemlock Mortality? *Journal of Freshwater Ecology* 23.1: 33-40.

Smith, F F, Webb R E, Boswell A L, Combs Jr. G F. 1970. A circular rotating cage for obtaining uniform oviposition by Liriomyza munda in exposed plants. *Journal of Economic Entomology* 63.2: 655-656.

Teale, S A, Letkowski S, Matusick G, Stehman S V, Castello J D. 2009. Quantitative, Nondestructive Assessment of Beech Scale (Hemiptera: Cryptococcidae) Density Using Digital Image Analysis of Wax Masses. *Environmental Entomology* 38(4): 1235-1240.

Tingley, MW, Orwig D A, Field R, Motzkin G. 2002. Avian response to removal of a forest dominant: consequences of hemlock woolly adelgid infestations. *Journal of Biogeography* 29.10-11: 1505-1516.

Toews M D, Pearson T C, Campbell J F. 2006. Imaging and automated detection of *Sitophilus oryzae* (Coleoptera: Curculionidae) pupae in hard red winter wheat. *Journal of Economic Entomology* 99:583–592.

USDA Forest Service 2012. Counties with established *A. tsugae* populations 2012. Distribution map available online at http://na.fs.fed.us/fhp/hwa/maps/2012.pdf.

Vose J M, Wear D N, Mayfield A E III. 2013. Hemlock woolly adelgid in the southern Appalachians: Control strategies, ecological impacts, and potential management responses. *Forest Ecology and Management* 291: 209-219.

Table 1. Linear regression parameter estimates to predict crawler density per cm² on Tanglefoot® papers based on ImageJ area measurements.

Variable	DF	Parameter Estimate	Standard Error	t	P
Stationary					
Intercept	1	-0.08	0.45	-0.18	0.8558
area	1	985.26	41.71	23.62	<0.0001
Rotating					
Intercept	1	0.73	0.28	2.56	0.0127
area	1	683.74	44.41	15.39	<0.0001

Table 2. Analysis of variance for regression of the crawler density per cm² on ImageJ area measurements for stationary and rotating branch Tanglefoot® paper.

Source	DF	F	P
Stationary			
Model	1	558.07	<0.0001
Error	73		
Corrected Total	74		
Rotating			
Model	1	237.00	<0.0001
Error	65		
Corrected Total	66		

Figure 1. Stationary (right) and rotating (left) suspended branches. Rotating sususpended branches were suspended above ground from a wooden frame attached to a rotating motor.

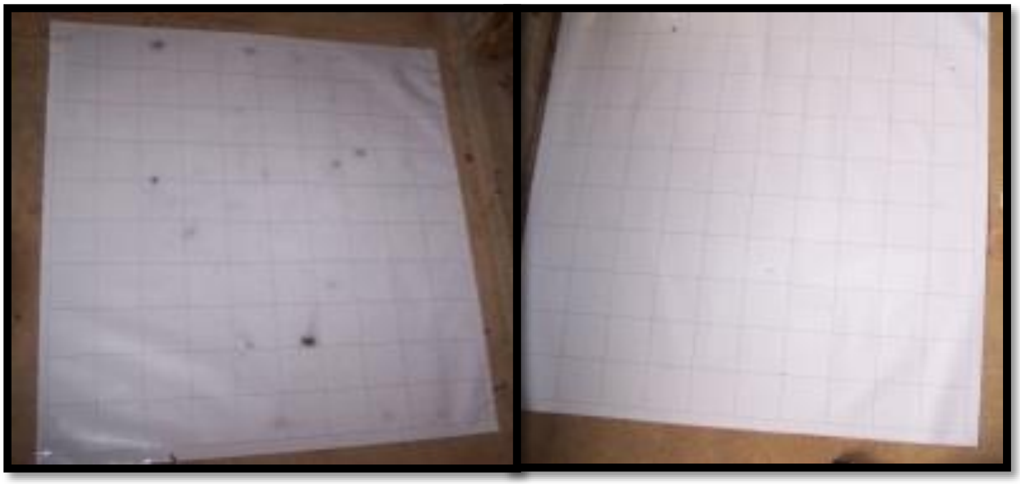


Figure 2. Stationary (right) and rotating (left) suspended branch Tanglefoot ® paper. Hot spots are visible on the stationary sheet where crawlers clustered below the branches. A faint ring pattern is present on the rotating sheet.

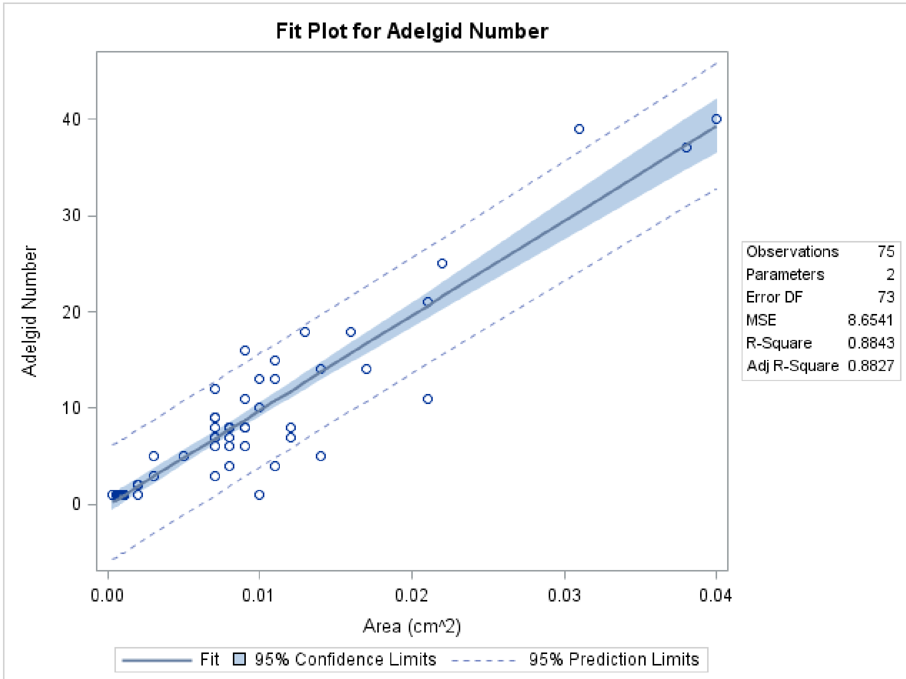


Figure 3. Stationary suspended branch linear regression fit plot. Confidence limits, prediction limits, and subsampled data points are illustrated.

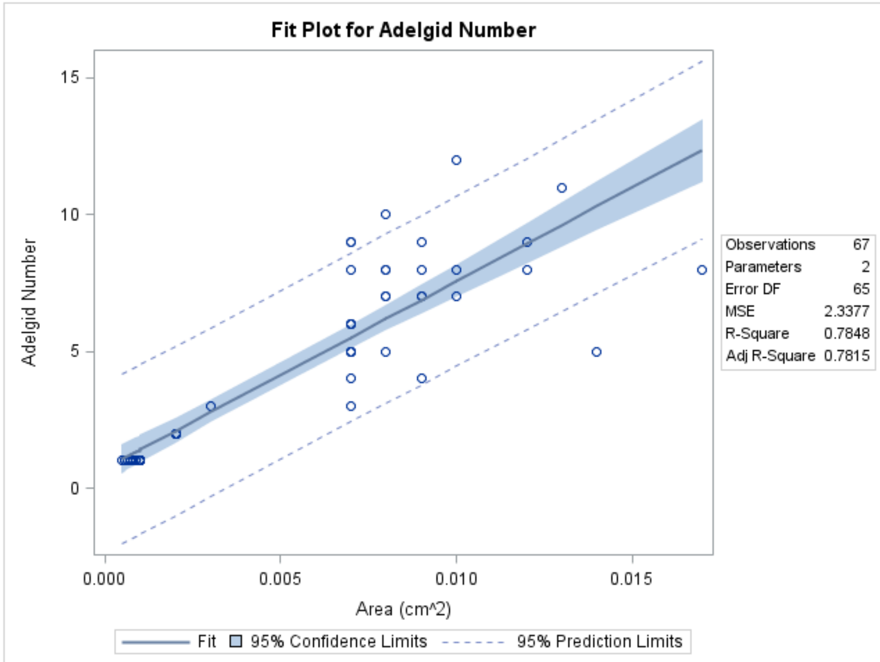


Figure 4. Rotating suspended branch linear regression fit plot. Confidence limits, prediction limits, and subsampled data points are illustrated.

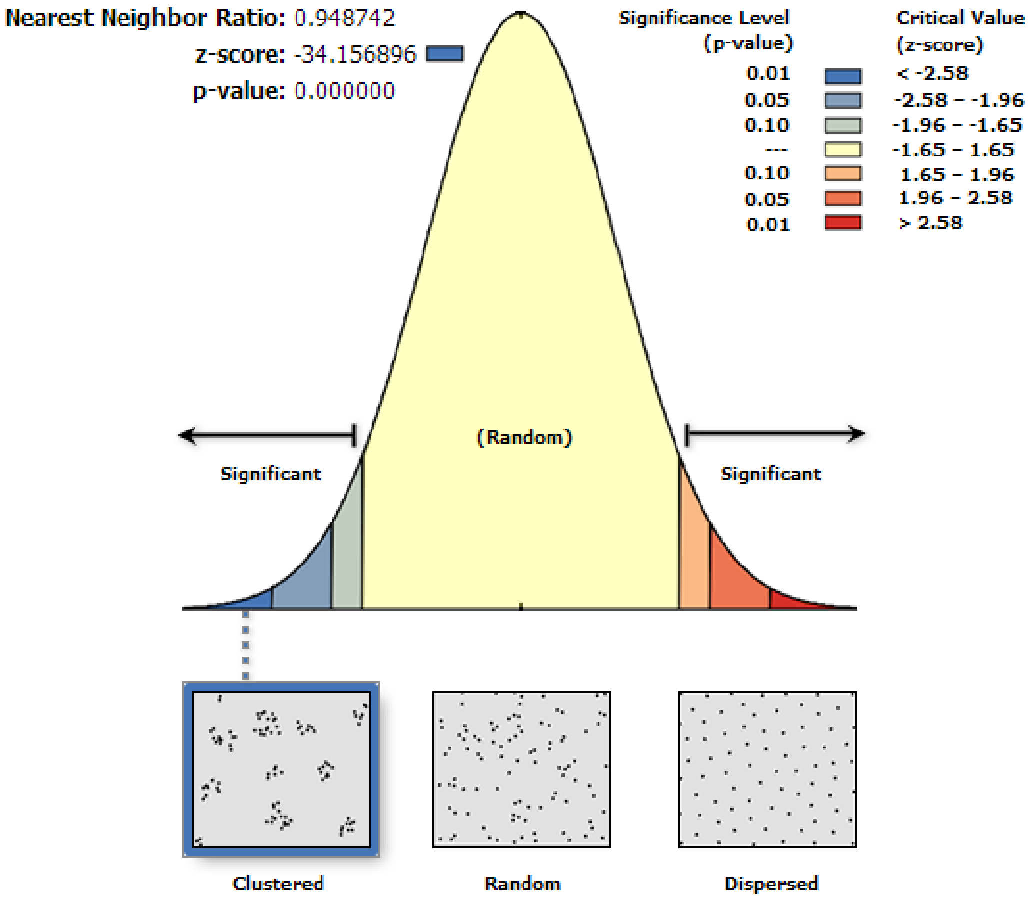


Figure 5. Average Nearest Neighbor output of progrediens crawler distribution for stationary suspended branch plot 1A.

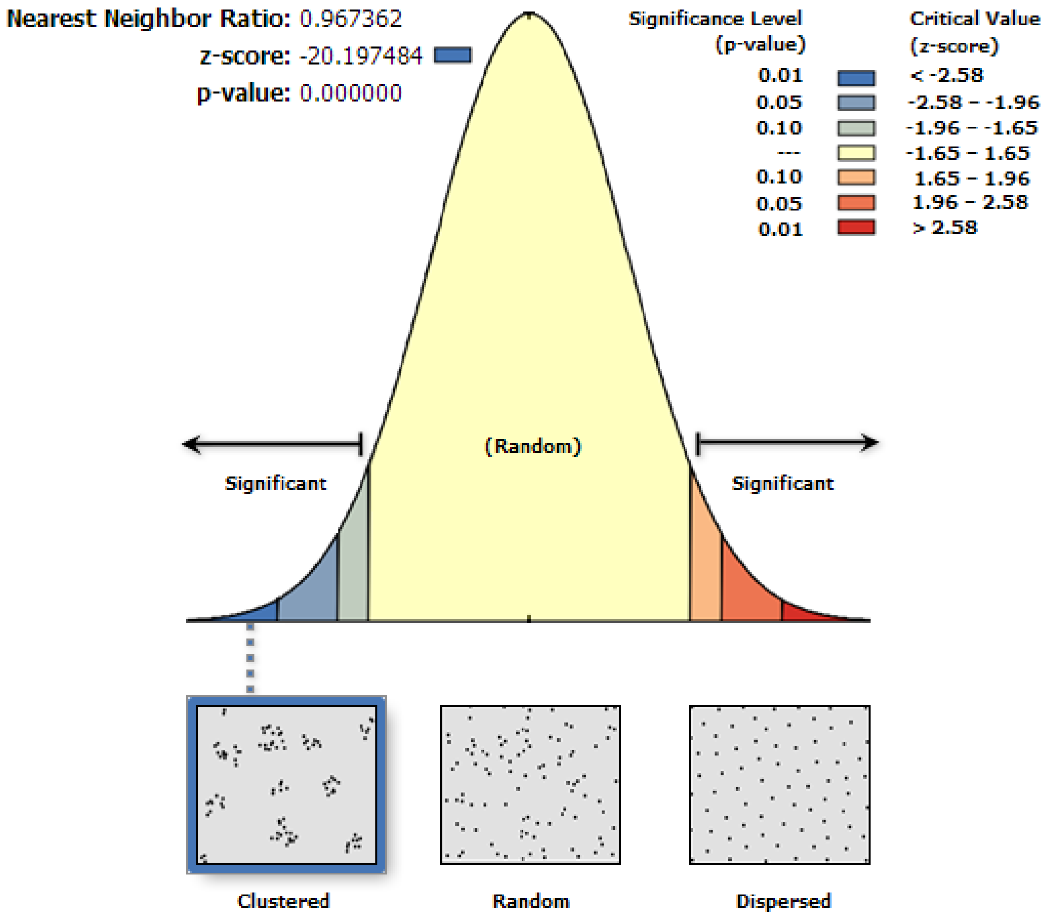


Figure 6. Average Nearest Neighbor output of progrediens crawler distribution for stationary suspended branch plot 2A.

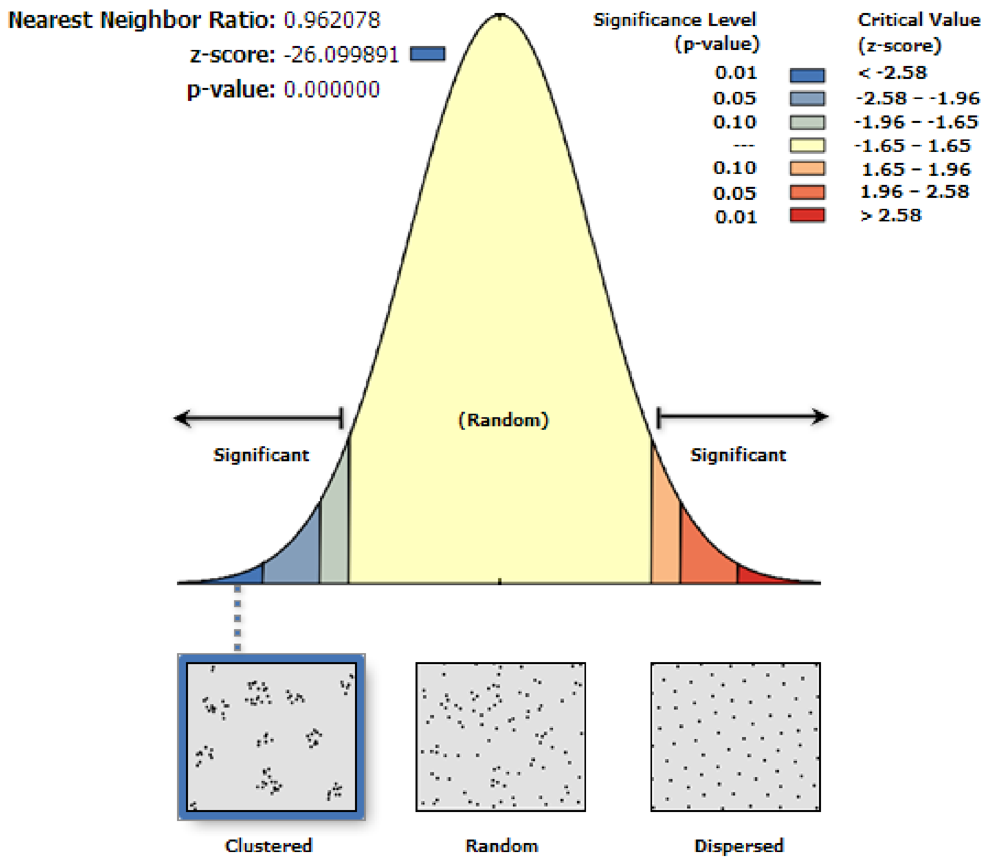


Figure 7. Average Nearest Neighbor output of progrediens crawler distribution for stationary suspended branch plot 3A.

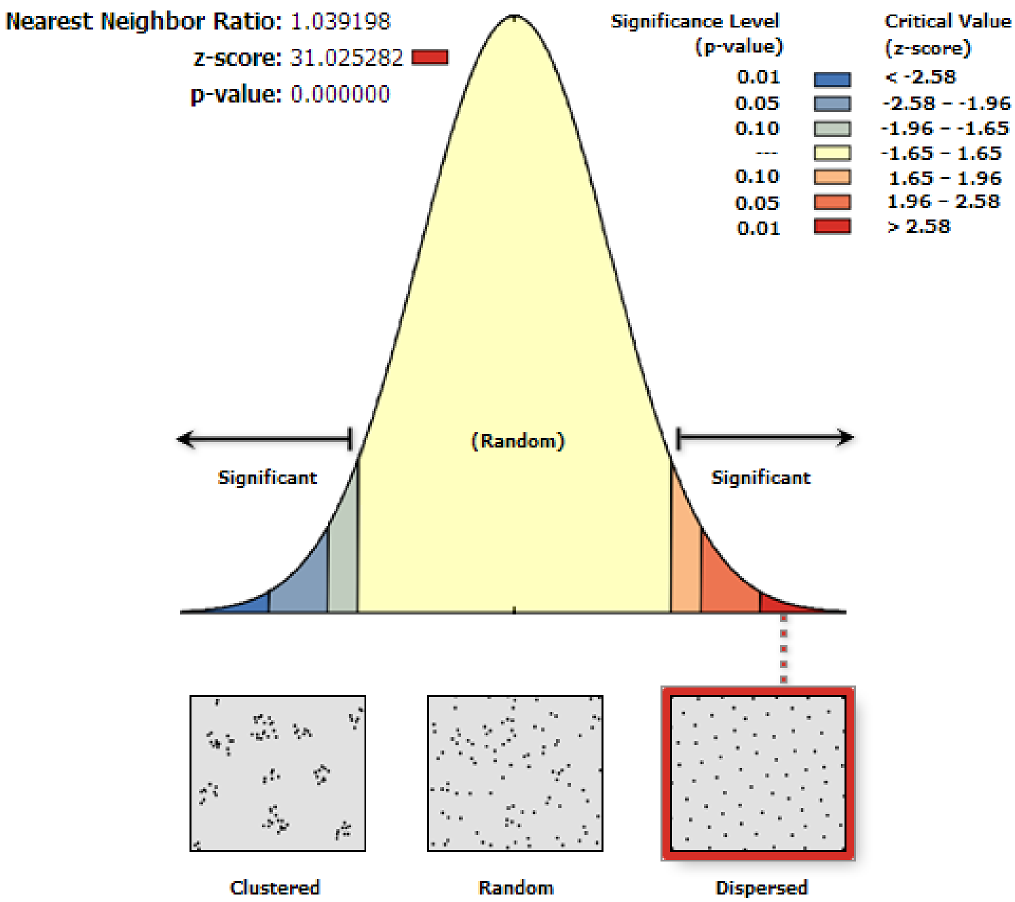


Figure 8. Average Nearest Neighbor output of progrediens crawler distribution for rotating suspended branch plot 1B.

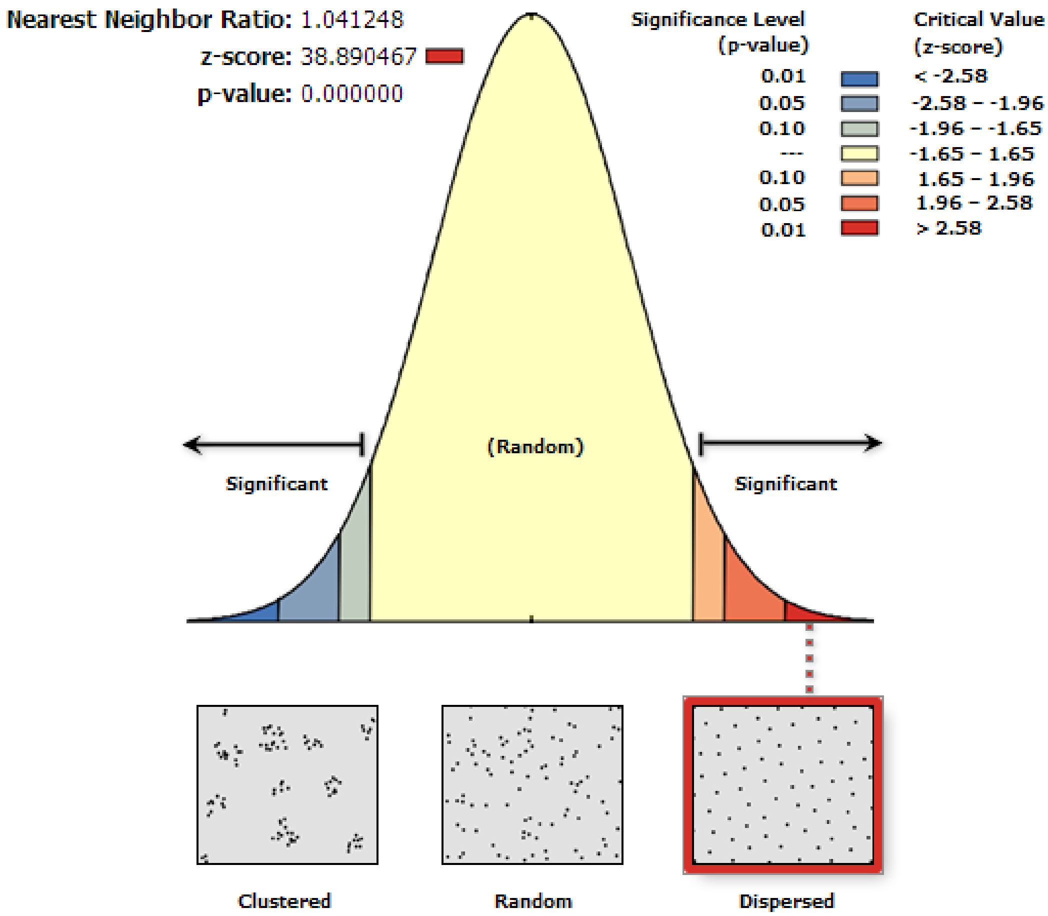


Figure 9. Average Nearest Neighbor output of progrediens crawler distribution for rotating suspended branch plot 2B.

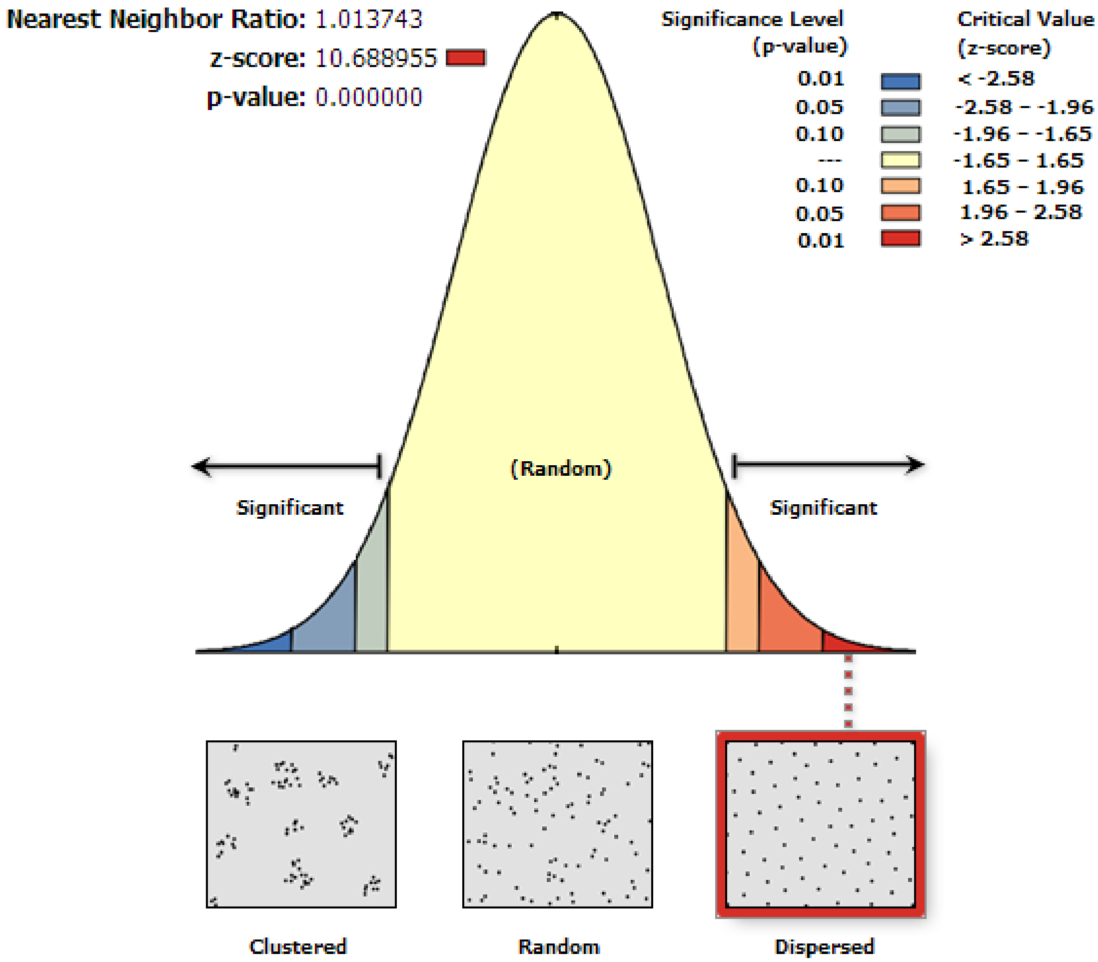
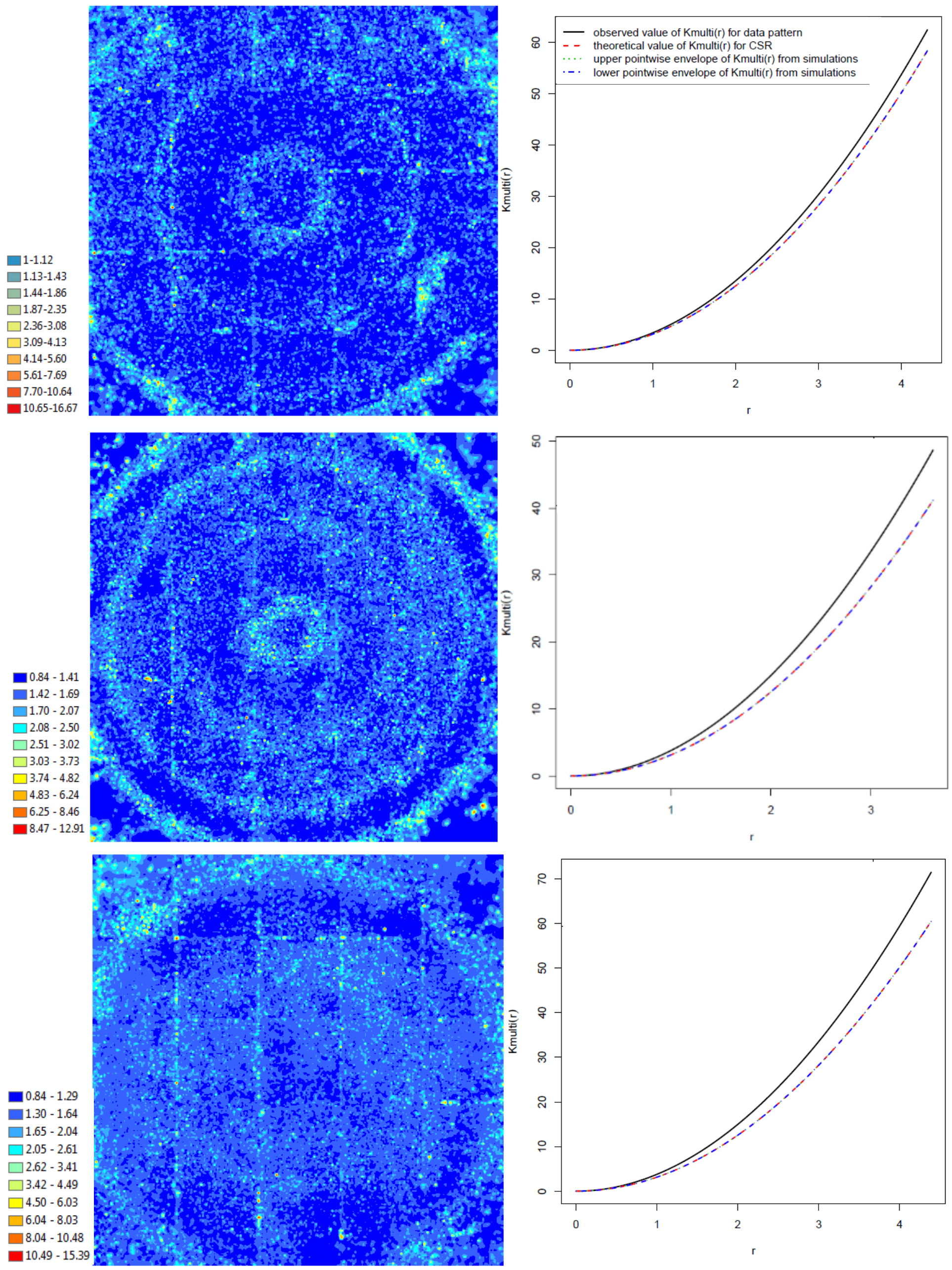


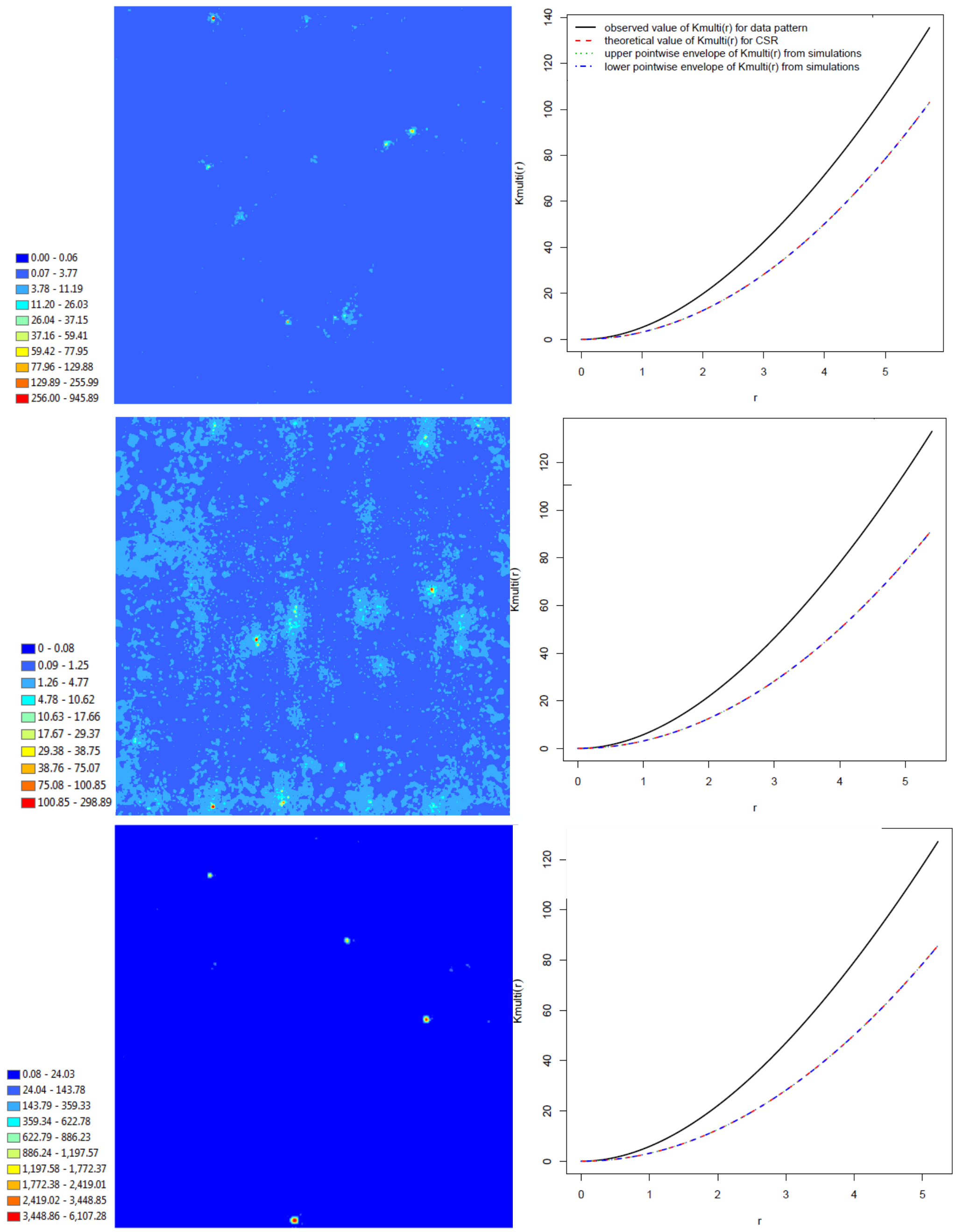
Figure 10. Average Nearest Neighbor output of progrediens crawler distribution for rotating suspended branch plot 3B.

Figure 11. Ripley's K results for rotating suspended branch plots 1B (top left), 2B (middle left) and 3B (bottom left) paired with density maps of crawlers on the 1m² sheets for 1B (top right), 2B (middle right), and 3B (bottom right). The graphs illustrate a small but significant degree of clustering. The graph scales were automatically generated by R.



1-1.12
1.13-1.43
1.44-1.86
1.87-2.35
2.36-3.08
3.09-4.13
4.14-5.60
5.61-7.69
7.70-10.64
10.65-16.67
observed value of Kmulti(r) for data pattern
theoretical value of Kmulti(r) for CSR
upper pointwise envelope of Kmulti(r) from simulations
lower pointwise envelope of Kmulti(r) from simulations
Kmulti(r)
r
0.84 - 1.41
1.42 - 1.69
1.70 - 2.07
2.08 - 2.50
2.51 - 3.02
3.03 - 3.73
3.74 - 4.82
4.83 - 6.24
6.25 - 8.46
8.47 - 12.91
Kmulti(r)
r
0.84 - 1.29
1.30 - 1.64
1.65 - 2.04
2.05 - 2.61
2.62 - 3.41
3.42 - 4.49
4.50 - 6.03
6.04 - 8.03
8.04 - 10.48
10.49 - 15.39
Kmulti(r)
r

Figure 12. Ripley's K results for stationary suspended branch plots 1A (top left), 2A (middle left) and 3A (bottom left) paired with density maps of crawlers on the 1m² sheets for 1A (top right), 2A (middle right), and 3A (bottom right). The graphs illustrate a large degree of significant clustering. The graph scales were automatically generated by R.


0.00 - 0.06
0.07 - 3.77
3.78 - 11.19
11.20 - 26.03
26.04 - 37.15
37.16 - 59.41
59.42 - 77.95
77.96 - 129.88
129.89 - 255.99
256.00 - 945.89
observed value of Kmulti(r) for data pattern
theoretical value of Kmulti(r) for CSR
upper pointwise envelope of Kmulti(r) from simulations
lower pointwise envelope of Kmulti(r) from simulations
Kmulti(r)
r
0 - 0.08
0.09 - 1.25
1.26 - 4.77
4.78 - 10.62
10.63 - 17.66
17.67 - 29.37
29.38 - 38.75
38.76 - 75.07
75.08 - 100.85
100.85 - 298.89
Kmulti(r)
r
0.08 - 24.03
24.04 - 143.78
143.79 - 359.33
359.34 - 622.78
622.79 - 886.23
886.24 - 1,197.57
1,197.58 - 1,772.37
1,772.38 - 2,419.01
2,419.02 - 3,448.85
3,448.86 - 6,107.28
Kmulti(r)
r

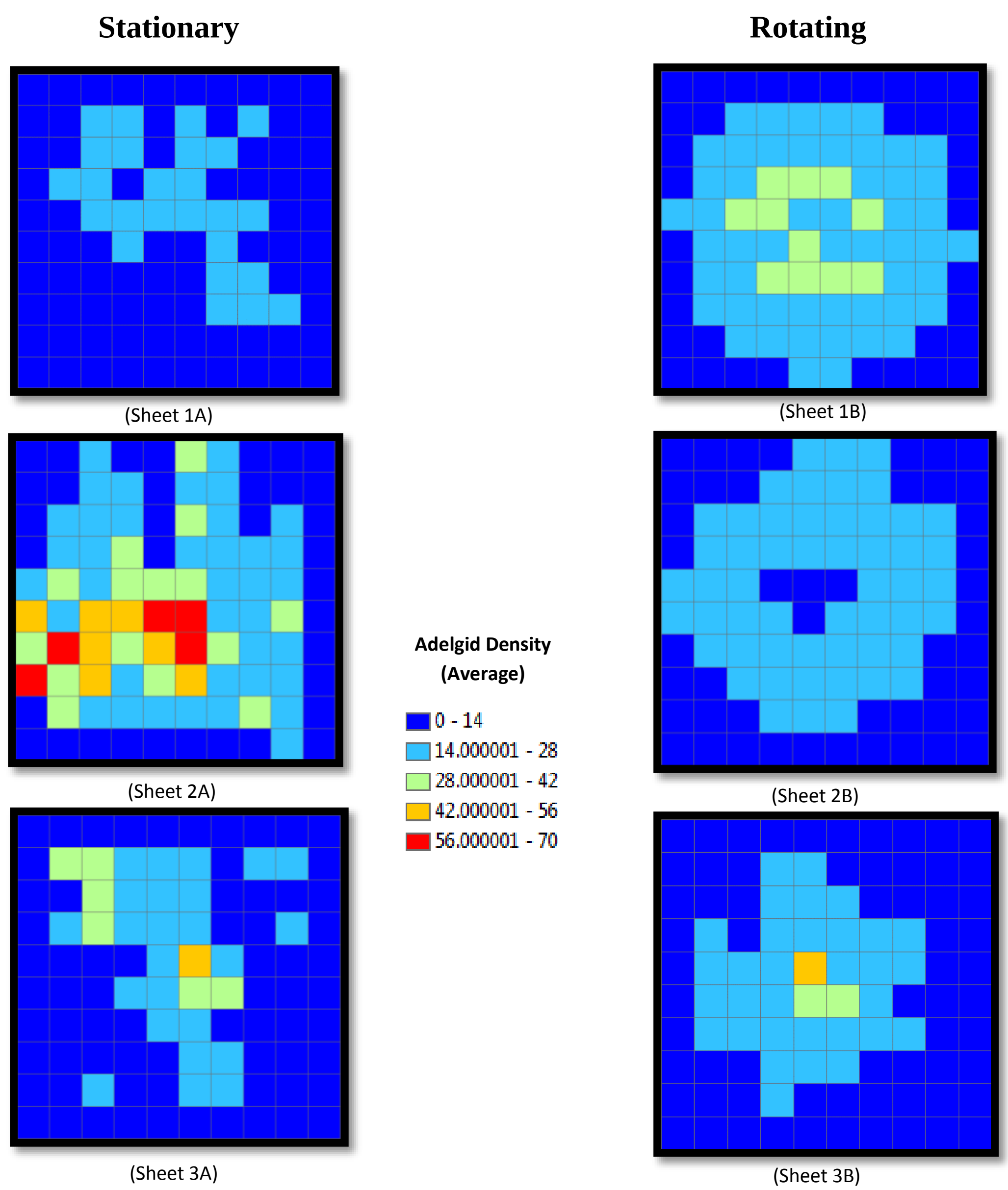


Figure 13. Spatial distribution (average density on m² sheets) of sistens crawlers shed onto Tanglefoot® sheets in May 2012 for three stationary (right) and three rotating (left) plots of suspended infested branch treatments.

CHAPTER IV
Observations on the Emergence of Hemlock Woolly Adelgid Progrediens Crawlers

ABSTRACT

Eastern (*Tsuga canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock populations in eastern North America are devastated by the invasive hemlock woolly adelgid (HWA, *Adelges tsugae* Annand). The purpose of this experiment was to determine the number of days required for peak crawler emergence from infested branches, the number of days required for complete crawler emergence from the peak, and the influence of hydration on the density of emerging crawlers. Adelgid density was analyzed for small scale stationary suspended branch infestations with the progrediens generation. The experiment was initiated near the beginning of a population emergence. It was not known if the population had peaked earlier in year on the sample trees. A peak in adelgid emergence was accomplished by day 5. Crawler emergence decreased during the next couple days, supporting the fact that a peak occurred. On day 10 the number of dispersing crawlers began to increase again, indicating a second peak. Hydrated branches had significantly fewer progrediens crawlers disperse than non-hydrated branches, with an average of 596 ± 65.1059 and 1,102.4 ± 158.7104 crawlers, respectively. Branches should be suspended for at least 5 days with non-hydrated infested branches for effective progrediens infestations.

INTRODUCTION

The hemlock woolly adelgid (HWA, *Adelges tsugae* Annand) is an insect native to Japan. The adelgid was brought to North America on southern Japanese hemlock (*Tsuga sieboldii* Carr.) nursery stock (Havill et al. 2006). In eastern North America the adelgid has become an invasive species and feeds on eastern (*T. canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock (Havill et al. 2006; Havill et al. 2007). The infestation began in Virginia and has spread north and south to 19 states (USDA Forest Service 2012). Since the insect was first documented on the east coast in the 1950s it has caused significant changes in hemlock ecosystems (McClure 1987; Havill et al. 2006; Havill et al. 2007).

HWA is transported passively by wind, birds, deer, and humans (McClure 1990). Population density of the adelgid is influenced by environmental conditions after crawler emergence. Initial infestations take place on healthy hemlock where the adelgid population density peaks in the first year. The decline in hemlock health leads to less new growth on the tree and a decrease in HWA population in the second year. Adelgid fecundity and survival declines when the insect has to feed on old growth. During the second year the sexupara population, the sexual stage of HWA, increases and disperses to other trees while the adelgid population on the tree decreases. Hemlock health improves in the third year due to lower HWA density. The adelgid population on the tree peaks again after the production of new growth and tree mortality can occur in as few as four years (McClure 1991).

Forest infestations may be best managed with biological controls and deploying resistant planting stock. The general lack of tolerance or resistance in eastern North American hemlock was likely influenced by the dominance of leaf defoliators and the absence of native adelgids and scale insects in the evolution of the hemlock. Selective pressure to prevent feeding by leaf defoliators may have significantly reduced or eliminated genes for deterring adelgid feeding (Montgomery and Lagalante 2008). The search for resistant eastern and Carolina hemlock is necessary to determine if natural resistance is present in the two species today. Some hemlock species exhibit tolerance or resistance to HWA feeding, including Southern Japanese hemlock, Chinese hemlock (*T. chinensis* (Franch.) E. Pritz.), hybrids of Chinese and Carolina hemlock, western hemlock (*T. heterophylla* (Raf.) Sarg.), and potentially Carolina hemlock (Montgomery et al. 2009; Havill et al. 2006; Oten 2011). Terpenoids play a role in plant insect interactions. With regard to adelgids, α-humulene and isobornyl acetate are likely involved in tree resistance and susceptibility, respectively (Montgomery and Lagalante 2008).

Tolerance or resistance testing for HWA involves introducing the progrediens or sistens generation to non-infested hemlock and allowing the crawlers to naturally disperse on the tree. Direct infestations and suspended branch infestations produce successful adelgid populations on hemlock. The time required for dispersal of crawlers to peak and to completely disperse from infested branches during the peak has not been thoroughly tested with regard to artificial infestation. Additionally, the use of hydrated and non-hydrated branches has also not been examined with regard to dispersal during artificial infestation (Butin et al. 2007; Jetton et al. in press). The first objective of this study was to determine the number of days required for peak progrediens crawler population emergence and the number of days required for complete population emergence from the peak. The second objective was to compare progrediens crawler emergence from infested branches with and without a water supply.

MATERIALS AND METHODS

Location and Experimental Design

Source Material-Infested Branches

Stationary suspended branch infestations were conducted in a small scale collection experiment using eggs of the progrediens generation. Infested branch material was collected with pole pruners from infested eastern hemlock in Asheville, North Carolina near the Forest Service Southern Research Station (35.611966,-82.562181) in February 2012. Branches 10 cm long were cut from the infested trees. The average number of ovisacs per branch and eggs per ovisac were estimated from fifty-two 4 cm branch samples. The 10 cm branches were immediately used for infestation.

Progrediens Dispersal (February 2012)

Research was conducted at North Carolina State University in Raleigh, NC (35.783948, -78.680387). *Celebrate It!* Michaels' cardboard boxes $10cm^3$ were used as small scale cubes for suspended branch infestation set up. The flaps on the top and bottom of the boxes were folded outward and two flaps from the top and the bottom were removed. The remaining top and bottom flaps were parallel to each other. A 25 cm^2 section of poultry wire was placed on top of each cube. The infestation took place in a temperature regulated building. The study had fifteen matched pairs. Each pair had one infested branch with a Panacea Products Corp. water tube (8.5 mL) and one infested branch without a water tube (Figure 1, Figure 2).

HWA infested branches were introduced February 21, 2012. The 10 cm eastern hemlock branches, including the main stem and side branches, were placed on the poultry wire with the bottom side of the needles facing toward the poultry wire. The branches were positioned at a diagonal from corner to corner. A 14 cm by 10 cm paper with 1 cm^2 grid lines was sprayed with Tanglefoot® and placed under the open bottom side of each cube. Needles were removed from the bottom of the branches in order to insert the stem into the water tube. Branches without water did not have a water tube. Water tubes were checked once a day and refilled if they were empty or less than ¼ full. There was an average of 15.83 ± 1.55 ovisacs per 4 cm branch and an average of 13.35 ± 0.86 eggs per ovisac on the infested branches.

Data Collection

Tanglefoot® paper was collected from the first five matched pairs every day for ten days beginning February 22, 2012, soon after collection of the material when the crawlers were expected to emerge, to document the daily emergence of crawlers. The papers were replaced every day with a new paper sprayed with Tanglefoot®. The Tanglefoot® paper was collected from the last ten matched pairs on day ten to determine the total emergence of crawlers. By day ten, March 2, 2012, a second peak had begun and data collection halted.

The density of progrediens crawlers on the Tanglefoot® paper was determined. Papers were cleared of needles and debris, scanned using the overhead scanner Zeutschel OS 12000 Bookcopy at the Natural Resources Library at North Carolina State University, and the images were saved as TIFF files. Next the TIFF images were viewed in Image J. Pictures were enhanced by 0.4%, converted to binary, and the total number of crawlers for each sheet was determined using the Cell Counter.

Statistical Analysis

For each Tanglefoot® sheet, the total number of crawlers emerging from hydrated and non-hydrated branches was counted. This data was analyzed using a paired t-test. The null hypothesis that there was no significant difference in the mean number of crawlers emerged from hydrated versus non-hydrated branches was tested. Data were analyzed using the TTEST procedure ($\alpha = 0.05$) of SAS Enterprise Guide 5.1 (SAS EG 5.1).

RESULTS

The data indicated that peak progrediens crawler emergence occurred on day 5 and emergence decreased after day 5 for both hydrated and non-hydrated branches. The results indicated a potential second peak beginning on day 10. The peak density on day 5 was 12 times the initial density on day 1 and 1.5-2.5 times the final count on day 10 (Figure 3). The density of crawlers emerging from hydrated branches was significantly different from the density of crawlers emerging from non-hydrated branches ($t=3.47$, $p=0.0070$, $df = 9$). Approximately twice the number of crawlers dispersed from non-hydrated branches than hydrated branches (Figure 4).

DISCUSSION

The results indicated that progrediens crawlers required at least 5 days for peak emergence, with a decrease in emergence beyond day 5. The peak in adelgid density appears to be followed by future peaks. HWA emergence began to increase slightly for branches with water on days 9 and 10 (Figure 1). Infested branches should remain above seedlings at least 5 days to allow for maximum emergence from one peak. Previous literature indicates that a large percentage of the emergence takes place in 16 days, and infested branches should be collected about 1 week prior to crawler emergence for the most effective infestation (Butin et al. 2007). The study by Butin et al. (2007) was conducted with hydrated infested branches and the effectiveness of adelgid emergence from branches cut 1 week before emergence may differ from non-hydrated infested branches. During this experiment the number of days before emergence when the branches were cut was not documented. Branches were collected at the time of year when the adelgid was known to start emergence. Future infestations should incorporate the data from this experiment along with previous knowledge regarding infested branch collection, and should explore the best time to collect branches for the most effective adelgid infestations with non-hydrated branches.

The density of crawlers emerging from non-hydrated branches was significantly greater than hydrated branches. The results indicated that future infested branches should not be hydrated. HWA is initially attracted to healthy hemlock and preferentially feeds on new growth. Adelgid population growth is hindered when the tree health declines and the production of new growth is slowed or halted (McClure 1991). The non-hydrated branches were less healthy than hydrated branches by day 10. Needles were browning and fell onto the sticky sheets (Figure 1). Hydrated branches continued to have green needles by day 10 (Figure 1). There may have been more dispersal from non-hydrated branches due to the crawlers search for healthy material while the crawlers on the hydrated branches were not pressured to search for other healthy material. The number of crawlers that remained on the branches after 10 days was not documented. Future experiments should record the number of crawlers that remain on the branches to determine the percent crawlers that dispersed.

The experiment utilized infested branches with 13.35 ± 0.86 eggs per ovisac. Previous studies have noted 50 to125 eggs per ovisac (Montgomery et al. 2009) and 45.7 ± 5.8 eggs per ovisac (Jetton et al. in press) on infested branches. The lower numbers of eggs per ovisac could be due to the timing of collection. Branches were collected in February when the total amount of eggs may not have been deposited. The study was utilized to determine the timing of emergence for infestations in March of the same year and an early analysis was necessary. Future examinations of the timing of crawler emergence from non-hydrated and hydrated branches should take place when maximum egg population density is present.

Extrapolating from the results, a total of approximately 441 and 238 crawlers dispersed per 4 cm^2 Tanglefoot® paper from non-hydrated branches and hydrated branches, respectively. The values were greater than the crawler density for the high ovisac treatment utilized by Jetton et al. (in press) in an analysis of the density of crawlers distributing from suspended infested branches over a 21 day period. Despite the low egg densities in the infested material, the number of crawlers dispersing from the small scale suspended branch experiment was greater than previous studies with the suspended branch technique.

The health of the crawlers from non-hydrated branches requires further research. HWA infestations to test for tolerance or resistance require healthy and reliable infestations. Fecundity and survival is negatively influenced by old growth in declining trees (McClure, 1991). The health of the crawlers may have been reduced as branch health declined during the 10 day experiment.

The results will improve the technique for artificial infestations with HWA. The experiment provides a timeline that may be used as a reference in large scale infestations. Previous studies have infested seedlings at different times of adelgid emergence (Jetton et al. in press; Butin et al. 2007; Montgomery et al. 2009). Jetton et al. (in press) left infested branches above seedlings for 21 days. Other studies utilized infested branches for 16 days before removing from hemlock trees (Butin et al. 2007). The results from this study indicate that at least 5 days are required for utilizing a peak in crawler emergence. The process for infesting mass numbers of seedlings may be quickened now that the emergence process has been documented.

The experiment was a small scale infestation above Tanglefoot ® paper. The number of crawlers emerging from the branches may not correlate directly to the number of crawlers infesting branches. Additional experiments should examine the suspended branch techniques at full scale above seedlings and determine the level of settled crawlers as well as the health and survival of the settled adelgid population. A study by Jetton et al. (in press) documented adelgid settlement on hemlock seedlings, but did not exhibit conditions required for mass infestations where many seedlings are placed below infested branches.

CONCLUSION

This study explored aspects of adelgid dispersal that had not been reported in previous literature. The small scale experiment indicated that suspended branch infestations should be conducted over at least a 5 day period with non-hydrated branches. Future research should focus on the health of adelgid populations after dispersal from non-hydrated branches onto seedlings. If adelgid population survival is severely hindered by the decline in branch health, the method of infestation will not be adequate for tolerance/resistance testing.

ACKNOWLEDGMENTS

We thank Fred Hain at North Carolina State University for providing laboratory space. This work is funded by USDA Forest Service Southern Research Station Cooperative Agreement No. 11-CA-11330129-064.

REFERENCES

Butin E, Preisser E, Elkinton J. 2007. Factors affecting settlement rate of the hemlock woolly adelgid, *Adelges tsugae*, on eastern hemlock, *Tsuga canadensis. Agricultural and Forest Entomology* 9: 215-219.

Havill N P and Foottit R G. 2007. Biology and Evolution of Adelgidae. *Annual Review of Entomology* 52: 325-349.

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

Jetton R M, Mayfield A E, Powers Z L. Development of a Rain Down Technique to Artificially Infest Hemlocks with the Hemlock Woolly Adelgid (Hemiptera: Adelgidae). In press.

McClure M S. 1987. Biology and Control of Hemlock Woolly Adelgid. *Bulletin of the Connecticut Agricultural Station* 851: 1-9.

McClure, M S. 1991. Density-Dependent Feedback and Population Cycles in *Adelges Tsugae* (Homoptera: Adelgidae) on *Tsuga Canadensis*. *Environmental Entomology* 20.1: 258-264.

McClure, M S. 1990. Role of Wind, Birds, Deer, and Humans in the Dispersal of Hemlock Woolly Adelgid (Homoptera: Adelgidae). *Environmental Entomology* 19.1: 36-43.

Montgomery M E, Bentz S E, Olsen R T. 2009. Evaluation of hemlock (*Tsuga*) species and hybrids for resistance to *Adelges tsugae* (Hemiptera: Adelgidae) using artificial infestation. *Journal of Economic Entomology* 102: 1247-1254.

Montgomery, M E, and Lagalante A F. 2008. The Role of Volatile Terpenoids in the Relationship of the Hemlock Woolly Adelgid and its Host Plants. *Fourth Symposium on Hemlock Woolly Adelgid in the Eastern United States*. 118-123.

Oten K. 2011. *Host-plant selection by the hemlock woolly adelgid, Adelges tsugae Annand: sensory systems and feeding behavior in relation to physical and chemical host-plant characteristics*. Ph.D. Thesis, Department of Entomology, North Carolina State University, Raleigh, NC, USA.

USDA Forest Service 2012. Counties with established *A. tsugae* populations 2012. Distribution map available online at http://na.fs.fed.us/fhp/hwa/maps/2012.pdf.

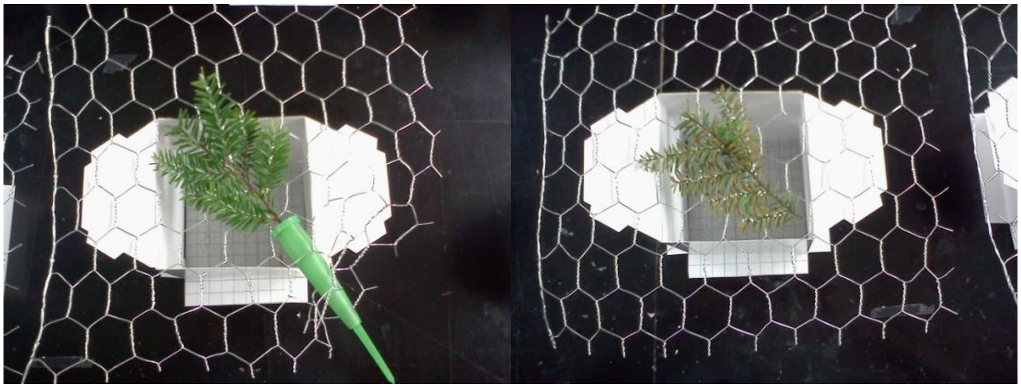


Figure 1. One pair of branches in the small scale stationary suspended branch study. One infested branch was hydrated (left) and one infested branch was not hydrated (right). Hydrated branch needles remained green throughout the study while non-hydrated branches experienced browning and needle loss.

Figure 2. The small scale stationary suspended branch infestation took place in a lab at room temperature. Each matched pair had one hydrated branch and one non-hydrated branch.

Average Hemlock Woolly Adelgid Dispersal per Day for 10 Days from Infested Branches with and Without Hydrated Branches

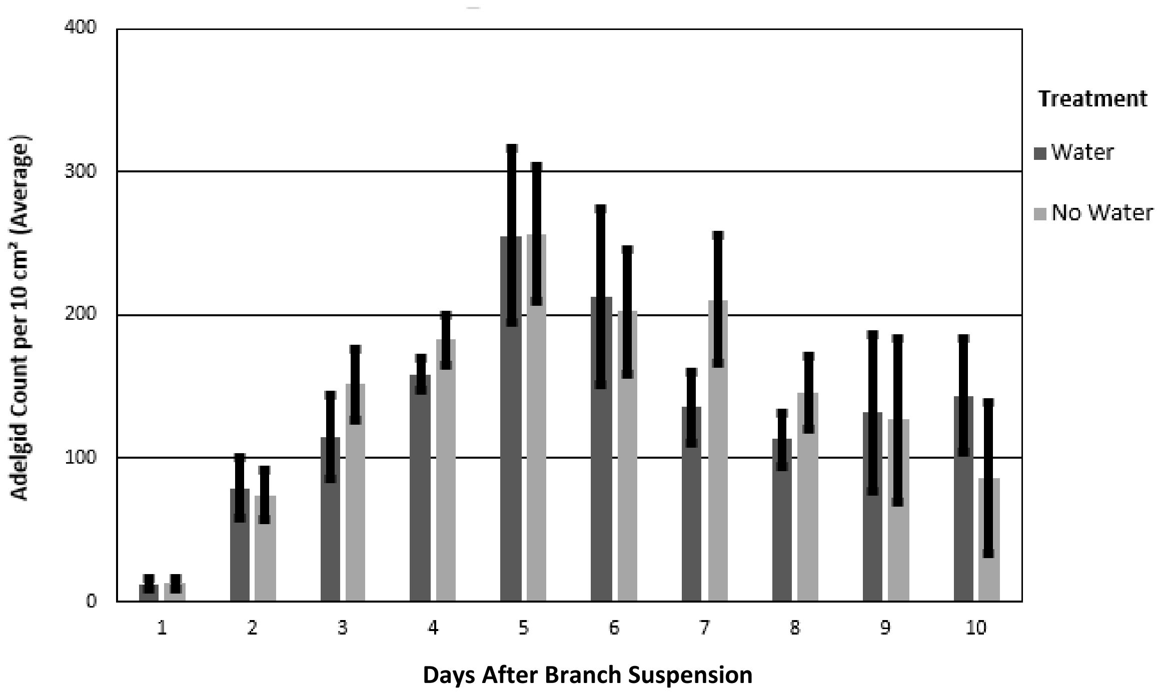


Figure 3. Average (SE) daily dispersal of crawlers from hydrated and non-hydrated infested eastern hemlock branches for a 10 day period.

Average Hemlock Woolly Adelgid Dispersal from Eastern Hemlock Infested Branches With and Without Hydrated Branches

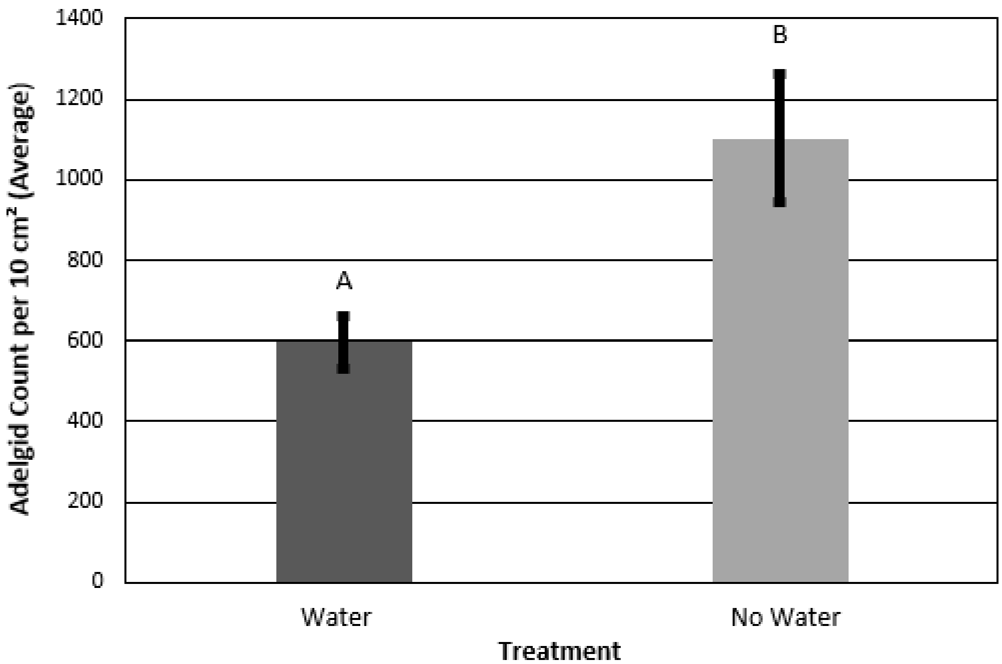


Figure 4. Average (SE) crawler density dispersing from hydrated and non-hydrated branches after 10 days. Letters indicate significance levels according to a paired t-test (α=0.05).

THESIS CONCLUSIONS

The hemlock woolly adelgid (HWA, *Adelges tsugae* Annand) has devastated hemlock forests in eastern North America causing decline in ecologically important eastern (*Tsuga canadensis* (L.) Carr.) and Carolina (*T. caroliniana* Engelm.) hemlock populations and dramatic changes in ecosystems. Biological controls and resistance breeding have become feasible methods for reducing and preventing hemlock decline in the forest setting. The results presented in the preceding chapters provide important improvements to the current methods for evaluating resistance that are necessary for evaluating mass numbers of seedlings efficiently and effectively. The study presented in Chapter II compared the effectiveness of a commonly used direct infestation technique with the new suspended branch technique. Infestations that took place in 2012 reiterated a common theme in previous literature where the infestations in March produced greater crawler densities than the infestations in May. Future research should focus on the March generation. The March 2012 direct infestations produced greater settled crawler densities than the suspended branch infestations, contradicting results from the March 2013 infestations. The 2012 data was influenced by high heat, and environmental conditions that may have decreased HWA populations. The 2013 seedlings were placed under the shade of a deciduous canopy and greater crawler densities settled on suspended branch seedlings than the directly infested seedlings. Other than environmental conditions the crawler distribution and/or timing of peak crawler emergence from the branches may have also influenced the results. The results suggest that the March suspended branch infestations were most effective at producing large numbers of settled crawlers under shaded conditions on eastern hemlock, and the high direct infestation was equally effective for Carolina hemlock. The suspended branch technique may be less labor intense than the direct infestation technique and would be appropriate for mass testing procedures. These conditions should be considered in future seedling tests.

The 2013 experiment also revealed that eastern hemlock had greater densities of settled crawlers than Carolina hemlock. The study did not explore HWA fecundity due to absence of winter survival, and although eastern hemlock appears to be a more appropriate host due to large crawler settlement, Carolina hemlock populations have been observed with greater HWA fecundity than eastern and western hemlock. Future research should examine the fecundity on Carolina hemlock compared to eastern and western hemlock.

The study presented in Chapter III focused on improving the suspended branch infestation method with regard to progrediens and sistens dispersal, an important component of resistant testing. The stationary suspended branch method utilized in previous literature and in Chapter II is known to produce hot spots were adelgids were clustered below branches, a distribution pattern that could lead to false resistance assessment of susceptibility in tested seedlings. Rotating the suspended branches produced a more distributed and less clustered pattern of dispersing crawlers. May sistens distribution data could not be statistically analyzed but produced a similar pattern to March progrediens for both suspended branch techniques. The log density of crawlers emerging in March was similar for both studies and significantly greater for the rotating suspended branch technique in May. The significance of the density data may have differed for March and May due to the two different sampling techniques and unintended branch disruption. March rotating suspended branch infestations are recommended for seedling infestations to reduce clustering. The rotating suspended branch technique was not tested on seedlings during the study and should be analyzed for crawler settlement and adelgid fecundity before implementing in a large scale resistance testing program.

During the study a linear regression equation was developed in order to determine the number of crawlers in a given area. A large portion of variability was explained by the model. The equation may be used in the future for estimating the number crawlers in an area between 0.000162 cm^2 and 0.04 cm^2 for stationary suspended branch experiments and between 0.000162 cm^2 and 0.018 cm^2 for rotating suspended branch experiments.

The rotating suspended branch technique can be altered to improve infestations. Fewer adelgids dispersed to the corners of the sheet than the middle and center of the sheet, indicating that we should consider placing seedlings in a circular pattern. Additional research should examine seedling placement options. Throughout the experiment needle loss hindered or prevented data collection and analysis. The rotation of seedlings instead of branches may reduce needle loss and branch disruption while rotating suspended branches and changing the motor battery. Additionally, the data collection with Image J was slowed because of the presence of needles and gridlines, two factors that should be removed for faster processing. Dirt and dust could have hindered analysis, appearing as crawlers in the program, and future studies should avoid dusty areas. Lastly, the area of an adelgid can be affected by pressure which may flatten the crawler and future studies should avoid unnecessary pressure applied when collecting and storing the sheets.

The data presented in Chapter IV explored the timing of crawler emergence, part of the infestation process that had not been statistically analyzed previously, and has advanced the technique for selecting resistant phenotypes. The small scale study examined crawler dispersal from hydrated and non-hydrated branches. The results indicated a peak in the population emergence on day 5 for both hydrated and non-hydrated branches, followed by the beginning of a potential second peak on day 10. The data provided a time reference for future studies, quickening the infestation process. The number of days required for a peak in crawler emergence is much shorter than the number of days branches have been left above seedlings in previous studies, possibly due to the emergence of crawlers from branches at the start of the experiment. The small scale study also revealed that hydrated branches produced fewer dispersing adelgids than non-hydrated branches. The results indicated that non-hydrated branches were better suited for larger infestations.

The experiment may be improved by counting the number of crawlers remaining on the branches at the end of 10 days. This data was not documented and should be recorded to determine the number of crawlers that settled on the branch. Resistance testing requires healthy and reliable infestations and the health of the crawlers should be examined as well. The next step is to compare hydrated and non-hydrated branch infestation on seedlings and observe HWA settlement and fecundity.