Post-release establishment and supercooling point assessment of *Laricobius osakensis*, a predator of the hemlock woolly adelgid

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Post-release establishment and supercooling point assessment of *Laricobius osakensis*, a predator of the hemlock woolly adelgid

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Abstract (Academic)

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an invasive pest from Japan that is causing significant hemlock mortality in the eastern U.S. The most promising control method is biological control. Beetles in the genus *Laricobius* are adelgid specialists. In 2005, *Laricobius osakensis*, was discovered in Japan and in 2010 was approved for release in the eastern United States and there have now been more than 60 releases. In 2014, the polar vortex resulted in significant HWA mortality in the eastern U.S., depleting the food source for *L. osakensis*, which resulted in low field recoveries of them. In the 2015-2016 field season, there were 14 larvae recovered from the field, and the 2016-2017 field season yielded 90 larvae. A significant correlation was found between *Laricobius* beetles recovered and HWA density, between HWA density and plant hardiness zone, and a negative correlation between *Laricobius* beetles recovered and average tree health. Understanding more about the supercooling point of *L. osakensis* gave insight to its ability to survive subfreezing temperatures as occurred in 2014. Comparison of the supercooling point between the northern and southern populations of *L. osakensis*, and to the other released HWA predator, *L. nigrinus*, will allow us to determine which biological control agent is best suited for release in the coldest regions. The overall mean supercooling points of northern *L. osakensis* was -13.52 ºC, southern *L. osakensis* was -13.42 ºC, and *L. nigrinus* was -13.57 ºC. There were no significant differences between species or populations.

Post-release establishment and supercooling point assessment of *Laricobius osakensis*, a predator of the hemlock woolly adelgid

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Abstract (Public)

The hemlock woolly adelgid (HWA) is an invasive insect species from Asia that is the most important pest of eastern and Carolina hemlock trees in the eastern U. S. *Laricobius osakensis* is a small beetle from Japan that feeds only on HWA, and been released since 2012 in the eastern United States to control HWA populations. It is important to determine if *L. osakensis* populations were able to survive and spread in the eastern United States, and if the predator has an effect on HWA populations and the health of hemlock trees. In 2014, extreme cold temperatures in Virginia resulted in wide-scale death of HWA populations, depleting the food source for *L. osakensis.* Consequently, only 17 *L. osakensis* beetles were found on hemlock trees near release sites; however, the following year, 147 beetles were found. The ability to survive extreme cold temperatures is important for selecting a biological control agent for release in such regions. We can find out information about how tolerant a species is to cold temperatures by determining the supercooling point, the temperature at which it cannot stop itself from freezing. In this study we compared the supercooling points of a northern and southern population of *L. osakensis,* as well as another previously released and established biological control agent of HWA, *Laricobius nigrinus*. I found that there was no significant difference in supercooling point between the different types of *Laricobius* beetles suggesting that none of these species or populations appears to be anymore cold tolerant than the other for release in the colder regions of the U. S.

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Chapter 1: Literature Review

1.1 Biology and Damage of the Hemlock Woolly Adelgid

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is a small invasive insect native to Asia and the western United States, causing significant mortality to hemlock trees in the eastern United States (Havill et al., 2006). This insect was first found in Richmond, VA in the early 1950s (Souto et al., 1996). It is now established throughout more than half of the native range of the eastern hemlock, ranging from the mountainous region of the southeast to New England (Morin et al., 2011). The pest's distribution appears to be limited by cold northern climates or at high elevations. The species is spread by birds and other wildlife, wind, and the movement of nursery stock (Evans and Gregoire, 2007; McClure, 1990). *Adelges tsugae* is known to kill both Carolina hemlock (*Tsuga caroliniana* Engelmann) and eastern hemlock (*Tsuga canadensis* (L.) Carriere). Susceptibility of both species is due to a combination of a lack of both host resistance and native natural enemies (Cheah and McClure, 2000; McClure, 1989; Wallace and Hain, 2000).

The impact of HWA on hemlock forests in the eastern U.S. is important because these trees are a long lived, late successional, climax species in eastern hardwood forests (Daley et al., 2007; Orwig et al., 2002). Due to the abundance of the eastern hemlock in riparian areas and their dense evergreen canopy, the loss of the eastern hemlock is having a major impact on forest processes in the eastern U.S. (Miniat et al., 2016; Webster et al., 2012; Orwig et al., 2012; Brantley et al., 2014). One major impact includes the change in hydrologic fluxes due to transpiration rate changes from the plant species replacing it (Orwig and Foster, 1998; Ward et

al., 2004). Sites where hemlocks were once dominant become more homogenous with an increase in *Acer, Quercus, Betula, or Rhododendron* species, and could also result in an increase in soil temperatures because of the opening of the canopy (Brantley et al., 2013; Orwig and Foster, 1998; Jenkins et al., 1999; Ward et al., 2004).

Infested trees can be killed in as little as 4 years, but some have been known to live in an unhealthy state for 10 years or more (McClure, 1991; Orwig et al., 2002; McAvoy et al., 2017). The rate of death is sometimes slow because HWA populations cycle in a density-dependent manner in relation to tree health. Densities increase on new growth of healthy trees, but when tree health declines, HWA numbers also decline, allowing trees to grow again, which allows the trees to support adelgids again, resulting in a downward health spiral for the trees (McClure, 1991). *A. tsugae* nymphs damage trees by attaching their stylets (mouthparts) to the base of the needles, and feeding on the fluids and nutrients from parenchyma cells, which serve as nutrient transfer and storage cells in xylem rays (McClure, 1987, 1991; Young et al., 1995). The feeding causes needles to become discolored and dehydrated, and inhibits production of new growth (McClure, 1987; McClure et al., 2001).

In Japan, where the population of HWA invading the eastern United States is native to, the adelgid requires two hosts: Tiger-tail spruce, (*Picea torano* (Siebold ex K.Koch) Koehne) (primary host) and hemlock trees (*Tsuga sieboldii* Carriere and *Tsuga diversifolia* Masters) (the secondary host), to complete a holocyclic life cycle (Havill and Foottit, 2007; McClure, 1987). The primary host is not present in the eastern United States, and no *Picea* spp. native to North

America is a suitable host (McClure, 1987). As a result, the HWA lifecycle in the eastern United States is anholocyclic, comprised of two parthenogenic generations on hemlock only, made up solely of wingless females (Havill and Foottit, 2007; McClure, 1987). These two generations are known as the sistens and the progrediens. The sistens are the overwintering generation that hatch in the early summer and go through an aestival diapause. When diapause is broken in the fall, they resume development and continue to feed on hemlock trees throughout the winter (McClure, 1987; McClure et al., 2001). When eggs deposited by sistens begin to hatch in April-May, there is one of two types of progeny: asexual progrediens, or, in rare cases, sexual sexuparae. Sexuparae are insignificant in the eastern United States because they fly in search of spruce species that are unavailable to them (McClure, 1987). The first instar of both asexual life stages are known as "crawlers", as they are the only mobile stage of the insect (McClure, 1990). Crawlers disperse by crawling, attaching themselves to birds or other animals, and by the wind (McClure, 1990). Progrediens are present in late spring and the eggs from adults hatch into sistens in late June (McClure, 1989). The hemlock wooly adelgid, *A. tsugae*, gets its name from the covering of wax filaments resembling wool that is produced to protect from predators (McClure, 1987; Jones et al., 2014). This "wool" is found on all life stages except for the eggs and mobile crawlers.

1.2 Management of HWA

Some methods of control for HWA include silviculture, breeding resistance in the hemlock trees, application of insecticides, and biological control (Cowles et al., 2006). The silvicultural approach involves taking preserved Carolina hemlock seeds and planting in other countries to reintroduce at a later date in order to preserve the species (Jetton et al., 2008). Breeding

resistance in eastern hemlocks involves attempting to identify individual trees that seem more tolerant to adelgid feeding, and then using these trees as a seed source for other regeneration projects (Bentz et al., 2008). Application of insecticides is the most successful short-term suppression method for HWA. The most commonly used insecticide is the neonicotinoid, imidacloprid, which binds to nicotinic receptor sites on insect nerves and disrupts the ability to function normally. Although imidacloprid has low toxicity to mammals and birds, there are concerns related to insecticide use near streams and aquatic environments (Cowles et al., 2006). Imidacloprid is applied by either soil treatments, stem injections, or foliar sprays (Silcox, 2002). Another promising insecticide is dinotefuran, also a neonicotinoid, and though more expensive than imidacloprid, it is useful when rapid control is of HWA is required (Cowles, 2009). Chemical control methods are usually limited to small-scale settings because of cost, effort, and practicality on the larger scale since not all trees are easily accessible.

1.3 Biological Control of Hemlock Woolly Adelgid

1.3.1 Biological control

The biological control program for HWA may be a very useful tool in helping save the hemlocks in the eastern United States (Havill et al., 2011). *A. tsugae* is an ideal candidate for biological control because all life stages but the crawler stage are sedentary, and the extended amount of time spent on trees makes them easily accessible to predators. Classical biological control, the importation and release of exotic predators that have co-evolved with a pest, with the intention of controlling the pest population (Caltagione, 1981), is the current approach used for HWA. However, to create a successful biological control program, there are several attributes that potential candidate species must have; including a significant impact and close association with

the target pest, a host range limited to HWA, originate in a climate similar to where it would be released, be tolerant to many environmental variables, and be phenologically synchronized with the life cycle of HWA (Cheah et al., 2004).

In the northwestern United States 55 species from 14 different families have been found on HWA infested trees (Kohler et al., 2008). Although a majority of these are generalist predators of non-adelgid prey, the most abundant predators found were *Laricobius* spp. (Coleoptera: Derondontidae) (Kohler et al., 2008). It was found that in the southeastern United States that HWA is preyed upon by generalist predators such as Coleoptera: Coccinellidae, Neuroptera: Chrysopidae and Hemerobiidae, and Diptera: Cecidomyiidae (Wallace and Hain, 2000). In its native range in Japan, HWA predators include: *Sasajiscymnus tsugae* (Coleoptera: Coccinellidae) Sasaji and McClure (1997), *Laricobius osakensis* (Coleoptera: Derodontidae) Montgomery and Shiyake (Montgomery et al., 2011), and *L. naganoensis* Leschen, of which very little is known (Fischer et al., 2014).

1.3.2 *Laricobius* species as predators and biological control agents

Beetles in the genus *Laricobius* have been identified for biological control because of their host-specificity (Lamb et al., 2011). They are members of the family Derodontidae, which are known for feeding on fungi and for indentations found along the lateral margins of their pronotum. The genera *Laricobius* is an exception, since they are predators and adelgid specialists, making them an ideal choice for the biocontrol of HWA (Bright, 1991; Hava, 2006). Species of *Laricobius* present in the eastern United States include: *L. rubidus* LeConte and *L. nigrinus* Fender. *L.*

rubidus is native to the eastern United States and its known host is pine bark adelgid, *Pineus strobi* (Hartig) (Clark and Brown, 1960). *L. nigrinus* is a native predator of HWA in western North America (Mausel et al., 2010) and was first introduced in the eastern United States in 2003 as a biological control agent (Lamb et al., 2006; Mausel et al., 2010).

Initially, the predators *Scymnus sinuanodulus* Yu & Yao (Coleoptera: Coccinellidae), *Sasajiscymnus tsugae* Sasaji (Coleoptera: Coccinellidae), and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) were evaluated for their potential as biological control agents (Mausel and Salom, 2013). Of these three predators, *L. nigrinus* has been the most successful in terms of establishment and spread. However, only two site-specific studies assessed its short-term impacts as a predator (Mausel et al., 2008; Mayfield et al., 2015). Long-term and large-scale impacts in terms of reduced adelgid populations and subsequent improvement in tree health are still needed and these assessments are currently ongoing (Heminger, 2017).

1.3.3 *Laricobius osakensis*

In 2005, *Laricobius osakensis* Montgomery and Shiyake, was discovered in Japan from sampling *Tsuga sieboldii* (Montgomery et al., 2011). Northern and southern populations of *L. osakensis* are found in Japan and are collected on southern Japanese hemlock, *Tsuga sieboldii* (Carriere) and northern Japanese hemlock, *Tsuga diversifolia* (Maxim.) (Mausel and Salom, 2013; Lamb et al., 2011). In March 2006, 300 beetles were collected in the Kansai (southern) region and shipped to the Beneficial Insect Quarantine Lab at Virginia Tech to be studied and reared for potential future releases (Vieira et al., 2011). In 2010 the species was approved for release in the

eastern United States (Mausel and Salom, 2013). *L. osakensis* has the potential to be a promising control agent, because it is a co-adapted predator of the HWA populations that are present in the eastern United States (Havill et al., 2006). An exclusion cage study performed in the Kansai region showed that *L. osakensis* controls HWA populations and is the primary predator of HWA in the winter and spring (Lamb et al., 2011). *L. osakensis* has several characteristics including its synchronous life cycle with HWA (Vieira et al., 2013) and faster reproduction rates than *L. nigrinus* (Vieira et al., 2012), highlighting its need to be further assessed as a biological control agent to HWA. *L. osakensis* has a lower temperature threshold and higher feeding rate, increased oviposition, and faster larval development rate than *L. nigrinus*.

Adult *L. osakensis* are between 2-3 mm in length. The antennae have eleven segments with a three-segmented club, and their elytra are serrate or striate and their abdomen has five visible sternites (Lamb et al., 2011). Male and female *L. osakensis* can be distinguished from each other: females have reddish elytra and are glossy, while males are black with almost no gloss (Lamb et al., 2011). However, *L. osakensis* are extremely difficult to distinguish from other *Laricobius* species, with their lack of ocelli, and lack of pronotal tooth being a primary diagnostic characteristic (Montgomery et al., 2011).

Both the adults and larvae of *L. osakensis* feed on HWA. Adults feed on the sistens nymphs and adults as well as progrediens eggs and early instars (Vieira et al., 2013). Both the larvae and adults display a Type II functional response for predation, meaning that predation consumption increases with prey availability at a decreasing rate to a maximum (Vieira et al., 2012). *L.*

osakensis is highly host-specific on HWA and prefer to feed and oviposit on HWA compared with other prey species and thus, should pose little risk to native fauna (Vieira et al., 2011). It has also been shown in laboratory studies to have no negative impact on *L. nigrinus* or *L. rubidus* (Story et al., 2012). In addition, *L. osakensis* cannot produce viable eggs from mating with either *L. nigrinus* or *L. rubidus*, even though the latter two species successfully hybridize (Fischer et al., 2015).

L. osakensis typically begins emerging in August (can begin earlier or later depending on location) and continues to do so through December, in synchrony with HWA sistens breaking diapause (Lamb et al., 2008; Vieira et al., 2013). Peak oviposition for females is in in mid-April (Vieira et al., 2013). Larvae feed mostly on HWA eggs, but 3rd and 4th instars will also feed and develop on progrediens nymphs. Most of the larvae reach maturity and drop into the soil to pupate by the end of May (Vieira et al., 2013).

Approval for release of *L. osakensis* in the U.S. was granted in 2010 by the USDA-Animal and Plant Health Inspection Service (APHIS), and releases began in fall 2012. Since approval, there have been over 60 *L. osakensis* releases (Roberts et al., 2011). Releases will continue on an annual basis with source beetles coming from both Virginia Tech and University of Tennessee Insectaries. It is essential that the establishment, dispersal, and impact of *L. osakensis* on HWA populations is determined.

In the first year of sampling (2013), a total of 22 adults and 212 larvae were recovered at the Little Meadows, VA site and Carnifex, WV site. In 2014, 7 adults were collected from Little Meadows and Carnifex, 8 adults at the newer Goshen and Saltville sites, but no larvae were recovered at any of the field sites (Mooneyham et al., 2016). Unsuitable and sustained cold temperatures are likely to be responsible for the low population numbers, as some places experienced 99% HWA mortality (McAvoy et al., 2017), depleting the food source for *L. osakensis. L. nigrinus* collections were significantly correlated to the number of HWA present at a site, and this could help explain the lack of recoveries for *L. osakensis* (Mausel et al., 2010; Mooneyham et al., 2016).

1.4 Supercooling Point

Insects may use different strategies to survive cold winter temperatures. These strategies include cyroprotective dehydration, vitrification, cold intolerance, freeze tolerance, and freeze avoidance (also known as freeze intolerance) (Storey and Storey, 2013; Sinclair, 2015). The cyroprotective dehydration strategy is used in some polar insects, and is a combination of high cryoprotectant levels to stabilize macromolecules, and extreme dehydration that makes it so there is very little liquid to freeze in their bodies (Sorenson and Holmstrup, 2011; Storey and Storey, 2013). Vitrification is a strategy that is used by an Alaskan bark beetle, and is a combination of extreme dehydration and the accumulation of antifreeze proteins and a high concentration of polyols (Sformo et al., 2010; Storey and Storey, 2013). Cold- intolerant (also known as chill-susceptible) insects are killed by cold temperatures without internal freezing, and these insects often use other strategies to survive the winter, such as harborage or migration (Bale, 1993; Storey and Storey 1988, Sinclair, 2015). Freeze-tolerant insects can survive internal ice formation through the

regulated freezing of up to about 65% (though up to 84% has been reported) of total body water in extracellular spaces (Figure 1.1) (Ramlov and Westh, 1993; Block et al. 1998; Storey and Storey, 2013). The ice formation in freeze tolerant insects is triggered by the action of specific ice nucleating agents or proteins (INA or INP's) (Storey and Storey 1991; Zachariassen and Kristiansen 2000; Dunman 2001). Freeze avoidant insects cannot survive internal freezing, but are able to depress the temperatures at which they freeze (the supercooling point) by the synthesis of antifreeze proteins, the accumulation of extremely high levels of carbohydrate cryoprotectants, and partial dehydration of body fluids (Figure 1.1) (Croswaithe et al., 2011 Storey and Storey, 1988; Storey and Storey, 2013). The antifreeze proteins reduce the nucleation potential of seed crystals, and the sugars from the cryoprotectants lower crystallization temperatures (Zachariassen, 1985; Ramlov and Lee, 2000; Holmstrup et al., 2002; Sinclair et al., 2003).

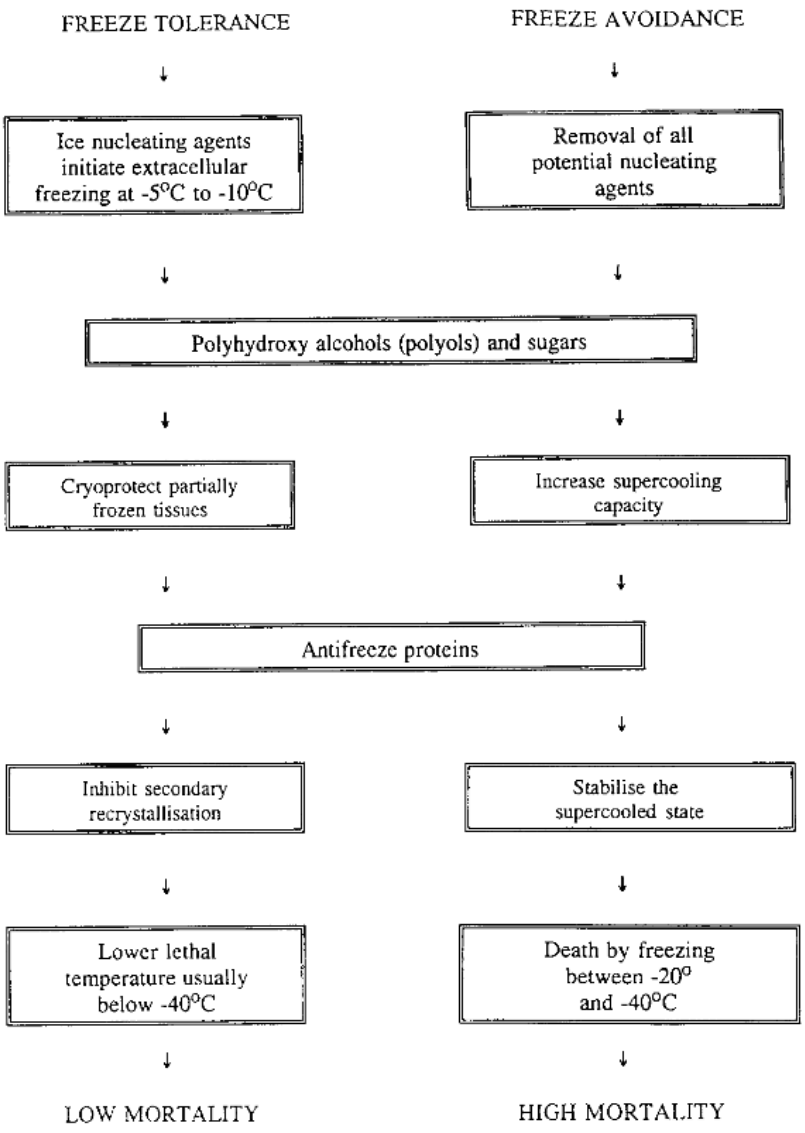

Figure 1.1: Comparison of the freeze tolerant and freeze intolerant cold weather survival strategies (from Bale, 1993)

The supercooling capability of an insect is a phenomenon where water and aqueous solutions remain unfrozen below their melting point (the temperature where the last ice crystal disappears when a frozen sample is warmed) (Leather, 1993; Renault et al., 2002). The supercooling point is the depressed temperature at which the ice crystals begin to form. A supercooling point can be

detected when ice forms in the tissue fluids of an insect, and a temperature spike occurs as a result of the release of latent heat of fusion (Lee and Lewis, 1985; Mausel et al., 2011). The supercooling point of an insect is dependent on the organism's native characteristics such as body composition, which is tightly linked to their physiological state (feeding status, diapause, life stage, metamorphosis, etc.). An insect's ability to avoid nucleation is influenced by many factors including: eliminating internal nucleation sites in the digestive track, accumulating antifreeze agents, dehydration, and the avoidance of contact with external ice (Danks, 1996; Danks 2000). All of these factors decrease the supercooling point of the insect, and as a result, help prevent the formation of ice in the hemolymph or gut (Zachariassen and Kristiansen, 2000). However, long periods of subzero temperatures can cause stress and mortality, even if it is above the SCP (Mausel et al., 2011; Block 1990).

In cold temperatures, most insects will eventually lose their ability to move, and enter a state known as a chill coma. This is a reversible state of paralysis that is used when a certain temperature threshold (critical thermal minimum (CTmin)) is crossed (Hazell and Bale, 2011; Sinclair, 2015). The CT min can provide useful information on the lower limit to insect function and help approximate insect cold tolerance, because insects cannot move, feed, evade predators, or reproduce in this state (Andersen et al., 2015b; David et al., 1998; Gibert et al., 2001; MacMillan and Sinclair, 2011a; Sinclair, 2015). Chill coma is usually not lethal, and as the insects are warmed, they enter a state called chill coma recovery, which is when movement is resumed (David et al., 1998; Gibert et al., 2001; MacMillan and Sinclair, 2011a, Sinclair, 2015).

Determining the supercooling point of *L. osakensis* (a freeze intolerant species) is of interest in order to determine if the low recovery numbers experienced in 2014 and 2015 were due to the beetles being intolerant of sustained low temperatures or if it was due to the lack of a primary food source (HWA). Since *L. osakensis* has a limited ability to feed on other prey species, it would be more difficult for them to survive when there are extreme fluctuations in HWA populations (Vieira et al., 2011). Cold is often a limiting factor in species distribution (Cira et al., 2015), and therefore cold temperature biology is an excellent way to predict a species distribution (Andersen et al., 2015). Acclimating lab reared insects to field conditions is important when determining the supercooling point, because an insect's physiology adapts as temperatures change, and this can have a large impact on an insect's cold tolerance (Salt, 1960; Cira et al., 2015). Cold hardiness is not a constant feature in insects, as it varies throughout the season and year. Therefore, monitoring should be done multiple times a season, and for multiple years (Danks, 2005). While the supercooling point of an insect does not necessarily indicate the cold hardiness of a species, it still is of value because it represents a physiological threshold that can indicate relevant variation in cold tolerance (Croswaithe et al., 2011).

The supercooling point of *L. osakensis* compared to that of other *Laricobius* species under the same conditions is of special interest. The northern and southern populations of *L. osakensis* are hypothesized to have different supercooling points, as was observed for *L. nigrinus* coastal versus inland strains (Mausel et al., 2011). As a result, the northern population has been released in northern parts of the United States and the southern population released in southern parts of the United States. It is important to test this hypothesis experimentally to determine if, in fact, there is a difference between beetles collected from two different locations in Japan. The coastal

strain of *L. nigrinus* had an average supercooling point of -16.9 °C (± 0.3), and the inland strain of *L. nigrinus* was between -18.6 °C (± 0.6) and -19.2 °C (± 0.7), it was also found that there were significant differences between the two strains (Mausel et al., 2011).

1.5 Research Rationale

Eastern and Carolina hemlock trees are important species currently threatened by the hemlock woolly adelgid. The loss of these trees will result in the change of forest processes, and a loss of habitat for certain (Orwig et al., 1998). Hemlock trees throughout most of the eastern United States are impacted by this invasive species, and can be killed in as little as four years from HWA feeding, which causes discoloration, dehydration, and the inhibition of new growth (McClure, 1987; McClure et al., 2001). Although there are several methods used to control HWA, biological control is the most promising and practical long-term solution. Hemlock woolly adelgid is an ideal candidate for biological control because it is mostly sedentary and easily accessible to predators. Beetles in the genus *Laricobius* were selected for their host-specificity, and most recently, the beetle *L. osakensis* has been used as biological control agent. *L. osakensis* was chosen because it is from the same region of Japan that the population of HWA on the eastern U.S. is from (Havill et al., 2006). It is host-specific, well synchronized with HWA, and has a high predation and oviposition rates (Vierra et al., 2013).

Laricobius osakensis has been released in the eastern U.S. since 2012. Recoveries of these beetles were common in the first year of post release monitoring, but not in the second. Poor recoveries in the second year have been considered due to a polar vortex in the 2013-2014 winter

(Mooneyham et al., 2016.) A subsequent polar vortex event occurred in the winter of 2014/2015, again killing high proportions of HWA. Two consecutive years of extreme cold temperatures dramatically decreased the density of HWA, making it difficult for the newly released biological control agent to colonize. HWA numbers rebounded during 2016 and 2017, as winter temperatures were closer to the average (McAvoy et al., 2017). Field monitoring efforts of both HWA and predator populations are important to determine if these beetles are able to establish, and learn more about the insect's ability to disperse. It is unknown if *L. osakensis* will make a significant difference on HWA densities once they establish.

It is unclear if the lack of *L. osakensis* recoveries were due to the loss of its main food source (HWA) or the fact that the beetles themselves could not withstand the extreme cold weather, or both following the polar vortex. Learning more about the supercooling point and cold tolerance of *Laricobius* beetles could provide insight as to what happened after that winter. Comparison of the northern and southern populations of *L. osakensis* and *L. nigrinus* would give insight as to which biological control agent may be suited best for release throughout the geographic range of HWA.

1.6 Research Objectives

The first objective of this study was to monitor the establishment of *L. osakensis* at various release sites in VA, TN, WV, OH, and PA, and, if established, characterize its dispersal from the central release areas. The second objective was to determine the supercooling point of *L.*

osakensis from fall to winter to help relate suitability of the predator populations to different plant hardiness zones in the region.

Objective 1A: To assess the establishment of *L. osakensis* at release sites in Virginia

The 2012- 2014 release sites were sampled regularly to assess the presence and density of *L. osakensis* in the field. This will help indicate if the beetle is able to establish, and be successful as a biological control agent.

Objective 1B: To assess the dispersal of *L. osakensis* at release sites in Virginia

Sampling trees around the release trees allows us to determine if *L. osakensis* is dispersing at the release sites. A steady dispersal rate can be a good indicator of the success of establishment at the release sites and helps us predict long-term spread of the predator. The release sites varied from roadside areas to dense forests, and impacts the ability to sample dispersal but may provide insight into which types of sites have more successful establishment.

Hypotheses:

1.1. H_o If *L. osakensis* is establishing, we will not see a relationship between the number of prey present in the stand and the number of *L. osakensis* recovered.

1.1. H_a If *L. osakensis* is colonizing, we will see a relationship between the number of prey present in the stand and the number of *L. osakensis* recovered.

1.2. H_o *L. osakensis* will not be found on trees outside of the release center

1.2. H_a *L. osakensis* will be found on trees outside of the release center

Objective 2: To determine the supercooling point of northern and southern populations of *L. osakensis*, and see how they compare to the supercooling points of *L. rubidus*, and *L. nigrinus*

Determination of about the supercooling point of *L. osakensis* may provide insight into the lack of field recoveries made in 2014. Comparison of the supercooling points of all the *Laricobius* species in this system, including the northern and southern *L. osakensis* populations, may provide some insight as to which biocontrol agent would be best suited for different geographic locations in the eastern U.S. Since supercooling points vary by location, year, and time of year, it is important to do a comparison of HWA and the *Laricobius* species at the same time taken from the same place to get the most accurate results. Evaluation of the supercooling point of several samples taken throughout the winter will enable us to determine if the supercooling point variability of these species.

Hypotheses:

2. 1. H_o The supercooling points will not differ between the southern and northern strains of *L. osakensis* collected and reared in the lab.

2. 1. H_a The supercooling points will differ between the southern and northern strains of *L. osakensis* collected and reared in the lab.

2. 2. H_o The supercooling points will not differ between *L. osakensis* and *L. nigrinus* collected and reared in the lab.

2. 2. H_a The supercooling points will differ between *L. osakensis* and *L. nigrinus* collected and reared in the lab.

2. 3. H_o The supercooling points will not differ throughout the winter.

2. 3. H_a The supercooling points will differ throughout the winter.

1.7 References:

Andersen, J. L., T. Manenti, J. G. Sorensen, H. A. MacMillan, V. Loeschcke, and J. Overgaard. 2015. How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. Funct. Ecol. 29: 55-65.

Bale, J. S. 1993. Classes of insect cold hardiness. Funct. Ecol. 7: 751-753

Bentz, S. E., M. E. Montgomery, and R. T. Olsen. 2008. Resistance of hemlock species and hybrids to hemlock woolly adelgid. In: B. Onken, and R. Reardon, comps. Fourth Symposium on hemlock woolly adelgid in the eastern United States; 2008 February 12-14; Hartford, CT. FHTET 2008-01. Morgantown, WV: U.S. Forest Service, Forest Health Technology Enterprise Team: 137-139.

Block, W. 1990. Cold tolerance of insects and other arthropods. Philosophical Transactions of the Royal Society of London B. 326: 613–633.

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

Brantley, S. T., C. F. Miniat, Ford; K. J. Elliott., S. H. Laester, and J. M. Vose. 2014. Changes to southern Appalachian water yield and stormflow after loss of a foundation species. Ecohydrology. 8: 518-528

Bright, D. E. 1991. Family Derodontidae: tooth-necked fungus beetles. Checkl. Beetles Canada Alaska. Agric. Canada Res. Branch, Publ. 195–196.

Caltagirone, L. E. 1981. Landmark examples in classical biological control. Annu. Rev. Entomol. 26: 213–232.

Carter, C. I. 1971. Conifer woolly aphids (Adelgidae) in Britain. Bull. For. Commn., Lond.

Cheah, C. A., and M. S. McClure. 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). Agric. For. Entomol. 2: 241–251.

Cheah, C., R. C. Reardon, and B. Onken. 2004. Biological control of hemlock woolly adelgid.

US Department of Agriculture, Forest Service, FHTET.

Cira, T. M., R. C. Venette, J. Aigner, T. Kuhar, D. E. Mullins, S. E. Gabbert. 2015. Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales. Environ. Entomol. 45: 484-491

Clark, R. C., and N. R. Brown. 1960. Studies of Predators of the Balsam Woolly Aphid, *Adelges piceae* (Ratz.)(Homoptera: Adelgidae), VII. *Laricobius rubidus* Lec.(Coleoptera: Derodontidae), a Predator of *Pineus strobi* (Htg.)(Homoptera: Adelgidae). Can. Entomol. 92: 237–240.

Cowles, R. S., and A.F. Lagalante. 2009. Activity and persistence of systemic insecticides for managing hemlock woolly adelgids. In Proceedings of the 20th US Department of Agriculture Interagency Research Forum on Invasive Species. USDA Forest Service General Technical Report NRS-P-51. 17-18.

Cowles, R. S., M. E. Montgomery, and C.-J. Cheah. 2006. Activity and residues of imidacloprid applied to soil and tree trunks to control hemlock woolly adelgid (Hemiptera: Adelgidae) in forests. J. Econ. Entomol. 99: 1258–1267.

Crosthwaite, J. C., S. Sobek, D. B. Lyons, M. A. Bernards, and B. J. Sinclair. 2011. The overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). J. Insect Physiol. 57: 166–173.

Daley, M. J., N. G. Phillips, C. Pettijohn, and J. L. Hadley. 2007. Water use by eastern hemlock (*Tsuga canadensis*) and black birch (*Betula lenta*): implications of effects of the hemlock woolly adelgid. Can. J. For. Res. 37: 2031–2040.

Danks, H. V. 2005. Key themes in the study of seasonal adaptations in insects I. Patterns of cold hardiness. Appl. Entomol. Zool. 40: 199–211.

David, R. J., P. Gibert, E. Pla, G. Petavy, D. Karan, and B. Moreteau. 1998. Cold stress tolerance in Drosophila: analysis of chill coma recovery in D. melanogaster. J. Thermal Biol. 23: 291-299.

Duman, J.G., 2001. Antifreeze and ice nucleator proteins in terrestrial arthropods. Annual Rev. Phys. 63, 327–357.

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

Fischer, M. J., C. C. Brewster, N. P. Havill, S. M. Salom, and L. T. Kok. 2015. Assessment of the potential for hybridisation between *Laricobius nigrinus* (Coleoptera: Derodontidae) and *Laricobius osakensis*, predators of the hemlock woolly adelgid (Hemiptera: Adelgidae). Biocontrol Sci. Technol. 25: 1467–1482.

Fischer, M. J., N. P. Havill, C. S. Jubb, S. W. Prosser, B. D. Opell, S. M. Salom, and L.T. Kok. 2014. Contamination delays the release of *Laricobius osakensis* for biological control of hemlock woolly adelgid: Cryptic diversity in Japanese *Laricobius* spp. and colony purification techniques. S.E. Naturalist 13:178–191.

Ford C.R., K. J. Elliot, B. D. Clinton, B. D. Kloeppel and J. M. Vose. 2011. Forest dynamics following eastern hemlock mortality in the southern Appalachians. Oikos. 121: 523-536.

Gibert, P., B. Moreteau, G. Pétavy, D. Karan, and J. R. David. 2001. Chill-coma tolerance, a major climatic adaptation among Drosophila species. Evolution. 55: 1063-1068.

Háva, J. 2006. A world catalogue of the family Derodontidae (Coleoptera). Pol. Pismo Entomol. 75.

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

Havill, N. P., M. E. Montgomery, and M. Keena. 2011. Hemlock woolly adelgid and its hemlock hosts: a global perspective. Implementation and status of biological control of the hemlock woolly adelgid. 3.

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

Hazell, S. P., and J.S. Bale. 2011. Low temperature thresholds: are chill coma and CTmin synonymous. J Insect Physiol. 57: 1085-1089.

Heminger, A. R. 2017. Establishment of *Laricobius nigrinus* (Coleoptera: Derodontidae) in Virginia and assessment of its impact on hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), throughout the eastern U.S. M.S. thesis. Virginia Tech, Blacksburg, VA. 93 pp.

Holmstrup, M., K. Hedlund, and H. Boriss. 2002. Drought acclimation and lipid composition in *Folsomia candida*: implications for cold shock, heat shock and acute desiccation stress. J. Insect Physiol. 48: 961–970.

Humble, L. M., and L. Mavin. 2005. Preliminary assessment of the cold tolerance of *Laricobius nigrinus*, a winter-active predator of the hemlock woolly adelgid from western Canada. In: Gottschalk, Kurt W., ed. Proceedings, 16th US Department of Agriculture interagency research forum on gypsy moth and other invasive species. 2005 January 18-21; Annapolis, MD. Gen. Tech. Rep. NE-337. Newtown Square, PA: US Department of Agriculture, Forest Service, Northeastern Research Station: 46.

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

Jetton, R.M., Dvorak, W.S. and 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. For. Ecol. and Mgmt, 255:3212-3221.

Jones, C. E., N. P. Havill, J. L. Hanula, and S. K. Braman. 2014. Post release recovery of hemlock woolly adelgid predators in the North Georgia mountains. J. Entomol. Sci. 49: 383–400.

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

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

Lamb, A., M. E. Montgomery, L. C. Vieira, S. Shiyake, and S. Salom. 2011. *Laricobius osakensis*, a hemlock woolly adelgid predator from Japan. Implement. Status of Biological Control of Hemlock Woolly Adelgid. Print. 90.

Lamb, A., S. Shiyake, S. Salom, M. Montgomery, and L. Kok. 2008. Evaluation of the Japanese *Laricobius* sp. n. and other natural enemies of hemlock woolly adelgid in Japan. In: Onken, Brad; Reardon, Richard, comps. Fourth Symposium on hemlock woolly adelgid in the eastern United States; 2008 February 12-14; Hartford, CT. FHTET 2008-01. Morgantown, WV: U.S. Forest Service, Forest Health Technology Enterprise Team: 29-36.Leather SR, Walters KFA & Bale JS (1993) The Ecology of Insect Overwintering, Cambridge University Press, Cambridge, 255.

Lee, R. E., and E. A. Lewis. 1985. Effect of temperature and duration of exposure on tissue ice formation in the gall fly, *Eurosta solidaginis* (Diptera, Tephritidae). Cryo-letters. 6: 25-34.

Leschen, R. A. 2011. World review of Laricobius (coleoptera: Derodontidae). Zootaxa. 2908: 1-44.

MacMillan, H. A., and B. J. Sinclair. 2011. Mechanisms underlying insect chill-coma. J. Insect Physiol. 57: 12-20.

Mausel, D.L., and S.M. Salom. 2013. Hemlock woolly adelgid: *Adelges tsugae* Annand (Hemiptera: Adelgidae). Pp. 167–187, *In* R. Van Driesche and R. Reardon (Eds.). The Use of Classical Biological Control to Preserve Forests in North America. USDA Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV. FHTET-2013-02.

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

Mausel, D. L., R. G. Van Driesche, and J. S. Elkinton. 2011. Comparative cold tolerance and climate matching of coastal and inland *Laricobius nigrinus* (Coleoptera: Derodontidae), a biological control agent of hemlock woolly adelgid. Biol. Control 58.2: 96-102.

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

woolly adelgid in Virginia. Environ. Entomol. 37: 1498–1507.

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

McAvoy, T. J., J. Régnière, R. St-Amant, N.F. Schneeberger, and S. M. Salom. 2017. Mortality and recovery of hemlock woolly adelgid (*Adelges tsugae*) in response to winter temperatures and predictions for the future. Forests. 8: 497.

McClure, M. S. 1987. Biology and control of hemlock woolly adelgid. Connecticut Agricultural Experiment Station New Haven, CT. 851.

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

McClure, M. S. 1990. Role of wind, birds, deer, and humans in the dispersal of hemlock woolly adelgid (Homoptera: Adelgidae). Environ. Entomol. 19: 36–43.

McClure, M. S. 1991. Nitrogen fertilization of hemlock increases susceptibility to hemlock woolly adelgid. J. Arboric. 17: 227–229.

McClure, M. S., S. M. Salom, and K. S. Shields. 2001. Hemlock wooly adelgid. For. Heal. Technol. Enterp. Team. US For. Serv. Publ. FHTET–2001–03. Morgantown, WV.

Miniat, Chelcy F.; Zeitlow, David; Brantley, Steven T.; Mayfield, Albert (Bud); Rhea, Rusty; Jetton, Robert; Arnold, Paul. 2016. Physiological responses of eastern hemlock (*Tsuga Canadensis*) to biological control and silvicultural release: implications for hemlock restoration. In: Stringer, Christina E.; Krauss, Ken W.; Latimer, James S., eds. 2016. Headwaters to estuaries: advances in watershed science and management -Proceedings of the Fifth Interagency Conference on Research in the Watersheds. March 2-5, 2015, North Charleston, South Carolina. e-General Technical Report SRS-211. Asheville, NC: U.S. Department of Agriculture Forest Service, Southern Research Station. 302 p.

Montgomery, M. E., S. Shiyake, N. P. Havill, and R. A. B. Leschen. 2011. A new species of *Laricobius* (Coleoptera: Derodontidae) from Japan with phylogeny and a key for native and

introduced congeners in North America. Ann. Entomol. Soc. Am. 104: 389–401.

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

Morin, R. S., S. N. Oswalt, R. T. Trotter III, and A. M. Liebhold. 2011. Status of hemlock in the eastern United States. USDA For. Serv. e-Science Update. SRS-038.

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

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

Orwig, D. A., J. R. Thompson, N. A. Povak, M. Manner, D. Niebyl, and D. R. Foster. 2012. A foundation tree at the precipice: *Tsuga canadensis* health after the arrival of *Adelges tsugae* in central New England. Ecosphere 3:10.

Ramlov, H., and R. E. Lee. 2000. Extreme resistance to desiccation in overwintering larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). J. Exp. Biol. 203: 783–789.

Ramløv, H., and P. Westh. 1993. Ice formation in the freeze tolerant alpine weta *Hemideina maori* Hutton (Orthoptera; Stenopelmatidae). Cryo-letters. 14:169-176

Salt, R.W.1960. Principles of insect cold hardiness. Annu. Rev. Entomol. 6: 55-74.

Sformo, T., K. Walters, K. Jeannet, B. Wowk, G.M. Fahy, B.M. Barnes, and J.G. Duman. 2010. Deep supercooling, vitrification and limited survival to–100 C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae. J. Exp. Biol, 213: 502-509.

Silcox, C. A. 2002. Using imidacloprid to control hemlock woolly adelgid. Proceedings Hemlock Woolly Adelgid in the Eastern United States. NJ Agricultural Experiment Station, New Brunswick, New Jersey. 280-287.

Sinclair, B. J., L. E. C. Alvarado, and L.V. Ferguson. 2015. An invitation to measure insect cold tolerance: methods, approaches, and workflow. J. Thermal Biol. 53: 180-197.

Sinclair, B. J., P. Vernon, C. J. Klok, and S. L. Chown. 2003. Insects at low temperatures: an ecological perspective. Trends Ecol. Evol. 18: 257–262.

Sorenson J.G., and M. Holmstrup. 2011. Cyroprotective dehydration is widespread in arctic springtails. J. Insect Physiol. 57: 1147-1153.

Souto, D., T. Luther, B. Chianese, S. M. Salom, T. C. Tigner, and R. C. Reardon. 1996. Past and current status of HWA in eastern and Carolina hemlock stands. Proc. First Hemlock Woolly Adelgid Review Charlottesville, Virginia, FHTET 96-10.

Storey, K. B., and J. M. Storey. 1991. Biochemistry of cryoprotectants. Insects at low temperature. Springer US. 64-93.

Storey, K. B., and J. M. Storey. 1988. Freeze tolerance in animals. Physiol. Rev. 68: 27–84.

Storey, K. B., and J. M. Storey. 2013. Molecular biology of freezing tolerance. Compr Physiol. 3: 1283-1308.

Story, H. M., L. C. Vieira, S. M. Salom, and L. T. Kok. 2012. Assessing performance and competition among three *Laricobius* (Coleoptera: Derodontidae) species, predators of hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). Environ. Entomol. 41: 896–904.

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

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

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

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

Ward, J. S., M. E. Montgomery, C. A.-J. Cheah, B. P. Onken, and R. S. Cowles. 2004. Eastern hemlock forests: guidelines to minimize the impacts of hemlock woolly adelgid. NA-TP-03-04. US Department of Agriculture, Forest Service, Northeastern Area State & Private Forestry, Morgantown, WV. 27: 1-27.

Webster et al. 2012. Effects of hemlock mortality on streams in the Southern Appalachian Mountains. Amer. Midland Naturalist. 168:112-131.

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

Zachariassen, K. E. 1985. Physiology of cold tolerance in insects. Physiol. Rev. 65: 799–832.

Zachariassen, K. E., and E. Kristiansen. 2000. Ice nucleation and antinucleation in nature. Cryobiology. 41: 257–279.

Chapter 2: Establishment in the eastern U.S. of *Laricobius osakensis* (Coleoptera: Derodontidae), a biological control agent for hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae)

Abstract:

The hemlock woolly adelgid (HWA) is an invasive species native to Japan, causing significant hemlock mortality in the eastern United States. Biological control is the most promising method of controlling HWA. Beetles in the genus *Laricobius* were chosen as biological control agents because they are adelgid specialists. *Laricobius osakensis* and the strain of HWA in the eastern United States are native to the same region of Japan. *L. osakensis* is phenologically synchronous with HWA, and is host-specific. It has been released as a biological control agent since 2012 with a total of 61 releases. In 2014, a polar vortex caused extensive mortality to HWA, which could explain the initial low recovery numbers of *L. osakensis.* The beetle's ability to survive and establish in the eastern United States is assessed here. The first year of this study yielded low recovery numbers of *L. osakensis*, as HWA populations were still rebounding from the polar vortex. In the second-year, 147 *L. osakensis* at 5 of 9 sites sampled were collected, coinciding with rebounding HWA populations. These results suggest the predator is successfully establishing at several of these sites.

Keywords: Classical biological control, monitoring, *Adelges tsugae*, *Laricobius osakensis*, *Tsuga canadensis*

2.1 Introduction:

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an insect pest native to Asia and the western United States. It is causing significant mortality to eastern and Carolina hemlock trees in the eastern United States (Havill et al. 2006). The spread of HWA has been perpetuated by birds and other wildlife, wind, and movement of nursery stock (Evans and Gregoire, 2007; McClure, 1990). Hemlock trees in the eastern United States are susceptible to HWA due to the lack of host resistance and native natural enemies (McClure, 1989; Cheah and McClure, 2000; Wallace and Hain, 2000).

Hemlock trees are are a long lived, late successional, climax species in eastern hardwood forests, making the impact HWA has on these trees of critical importance (Orwig et al., 2002; Daley et al., 2007). The loss of hemlock trees results in major impacts on forest processes. These impacts include changes in hydrologic fluxes**,** a transition to a more homogenous forest with an increase in *Acer, Quercus, Betula,* and *Rhododendron* species, and an increase in soil temperatures due to the opening of the canopy (Orwig and Foster, 1998; Orwig et al., 1998; Jenkins et al., 1999; Ward et al., 2004; Brantley et al., 2013).

HWA feeding results in needle discoloration, dehydration, formation of false growth rings, and the prevention of new growth (McClure, 1987; McClure et al., 2001). Hemlock wooly adelgid feeding induces hypersensitive responses in hemlock trees, killing the tissue around the feeding insect. In addition, formation of false growth rings interferes with solute transport, putting the tree in a constant state of water stress and inhibiting photosynthesis (Preisser et al., 2008; Gonda-King et al., 2012; Domec et al., 2013). This defensive response from the tree has very little effect

on HWA, and it is even suggested that HWA may benefit from this response, as they contain enzymes that could be used to enhance host feeding (Preisser et al., 2008).

Biological control has the potential to contribute in a sustainable way to the management of HWA and improve the health of hemlocks in the eastern United States. All the life stages of *A. tsugae* except the crawler stage are sedentary and thus this extended amount of time spent on trees makes them easily accessible to predators. For a biological control agent to successfully suppress HWA populations, it should meet the following criteria: (i) show a significant impact and close association with the target pest; (ii) have a host range limited to HWA; (iii) originate in a climate similar to where it would be released; (iv) be tolerant to many environmental variables; and (v) be phenologically synchronized with the life cycle of HWA (Cheah et al., 2004).

Beetles in the genus *Laricobius* have been identified for biological control for their host specificity (Lamb et al., 2011). *Laricobius rubidus* and *L. nigrinus* are both native to the United States. *L. rubidus* is native to the eastern United States, and feeds on pine bark adelgid, *Pineus strobi* (Hartig) (Clark and Brown, 1960), though it also can feed and develop on HWA (Zilahi-Balogh et al. 2005). *L. nigrinus*, is a native predator of HWA in western North America (Mausel et al. 2010), and was first introduced in the eastern United States as a biological control agent in 2003 (Lamb et al., 2006; Mausel et al., 2010). To date, *L. nigrinus* has successfully established and spread in the eastern United States (Mausel et al., 2010; Mayfield, 2015).

Laricobius osakensis Montgomery and Shiyake, was discovered in Japan in 2005 from sampling *Tsuga sieboldii* (Carriere) (Montgomery and Shiyake, 2011). *L. osakensis* has the potential to be

a promising control agent, because it is a natural predator of the HWA strain present in the eastern United States (Havill et al., 2006), it has a synchronous life cycle with HWA (Vieira et al., 2013), and has greater reproduction rates than *L. nigrinus* (Vieira et al., 2012). *L. osakensis* also has a greater feeding rate, increased oviposition, and faster larval development rate than *L. nigrinus.* Both the adults and larvae of *L. osakensis* feed and are highly host-specific to HWA. These species prefer to feed and oviposit on HWA over all other prey species in laboratory tests, posing little risk to native fauna (Vieira et al., 2011). It has also been shown in laboratory studies that *L. osakensis* has no negative impact on *L. nigrinus* or *L. rubidus* (Story et al., 2012), and cannot produce viable eggs from attempted mating with either *L. nigrinus* or *L. rubidus*, even though the latter two species can successfully hybridize (Fischer et al., 2015).

Releases of *L. osakensis* were initiated in the fall of 2012 following removal from quarantine granted in 2010 by the USDA Animal and Plant Health Inspection Service (APHIS). There have been releases at 61 sites since 2012 (Roberts et al., 2011). Previous collections of *Laricobius osakensis* from the original release sites found that there were 224 beetles recovered in the 2012-2013 field season at Mountain Lake site, near Pembroke, VA, but only two in the 2013-2014 field season. Twelve beetles recovered in the 2012-2013 field season at the Carnifex, WV site, and only five in the 2013-2014 field season. One beetle was found in the 2013-2014 field season at the Goshen, VA site (Mooneyham et al., 2016). As releases continue, it is important to determine the success of the insect in establishing, its dispersal potential, and impact on HWA populations. These findings provide and may predict the long-term spread of the predator giving insight into the type of site that may provide the best habitat for successful establishment. We hypothesize that *L. osakensis* will be able to establish on HWA in the eastern United States. The

objective of this study was to sample for the presence and density of *L. osakensis* at 10 sites where *L. osakensis* was released between 2012 and 2016.

2.2 Methods:

2.2.1 Field Sites:

Six local field sites in southwest Virginia and West Virginia were sampled regularly from October 2015- April 2016 and October 2016- April 2017. The first two sites each had 500 beetles released near Mountain Lake, VA, and Carnifex, WV in 2012. In 2013, two more field releases of 2000 beetles each were made in Saltville and Goshen, VA. In 2014, two more releases were made: 1000 beetles were released at the Powhatan Boy Scout Camp and 628 beetles were released at Hungry Mother State Park. In 2015, a 2nd release of 300 beetles was made in a different location in Hungry Mother State Park (Figure 2.1). Other field sites (listed in Table 2.1) were sampled once in the fall and spring, or were sampled by collaborators. All of these sites were selected for high HWA density with perceived building populations at time of release (Mooneyham et al., 2016). Sites with a high percentage of new shoot growth were considered areas where populations could build.

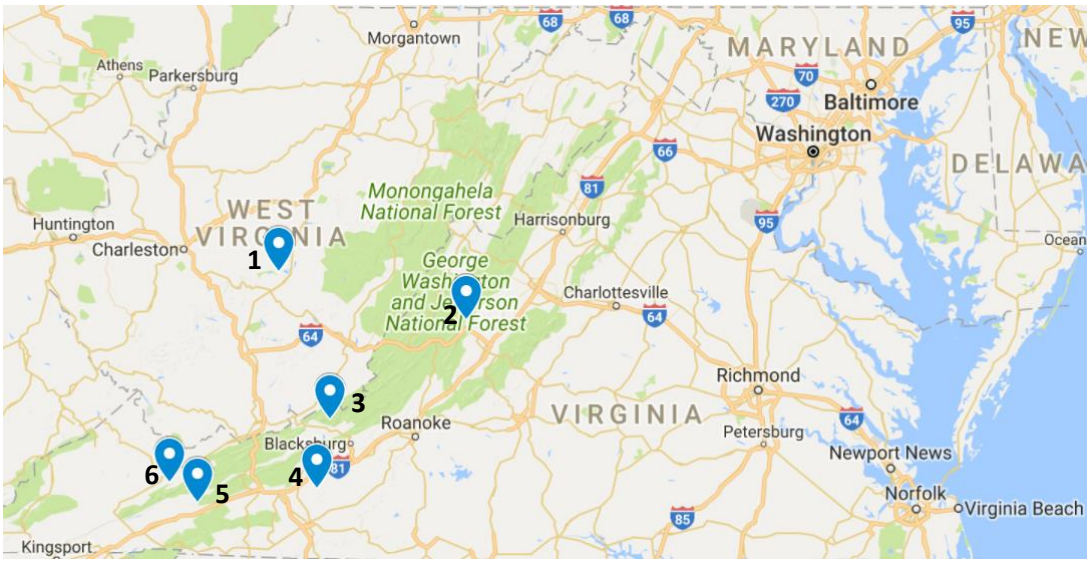

Figure 2.1: Map of locations of six local *Laricobius osakensis* release sites that were sampled regularly. Pins are numbered by field site. 1. Carnifex WV; 2. Goshen, VA; 3. Mountain Lake, VA; 4. Powhatan Boy Scout Camp, VA; 5. Hungry Mother State Park, VA; 6. Saltville, VA

2.2.2 General Field Monitoring Methods:

Tree health and HWA predator densities were monitored at each release site to determine if there were signs of predator establishment (Table 2.1). Tree health was monitored once a year at each site for each release tree, and the percentages of live crown ratio, live branches, tips alive, new foliage, and crown density were recorded. Hemlock woolly adelgid density was recorded on each tree where beat sheet sampling took place. Four branches were randomly selected from the tree, and on these branches, ten branchlets/twigs were selected to determine how many have HWA. The number of HWA and the length of the branchlet was recorded and averaged to determine the number of HWA/cm.

2.2.3 Beat sheet sampling method:

Colonization at each site was tracked monthly from October to May for two years (2015-2017). During this time, the beat sheet collection method was performed. The beat sheet was made of a 71 cm^2 canvas sheet secured to an X-frame made of PVC piping. Beat sheet sampling took place in the "plot center" of release and surrounding trees. The sheets were placed under lower canopy branches with high HWA populations, and tapped ten times with a stick. The sheets were examined to identify if any predators had dropped onto the sheets. If any were found, they were returned to the hemlock tree unharmed. The tree number (when applicable), the number of *L. osakensis* recovered, and site conditions were then recorded. Beat sheet sampling only took place on days above 0°C, and when it was not windy, snowing, or raining, as these are the worst conditions to find *Laricobius* species, and when they would be least active (Zilahi-Balogh et al., 2003). Beat sheet sampling is less effective in the winter, due to colder temperatures, than it is in the spring and fall, often producing false negatives in recovery numbers (Zilahi-Balogh et al., 2003; Mausel et al., 2010).

2.2.4 Larval Branch clipping method:

Clipping infested branches in the spring for collecting *Laricobius* larvae is a more accurate method than beat sheeting (Mausel et al., 2010). Branch clippings were collected from each site for each of two years, either in March or April, depending on when HWA oviposition was at its peak (Mooneyham et al., 2016). Eastern redbud (*Cercis canadensis* L.) flowering can

Table 2.1: *Laricobius osakensis* release sites in Virginia and West Virginia, post-release sampling year, and average health index variables (± SD) of hemlock trees.

Site	Year	Avg. % Live crown ratio	Avg. % live branches	Avg. % tips alive	Avg. % new growth	Avg. % crown density	Overall Avg. Tree health
Shenandoah National Park	2015	84.0±15.2	77.0±8.4	72.0±13.0	52.0±17.9	64.0±11.4	69.8±12.3
	2016	64.0±20.7	74.0±13.4	62.0±4.5	20.0±0.0	54.0±24.1	54.8±20.7
Hungry Mother State Park	2015	64.3±11.3	61.4±10.7	55.7±7.9	41.4±10.7	54.3±18.1	55.4±8.8
	2016	65.7±18.1	62.9±17.0	57.1±7.6	10.0±0.0	57.1±24.3	50.6±23.0
Powhatan Boy Scout Camp	2015	22.9±12.2	36.4±17.0	33.6±19.1	3.9±2.8	17.0±9.5	22.8±13.2
	2016	35.0±22.0	41.3±18.7	40.0±15.6	7.5±10.3	31.0±16.4	31.0±13.7
Mountain Lake	2015	88.8±10.3	78.8±16.5	78.8±16.5	39.4±14.8	87.5±2.9	74.7±20.3
Carnifex, WV	2015	90.0±10.0	85.0±8.7	86.7±15.3	86.7±15.3	90.0±10.0	87.7±2.2
	2016	95.0±7.1	90.0±14.1	90.0±14.1	86.7±15.3	95.0±7.1	91.3±3.6
Goshen	2015	55.6±15.9	62.5±16.9	66.3±16.0	56.3±30.7	55.6±12.7	59.3±4.9
	2016	72.0 ±10.9	66.0±8.9	72.0±4.5	20.0±0.0	68.0±8.4	59.6±22.3
Saltville	2015	63.8±11.9	65.0±12.0	66.9±11.6	48.8±3.5	50.0±16.0	58.9±8.8
	2016	60±10.7	66.25±13.0	68.75±15.5	37.5±3.5	50±19.1	56.5±12.9

be used as a phenological indicator to begin larvae sampling (Mausel et al. 2010). Eggs and larvae were sampled by selecting branches with HWA ovisacs between 2-3 mm in diameter, which indicates peak egg abundance (Zilahi-Balogh et al. 2002). Branch sections of 20-30 cm were clipped using hand pruning shears. Samples were placed into individually labeled bags, taken back to the Virginia Tech Insectary in Blacksburg, VA, and placed into rearing funnels (Salom et al., 2012). Once larvae completed development, pre-pupae began to drop into mason jars attached to the rearing funnels. The funnels were inspected several times a week to see if any larvae had dropped into the mason jars, and the number of larvae were recorded for each tree's branch clippings (Lamb et al., 2005; Salom et al., 2012). Any larvae found throughout this process were placed in a vile of 99% ethanol. Genetic testing was used to determine species, since *Laricobius* larvae are morphologically indistinguishable among species. A mitochondrial cytochrome oxidase I (COI) gene was used for species identification (Davis et al., 2011; Mooneyham et al., 2016). A partial cytochrome oxidase subunit I (COI) was amplified for *Laricobius* larvae recovered over the field season. The amplification of the COI gene was performed according to the protocols described by Davis et al. (2011) and Fischer et al. (2014).

2.2.5 Dispersal Study Methods:

Dispersal was assessed at sites where releases were performed at least two years prior to the study and had a high density of HWA present. Beat sheet sampling was performed on release trees in the "plot center," and at approximately 30, 90, and 180 m from the plot center in each cardinal direction (where applicable). In the spring, branches infested with HWA were clipped from trees approximately 30, 90, and 180 m from the plot center in each cardinal direction. These branches were labeled by distance from the release tree, and brought to the Virginia Tech

Insectary, Blacksburg, VA. Any larvae collected were put in ethanol and saved for genetic testing. The exact distances and directions from the release trees vary on a site to site basis, since there were not always trees 180 m from the release tree.

2.2.6 Statistical Analysis:

Simple correlation analyses (nonparametric Spearman's Rank Correlation) were used to measure association of both *L. osakensis* larvae and *Laricobius* spp. with different variables including HWA density at sites, tree health, number of beetles released, years since release, and plant hardiness zone. A nonparametric test was used to account for the uneven distribution of data. A correlation analysis was also used to determine an association between HWA density and tree health, and between HWA density and plant hardiness zone. We used JMP Pro software by SAS version 12.1 (Cary, NC). Because of a lack of distance related collections, no statistical analysis was performed on dispersal data.

2.3 Results:

2.3.1 Recovery of *L. osakensis*

The total number of *Laricobius* adults collected during the first field season (Fall 2015 - Spring 2016) was three beetles, all of which came from the new Hungry Mother State Park release site. All other sites that were beat sheet released in early December 2015 sampled regularly (old Hungry Mother site, Powhatan Boy Scout Camp, Goshen, and Saltville) yielded no adults. During the 2016- 2017 field season, a total of 57 adult beetles were found through beat sheet sampling. Twelve adults were found at Hungry Mother State Park site, three at the Powhatan Boy Scout Camp, 41 at Trout's Run, and one at Shenandoah National Park. *Laricobius* adults

recovered during this study were returned to the trees unharmed since the adult recoveries were low. The percentage of *L. osakensis* from adult recoveries were estimated based on the number of *L. osakensis* found in larval recoveries later in the spring. The local sites (Hungry Mother, Boy Scout Camp, Goshen, Saltville, Carnifex, and Mountain Lake) were all sampled for adults 6-7 times throughout the course of each field season. The remaining sites, all further away, were sampled once in the fall and once in the spring.

In the first year of larval collections (Spring 2016), a total of 88 larvae were found. Definitive genetic analysis identification was achieved on 77 of them. From these, 14 were found to be *L. osakensis*. Eight *L. osakensis* larvae were found at the new Hungry Mother Release site, two in Shenandoah National Park, two at the Powhatan Boy Scout Camp, and two in Great Smoky Mountain National Park (this site is no longer monitored due to damage from a forest fire) (Table 2.2). No *L. osakensis* was found from other sites where larval branch clippings were collected, including Goshen, old Hungry Mother State Park site, Racine, Grandview, and South Cherokee National Forest. Other *Laricobius* larvae were collected, yielding a total of 41 *L. rubidus* and 22 *L. nigrinus* from varying sites. Larval branch clipping collections occurred once in the spring at each field site.

In the second year of larval collections (Spring 2017), a total of 318 larvae were collected. Conclusive genetic analysis identification was achieved on 255 of the 318 larvae. From these larvae, a total of 90 were found of be *L. osakensis.* Forty-six were from Trout's Run, 11 from Powhatan Boy Scout Camp, 12 from the old Hungry Mother release site, and 21 from Goshen (Table 2.2). No *L. osakensis* were recovered from other sites where larval branch clippings were

collected, including new Hungry Mother release site, Shenandoah National Park, South Cherokee National Forest, and Grandview. Larval branch clipping collections occurred once in the spring at each field site. The second year's larval collection also yielded a total of 103 *L. nigrinus* and 62 *L. rubidus* from varying release sites (Table 2.2). There was a significant positive correlation between *Laricobius* spp. recovered and HWA density, a negative correlation between average tree health and the number of *L. osakensis* larvae and *Laricobius spp.*, and a significant correlation between HWA density and plant hardiness zone (Table 2.3).

2.3.2 Dispersal Study

Sampling for dispersal in 2016 yielded no *L. osakensis*. In 2017, 19 *Laricobius osakensis* larvae were found on sample trees. At Powhatan Boy Scout Camp (2014 release), the furthest beetles found were 30 m outside release center. At Goshen (2013 release) the furthest a beetle was found was 30 m outside release site. At the old Hungry Mother State Park (2014 & 2015 release) site the furthest a beetle was found was 90 m outside of the release site. This dispersal study was on a smaller scale, and as a result, most beetles were found within close proximity to release center.

Table 2.2: 2015-2016 & 2016-2017 Field collections of *Laricobius* spp., including information on site location, plant hardiness zone of the field site, year of beetle release, number of beetles released, and HWA density (adelgids/cm) at time of sampling.

Site	Year of Release	Number Released	Plant hardiness zone[2]	Field Season	HWA Density (adelgid/cm)	*Laricobius* adults (percent estimated *L. osakensis*)[3]	*Laricobius osakensis* larvae	*Laricobius nigrinus* larvae	*Laricobius rubidus* larvae
Mountain Lake (VA)	2012	500	5b	2015-2017	~0	0	0	0	0
Carnifex (WV)	2012	500	6a	2015-2017	~0	0	0	0	0
Saltville (VA)	2013	2000	5b	2015-2017	~0	0	0	0	0
Goshen (VA)	2013	2000	6b	2015-2016	1.4 ± 1.0	0	0	0	7
				2016-2017	1.1 ± 0.6	0	21	2	38
Hungry Mother (VA)	2014	628	6b	2015-2016	1.7 ± 0.8	3	8	2	0
				2016-2017	1.6 ± 0.8	12 (13%)	12	62	16
Boy Scout Camp (VA)	2014	1000	6b	2015-2016	1.0 ± 0.4	0	2	0	26
				2016-2017	1.2 ± 0.6	3 (19%)	11	39	8
Shenandoah National Park (VA)[1]	2015	500	6b	2015-2016	1.9 ± 0.7	0	2	3	3
				2016-2017	1.8 ± 0.5	1	0	0	0
Great Smoky Mountain National Park (TN)	2014	921	6b	2015-2016	1.3 ± 0.3	0	2	0	0
Trouts Run (PA)[1]	2015	1021	6a	2016-2017	1.6 ± 0.4	41 (100%)	46	0	0
South Cherokee National Forest (TN)	2014	781	7a	2015-2016	1.9 ± 0.6	0	0	0	5
				2016-2017	1.8 ± 0.6	0	0	0	0

[1] Released beetles were a mix of three *Laricobius* spp. with ~34% being *L. osakensis.*

[2] Plant hardiness zone temperatures; Average annual extreme minimum temperature 1976-2005; 5b: -15 to -10 ^{o}C, 6a: -10 to -5 ^{o}C, 6b: -5 to 0 ^{o}C, 7a: 0 to 5 ^{o}C.

[3] Estimated percent of *L. osakensis* adults based on identified larval recoveries

Table 2.3: Simple correlation analysis using a nonparametric Spearman's rank correlation between different factors measured. Comparisons include *Laricobius* recoveries to HWA density, average tree health, plant hardiness zone, number of beetles released, and years since release. Correlation analysis was also assessed for HWA density compared to plant hardiness zone and tree health.

Factors	Correlation	Correlation value	Spearman p	p value
L. osakensis larvae & HWA density	No significant correlation	r= 0.2934	0.2879	0.247
Laricobius spp. & HWA density	Positive correlation	r= 0.3411	0.4829	0.042
L. osakensis larvae & avg. tree health	Negative correlation	r= -0.5435	-0.5588	0.045
L. osakensis larvae & plant hardiness zone	No significant correlation	r= 0.0622	0.2062	0.412
L. osakensis larvae & yrs since release	No significant correlation	r= -0.1210	-0.2587	0.300
L. osakensis larvae & # beetles released	No significant correlation	r= -0.0316	0.1915	0.447
HWA density & plant hardiness zone	Positive correlation	r= 0.8182	0.8298	0.001
HWA density & tree health	No significant negative correlation	r= -0.4789	-0.5032	0.066

2.4 Discussion:

Beat sheet sampling produced low numbers throughout both years of sampling, with only 60 beetles (41 of which were collected at Trout's Run) collected despite monthly field visits. Studies have shown that beat sheet sampling with small, hard to find insects is difficult and can produce false negatives (Venette et al., 2002; Mausel et al., 2010). Beat sheet sampling is less effective in the winter (Zilahi- Balogh et al., 2003), which could explain some of the lack of recoveries in this study. The larval branch clipping sampling method was much more successful in finding our target predators.

The low recovery numbers in the first year of field work was likely attributed to the below average winter temperatures in the 2013-2014 season. The below average temperatures resulted in a significant decrease in adelgid density throughout the range of HWA in the eastern U.S. (McAvoy et al., 2017). The low adelgid populations most likely made it difficult for *L. osakensis* to colonize. The following year, the adelgid populations were able to rebound, coinciding with increased winter temperatures, and as a result, the numbers of *L. osakensis* collections over the course of the study correlated to this increase in HWA. The increased *L. osakensis* populations suggests that this agent is establishing at some of the release sites. As HWA populations increase, the number of *L. osakensis* should continue to increase as well. Fischer et al. (2015) supports this hypothesis, as they reported increasing *L. nigrinus* populations in comparison to decreasing *L. rubidus* populations over time.

There was a negative correlation between tree health and *Laricobius* recoveries, and while not significant, there was a weak negative correlation between HWA density and tree health. This suggests that as tree health decreases, our likelihood of finding *Laricobius* beetles increases, and that as HWA density decreased, the health of the tree increased. These results may indicate that trees with higher HWA density, and therefore decreased tree health, are more likely to yield more *Laricobius* beetles, and not that these predators are more likely to thrive on unhealthy trees. The negative correlation between HWA density and tree health is expected because trees with low HWA density were expected to be healthier than those with high HWA densities, but also because HWA feeds in cycles (Sumpter et al., 2018). The cyclical feeding pattern occurred when the health of a tree declined, HWA populations decline due to lack of available feeding sites (new growth) (McClure, 1991). When the health of the tree rebounds, availability of feeding

sites increases for the adelgids (McClure, 1991). The relationship in tree health and adelgid populations is a density-dependent reciprocal feedback loop, with observed lag effects, often resulting in tree health decline over time (Sumpter et al., 2018). Extreme cold temperatures associated with the polar vortex reduced HWA densities (McAvoy et al., 2017), and gave an opportunity for the trees to rebound from this pest. The significant correlation between plant hardiness zone and HWA density indicated that trees in colder plant hardiness zones had lower densities of HWA. Trees from colder plant hardiness zones, like the Carnifex site, the Mountain Lake site, and the Saltville site have had almost no HWA since the polar vortex occurred. This suggests that the HWA populations struggled to rebound at these sites due to cold temperatures, and has allowed the trees at these locations to experience sustained growth for longer periods of time, leading to overall healthier trees (Table 2.1).

Some of the 2015 *L. osakensis* releases were accidentally contaminated with *L. nigrinus* and *L. rubidus*. Occasionally, HWA were collected for insect rearing where other *Laricobius* spp. were unknowingly present, and despite efforts to clean off branches from predators, some slip through. The composition of *Laricobius* spp. is generally unknown until genetic testing is done on the larvae following rearing. Therefore, when releases take place, there were times when the released composition of beetles was mixed. Subsequent collections often lead to multiple *Laricobius* spp. recoveries. Specifically, the contaminated *L. osakensis* releases were a combination of the F3 and F5 cohorts. The F3 Parents (99 total): *L. osakensis* 23%, *L. nigrinus* 57%, *L. rubidus* 6%, and unknown 13%. The F5 Parents (220 total beetles): *L. osakensis* 40%, *L. nigrinus* 46%, *L. rubidus* 5%, and unknown 8%. In total, the parents of the beetles released in 2015 were only ~34% *L. osakensis*. The beetles recovered in the 2015-2016 field season were

18% *L. osakensis*. This contamination will impact the results of future field monitoring data at these release sites. The Goshen and Boy Scout Camp release sites had two and 39 *L. nigrinus,* respectively. Since there was no documented contamination at these release sites, it suggests: 1. the *L. nigrinus* dispersed from other release locations; 2. there were undocumented releases at sites nearby; or 3. that previous *L. osakensis* colonies had been unknowingly contaminated. The closest *L. nigrinus* site to the Goshen release site was approximately 33 km away (Roberts et al. 2011), and the release was made in 2012. The closest *L. nigrinus* site to the Boy Scout Camp was ca 35 km away, and had releases in both 2010 and 2014 (Roberts et al., 2011). Due to *L. rubidus* being native to the field sites, it is not surprising to have the species appear in our samples. Our field sites commonly had white pine with pine bark adelgid, the preferred host for *L. rubidus* (Mausel et al., 2008, Wantuch et al. 2017).

Dispersal rates of *L. osakensis* and *L. nigrinus* were predicted to be similar to one another. Davis et al. (2012) found that *L. nigrinus* dispersed at a rate of 30.6 – 39.2 m/yr, but we obtained insufficient data for *L. osakensis* to make any type of comparison from this study. Due to the variation in field sites, some lacked hemlock trees with HWA at distances sampled, or there was an accessibility issue due to main roads and rivers limiting sampling. This study focused on local-scale dispersal of this insect, and perhaps future studies can monitor for further spread of these insects at longer distances and yield different results. Dispersal could be greater than these data suggested due to the low recovery numbers and the previously mentioned factors. One might predict that with increasing HWA populations, *L. osakensis* beetles will continue to establish and the dispersal rate of *L. osakensis* is likely to be similar to that of *L. nigrinus.*

L. osakensis appears to be established at Hungry Mother State Park, Goshen, and Powhatan Boy Scout Camp release sites. Some of the earlier release sites, including Mountain Lake, Saltville, and Carnifex had very low adelgid numbers, which explained the lack of *L. osakensis* recoveries. Continued sampling at all of the release sites is necessary to verify establishment. Our objective is to ensure successful establishment and spread of *L. osakensis* to control HWA. Upon successful establishment, we hope to find sites suitable for field insectaries. This will provide more natural conditions for rearing predators, as beetles will be better adapted to the environment, healthier, and more robust (Mausel et al., 2010). The objective is to build up predator populations for harvesting and redistributing on a consistent basis.

2.5 References

Bentz, S. E., M. E. Montgomery, and R. T. Olsen. 2008. Resistance of hemlock species and hybrids to hemlock woolly adelgid. In: B. Onken, and R.Reardon, comps. Fourth Symposium on hemlock woolly adelgid in the eastern United States; 2008 February 12-14; Hartford, CT. FHTET 2008-01. Morgantown, WV: U.S. Forest Service, Forest Health Technology Enterprise Team: 137-139.

Caltagirone, L. E. 1981. Landmark examples in classical biological control. Ann. Rev. Entomol. 26: 213–232.

Cheah, C. A., and M. S. McClure. 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). Agric. For. Entomol. 2: 241–251.

Cheah, C., R. C. Reardon, and B. Onken. 2004. Biological control of hemlock woolly adelgid. US Department of Agriculture, Forest Service, FHTET 2014-05.

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

Cowles, R. S., M. E. Montgomery, and C.-J. Cheah. 2006. Activity and residues of imidacloprid applied to soil and tree trunks to control hemlock woolly adelgid (Hemiptera: Adelgidae) in forests. J. Econ. Entomol. 99: 1258–1267.

Daley, M. J., N. G. Phillips, C. Pettijohn, and J. L. Hadley. 2007. Water use by eastern hemlock (*Tsuga canadensis*) and black birch (*Betula lenta*): implications of effects of the hemlock woolly adelgid. Can. J. For. Res. 37: 2031–2040.

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

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

Domec, J. C., L. N. Rivera, J. S. King, I. Peszlen, F. Hain, B. Smith and J. Frampton. 2013. Hemlock woolly adelgid (*Adelges tsugae*) infestation affects water and carbon relations of eastern hemlock (*Tsuga canadensis*) and Carolina hemlock (*Tsuga caroliniana*). New Phytologist. 199:452-463.

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

Fischer, M. J., C. C. Brewster, N. P. Havill, S. M. Salom, and L. T. Kok. 2015. Assessment of the potential for hybridization between *Laricobius nigrinus* (Coleoptera: Derodontidae) and Laricobius osakensis, predators of the hemlock woolly adelgid (Hemiptera: Adelgidae). Biocontrol Sci. Technol. 25: 1467–1482.

Fischer, M. J., N. P. Havill, C. S. Jubb, S. W. Prosser, B. D. Opell, S. M. Salom, and L. T. Kok. 2014. Contamination delays the release of *Laricobius osakensis* for biological control of Hemlock Woolly Adelgid: Cryptic diversity in Japanese *Laricobius* spp. and colonypurification techniques. S.E. Naturalist 13:178–191.

Gonda-King, L., L. Radville, and E. L. Preisser. 2012. False ring formation in eastern hemlock branches: Impacts of hemlock woolly adelgid and elongate hemlock scale. Environ. Entomol. 41: 523-31.

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

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

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

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

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

Lamb, A., M. E. Montgomery, L. C. Vieira, S. Shiyake, and S. Salom. 2011. *Laricobius osakensis*, a hemlock woolly adelgid predator from Japan. Implementation and Status Biological Control of the Hemlock Woolly Adelgid. Print. 90.

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

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

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

McAvoy, T. J., J. Régnière, R. St-Amant, N.F. Schneeberger, and S. M. Salom. 2017. Mortality and recovery of hemlock woolly adelgid (*Adelges tsugae*) in response to winter temperatures and predictions for the future. Forests. 8: 497.

McClure, M. S. 1987. Biology and control of hemlock woolly adelgid. Connecticut Agricultural Experiment Station New Haven, CT. 851.

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

McClure, M. S. 1990. Role of wind, birds, deer, and humans in the dispersal of hemlock woolly adelgid (Homoptera: Adelgidae). Environ. Entomol. 19: 36–43.

McClure, M. S., S. M. Salom, and K. S. Shields. 2001. Hemlock woolly adelgid. USDA Forest Service Publ. FHTET–2001–03. Morgantown, WV.

Miniat, C. F., D. Zeitlow, S. T. Brantley, A. Mayfield, R. Rhea, R. Jetton, and P. Arnold. 2016. Physiological responses of eastern hemlock (*Tsuga Canadensis*) to biological control and silvicultural release: implications for hemlock restoration. In: Stringer, C. E., K. W.

Krauss, and J. S. Latimer, eds. 2016. Headwaters to estuaries: advances in watershed science and management -Proceedings of the Fifth Interagency Conference on Research in the Watersheds. March 2-5, 2015, North Charleston, South Carolina. e-General Technical Report SRS-211. Asheville, NC: U.S. Department of Agriculture Forest Service, Southern Research Station. 302 p.

Montgomery, M. E., S. Shiyake, N. P. Havill, and R. A. B. Leschen. 2011. A new species of *Laricobius* (Coleoptera: Derodontidae) from Japan with phylogeny and a key for native and introduced congeners in North America. Ann. Entomol. Soc. Am. 104: 389–401.

Mooneyham, K., Loke T. Kok, K. S. M. Salom. 2016. Release and colonization of *Laricobius osakensis* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid, *Adelges tsugae*. N.E. Naturalist. 23: 141–150.

Morin, R. S., S. N. Oswalt, R. T. Trotter III, and A. M. Liebhold. 2011. Status of hemlock in the eastern United States. USDA For. Serv. e-Science Updat. SRS-038.

Orwig, D. A., and D. R. Foster. 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. J. Torrey Bot. Soc.1: 60–73.

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

Preisser, E. L., K. L. F. Oten, and F. P. Hain. 2014. Hemlock woolly adelgid in the eastern United States: What have we learned? S.E. Naturalist 13: 1-15.

Roberts, A., A. Lamb, B. Onken, and S. Salom. 2011. HWA predator release and monitoring database. *In* R. Reardon and B. Onken (eds.), Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. USDA Forest Service, FHTET-2011-04.

Salom, S. M., L. T. Kok, A. B. Lamb, and C. Jubb. 2012. Laboratory rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A predator of the hemlock woolly adelgid (Hemiptera: Adelgidae). Psyche 2012: 1-9.

Souto, D., T. Luther, and B. Chianese. 1996. Past and current status of HWA in eastern and Carolina hemlock stands. Proc. first hemlock woolly adelgid Rev. Charlottesville, Virginia, USA. 12 Oct. 1995.FHTET 96-10 9–15.

Story, H. M., L. C. Vieira, S. M. Salom, and L. T. Kok. 2012. Assessing performance and competition among three *Laricobius* (Coleoptera: Derodontidae) species, predators of hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). Environ. Entomol. 41:

896–904.

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

Venette R. C., R. D. Moon, and W. D. Hutchison. 2002. Strategies and statistics of sampling for rare individuals. Ann. Rev. Entomol. 47: 143-174.

Vieira, L. C., A. B. Lamb, S. Shiyake, S. M. Salom, and L. T. Kok. 2013. Seasonal Abundance and Synchrony Between *Laricobius osakensis* (Coleoptera: Derodontidae) and Its Prey, *Adelges tsugae* (Hemiptera: Adelgidae), in Japan. Ann. Entomol. Soc. Am. 106: 249–257.

Vieira, L. C., T. J. Mcavoy, J. Chantos, A. B. Lamb, S. M. Salom, and L. T. Kok. 2011. Host Range of *Laricobius osakensis* (Coleoptera: Derodontidae), a New Biological Control Agent of Hemlock Woolly Adelgid (Hemiptera: Adelgidae). Environ. Entomol. 40: 324–332.

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

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

Wantuch, H.A., T.P. Kuhar, and S.M. Salom. 2017. Phenology of the pine bark adelgid, *Pineus strobi* (Hemiptera: Adelgidae), in white pine forests of southwestern Virginia. Environ. Entomol. 46: 1195-1201.

Ward, J. S., M. E. Montgomery, C. A.-J. Cheah, B. P. Onken, and R. S. Cowles. 2004. Eastern hemlock forests: guidelines to minimize the impacts of hemlock woolly adelgid. U.S. Dep. Agric. –FS Northeastern Area State and Private Forestry Publication NA-TP-03-04. Morganstown, WV.

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

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

Chapter 3: Seasonal assessment of supercooling points for *Laricobius osakensis* and *Laricobius nigrinus*, predators of the hemlock woolly adelgid

Abstract

The hemlock woolly adelgid (HWA) is an invasive insect causing significant hemlock mortality in the eastern United States. The geographic spread of this insect has been limited by cold temperatures, specifically sustained extreme cold temperatures. In 2014, a polar vortex led to a decline in HWA densities throughout the eastern U.S. This decline led to a decrease in densities of *Laricobius osakensis,* a biological control agent of HWA. It was uncertain if the decline of *L. osakensis* densities was due to the extreme cold weather event, a lack of its primary host HWA, or both. Laboratory experiments were conducted to assess the survival and cold hardiness of insects during winter. The supercooling point temperature is the lowest temperature to which a species can avoid freezing of body fluids. Identification of this lethal temperature may be useful in providing best management strategies for choosing the best suited biological control agent for a particular region of the country. Our objective was to determine the supercooling point (SCP) of two distinct populations of *L. osakensis*, collected from northern and southern Japan, and another introduced biological control agent, *Laricobius nigrinus*. The SCP of both the northern and southern populations of *L. osakensis* and *L. nigrinus* were measured from November 2016 to February 2017. The overall mean supercooling points of the northern population of *L. osakensis* was -13.5 ^{o}C (± 0.5), the southern population of *L. osakensis* was -13.4 ^{o}C (± 0.5), and the mixed population *L. nigrinus* was -13.5 ^{o}C (± 0.5). No significant SCP differences between northern or southern populations or species were found. The results suggest that supercooling points should not be a factor when selecting *Laricobius* spp. biological control agents for a particular location.

There were significant differences between the average SCPs of all species in February compared to the average SCPs of all species in the other months.

Keywords: Supercooling point, cold tolerance, *Laricobius osakensis*, *Laricobius nigrinus*

3.1 Introduction:

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an invasive insect that has caused significant eastern hemlock, (*Tsuga canadensis,* (L.) Carriere), and Carolina hemlock, (*Tsuga caroliniana* Engelmann), damage in a large portion of the eastern United States (Havill et al. 2006). Though this pest is active in the winter, the spread has been limited by cold temperatures. In the winter of 2014, extreme and sustained cold temperatures occurred in the eastern United States during a "polar vortex." This event led to a drastic decrease in HWA densities (McAvoy et al., 2017; Tobin et al., 2017). The HWA biological control agent, *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) had a decrease in density concurrent with its host, HWA (Mooneyham et al., 2016). This event came two years after the initial releases of this insect, making it difficult to determine if difficulty in their establishment was due directly to the extreme temperatures or indirectly due to loss of prey.

A majority of insect winter survival can be split into three categories: (i) chill-susceptible insects (ii) freeze-tolerant insects, (iii) freeze-intolerant (also known as freeze avoidant) insects (Figure 3.1). Chill-susceptible insects perish before freezing, and are likely found in temperate areas (Bale, 1993; Sinclair, 2015). Freeze-tolerant insects are able to survive internal ice formation through the regulated freezing of their body water, with ice formation potentiated by the action of ice nucleating agents (INA's) and ice nucleating proteins (INP's) (Ramlov and Westh, 1993;

Block et al. 1998; Storey and Storey, 2013, Dunman 2001). Freeze intolerant insects are killed by freezing, so they have to depress the temperature at which their body fluids freeze (the supercooling point) (Croswaithe et al., 2010). The supercooling point is the depressed temperature at which the ice crystals begin to form, and can be detected when ice forms in an insect, resulting in a temperature spike from the release of latent heat of fusion (Renault et al., 2002; Mausel et al., 2011). To avoid mortality, freeze intolerant insects must maintain their body fluids below their melting point by removing ice nucleators that may initiate ice formation, by synthesizing antifreeze proteins to reduce the nucleation potential of seed crystals, and by accumulating extremely high levels of carbohydrate crytoprotectants (most often glycerol) that lower crystallization temperature (Zachariassen, 1985; Ramlov, 2000; Holmstrup et al., 2002; Sinclair et al., 2003). All of these factors decrease the supercooling point of the insect, and as a result helps to prevent the formation of ice in the hemolymph or gut (Zachariassen and Kristiansen 2000).

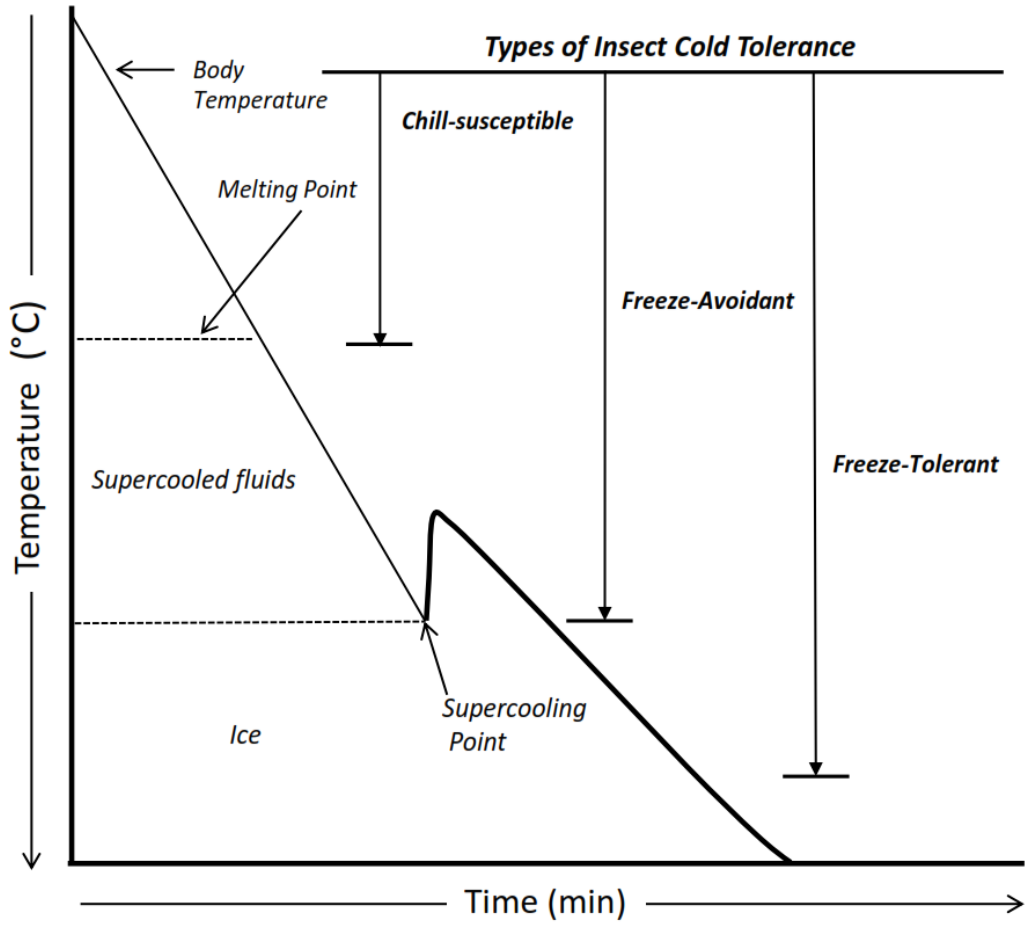

Figure 3.1: Graph displaying the three main types of cold tolerance and their freezing points (Sinclair et al. 2015).

This study was designed to examine the SCP's of *L. osakensis* to determine if the low recovery numbers were due to the beetles being intolerant of low sustained temperatures, or a lack of a primary food source (HWA). *L. osakensis* has a limited ability to feed on other prey species, making it difficult for them to survive when there are extreme fluctuations in HWA populations (Vieira et al., 2011).

Temperature biology is an excellent way to predict species distribution, since cold temperatures are often a limiting factor to their success (Andersen et al., 2015). When determining the SCP, lab reared insects should be acclimated to field conditions since insect's physiology adapts as temperatures change, impacting its cold tolerance (Salt, 1961; Cira et al., 2016). Cold hardiness is not a constant feature in insects as it may vary throughout the season and year. As a result, SCPs needs to be taken multiple times throughout the year (Danks, 2005). Special interest was given to how the SCP of both *L. osakensis* populations compared to one another in this study. These populations are hypothesized to have different cold tolerances, since they come from different climates in Japan. As a result, the northern population has been released in northern parts of the United States, and the southern population in southern parts of the United States. The populations of *L. osakensis* were also compared to the SCP of another biological control agent of HWA, *L. nigrinus*, (combination of coastal and interior strains) under the same environmental conditions. Mausel et al. (2011) determined the supercooling points of the coastal and inland strain of *L. nigrinus*. The coastal strain of *L. nigrinus* was collected from the Puget Trough area (including British Columbia, Victoria, and Seattle, Washington), which has a mild maritime climate (Mausel et al., 2011). The interior strain of *L. nigrinus* was collected from the Rocky Mountain area (Coeur d'Alene and Moscow Idaho), which has a colder and snowier continental climate (Mausel et al., 2011). Even though the SCP of *L. nigrinus* have been studied in the past, it is important to compare *L. nigrinus* SCP to northern and southern population *L. osakensis* using the same field conditions. This will allow for a more accurate comparison of the species, since time of year, local temperature, and extreme weather events all impact insect supercooling points. The comparison of SCPs of different *Laricobius* spp. and populations is intended to provide an additional indicator as to which biocontrol agent would be best suited for this region

of the country, as it has been found that mismatched biological control agents can fail to become established (Mausel et al., 2011; Hopper et al., 1993). Since SCP varies by location, year, and time of year, we compared the *Laricobius* spp. and populations under identical environmental conditions from November 2016 – February 2017.

3.2 Methods:

3.2.1 Colony rearing and acclimating beetles to field conditions.

A total of 284 southern population *L. osakensis* beetles (F1), 200 northern population *L. osakensis* (F1), and 200 *L. nigrinus* (F2) (mixed populations) were used in this study. The original southern population of *L. osakensis* was collected in the Osaka region of Japan in 2015, and are reared in the insectary at the University of Tennessee in Knoxville, TN (Table 3.1). The original beetles of the northern population of *L. osakensis* were collected from the northern mountain areas of Japan in 2015, and reared at the insectary at Virginia Tech in Blacksburg, VA (Table 3.1).

When HWA insects broke diapause in the late fall of 2016, the lab reared northern and southern populations of *L. osakensis* and *L. nigrinus* were removed from the Virginia Tech Insectary and placed onto hemlock trees at the Prices Fork Research Center (Blacksburg, VA). Beetles were placed in fine nylon mesh (104 x 94 mesh/square inch) 1 m x 66 cm x 66cm cages (MegaView Science, Taichung, Taiwan) that were zip tied to hemlock tree branches (Figure 3.2). Each month from November 2016 – February 2017, twenty beetles of each species and population were collected from the cages for SCP determination. Due to the low density of HWA at this

location, branches with high HWA densities were placed in small cups full of floral foam and zip tied to the branches inside of the mesh cages. Ten beetles were introduced into each cage and labeled by species. As HWA density decreased in the mesh cages, branches with higher HWA density were added or the branches were clipped, the beetles were collected, and then put into new mesh cages with fresh food.

The *Laricobius* spp. were acclimated to environmental conditions at Prices Fork Research center for two weeks before the first batch of supercooling point lab work was conducted in November 2016. Beetles were collected by clipping the branches two days prior to laboratory analysis, and beetles were collected from the cages at the Virginia Tech Insectary. Only living beetles were used for determining SCP. Missing or dead beetles were recorded. Beetles collected from field cages were kept in plastic containers without food for 48 h at 0° C before SCP analysis (Mausel et al., 2011).

Table 3.1: Locations where the northern and southern populations of *Laricobius osakensis* adults were collected from in Japan in 2015.

Population	City	Location	Elevation	Number Collected
Northern	Nagano	N 36.68213, E 138.50032	1720 m	85
Northern	Gunma	N 36.81287, E 139.34099	1550 m	248
Northern	Tochigi	N 36.80325, E 139.42031	1485 m	134
Southern	Nara	N 34.41465, E 135.51145	290 m	2
Southern	Kyoto	N 34.04233, E 135.45733	150 m	14

Southern	Kobe	N 34.44392, E 135.10750	440 m	5
Southern	West of Kyoto	N 34.57570, E 135.36691	375 m	67

Figure 3.2: Experimental set up of mesh cages (1 m long) on the hemlock tree branches at the Virginia Tech Price's Fork Research Center. Cages contained one of three treatments: northern or southern populations of *L. osakensis* or *L. nigrinus* beetles. Ten beetles were placed into each cage. Supercooling point was determined for all species once a month (Nov. 2016 – Feb. 2017).

3.2.2 Determination of beetle supercooling points:

Groups of four *Laricobius* adults were placed in specially constructed thermocouple arenas and placed on an electronic cooling plate (Thermoelectronics; Stir-Kool SK-1), used to generate a

controlled slow-freezing environment. The cooling plate was linked to a constant temperature water bath (Isotemp; Fischer Scientific) (1:1 u/v H_2O: 95% EtOH) held at -26 ^oC. Precision fine wire thermocouples (Type T 0.13 x 0.03 mm copper constantan, Omega Engineering, Inc) were covered with a thin layer of zinc oxide thermal grease (Zinc oxide/ type B emersion oil) for improved connection. The ideal cooling rate was achieved by stacking different materials for the cooling plate calibration. The first layer was a piece of cardboard, followed by the thermocouple arena, foam insulation, a layer of corrugated cardboard, an aluminum weight, and then enclosed in an insulated cover. The apparatus was calibrated to achieve a maximum low temperature of -25 °C. After placing insects on the thermocouples, cooling was initiated around 25 °C declining at a rate of about -0.5 °C/min until the SCP was reached (Croswaithe et al., 2011). Cool-plate settings were adjusted when needed to keep the cooling rate consistent. The supercooling point was recorded for each species, and later compared to one another. The temperature recording equipment was a PC based system with an 8-channel high-speed thermocouple interface (Omega OM-DAQ_USB-2401 Multiple Channel USB Data Acquisition Module). This equipment included connections to eight pairs of 0.0762 mm copper/constantan fine wire thermocouples, and the use of OM-DAQ recording software.

3.2.3 Statistical Analysis

The data set was not normally distributed, and therefore it was normalized using a Box-Cox Transformation. Main factors (month and species) and interactions between factors were analyzed using standard least squares (SLS) to determine significance of differences ($P<0.05$). Means were separated using a Student's t-test. Analyses were performed using JMP Pro software by SAS version 12.1 (Cary, NC).

3.3 Results:

During the acclimation portion of the experiment, there was a high rate of mortality for all three groups of beetles in the fine mesh cages during the acclimation period to outside environmental conditions. The mortality of southern population of *L. osakensis* was 157 (45%), the mortality of the northern population was 114 (57%), and *L. nigrinus* experienced a mortality of 104 (52%) throughout the course of the experiment. The average temperature beetles were exposed to in the field in November before testing was 5.5 ^{o}C, in December was 4.1 °C, in January was 3.3 °C, and in February was 4.9 °C.

The average SCP (± S.E.) of the northern population of *L. osakensis* was -13.5 °C (± 0.5) with temperatures that ranged from -5 ^{o}C to -23 ^{o}C, the southern population of *L. osakensis* was -13.4 °C (± 0.5) with temperatures that ranged from -5 ^{o}C to -22 ^{o}C, *L. nigrinus* was -13.6 ^{o}C (± 0.5) with temperatures that ranged from -6 ^{o}C to -21 ^{o}C. Figure 3.3 shows the supercooling points of northern population of *L. osakensis* in January, the supercooling points can be seen on the thermal curve right before the temperature spike produced by the release of latent heat of fusion. There were no significant differences between supercooling points between the different species and populations. The supercooling points for each month varied numerically throughout the course of the experiment (Figure 3.4). No overall changes in supercooling points were observed from November to January, but there was a significant difference in average supercooling points in February compared to the other three months (Fig. 3.4). Overall, there appears to be a general trend toward higher temperatures from November to February. In November the average (± S.E.) SCP was -14.1 °C (± 0.6), December was -13.9 ^{o}C (± 0.5), January was -13.6 °C (± 0.5), and February was -11.5 °C (± 0.6).

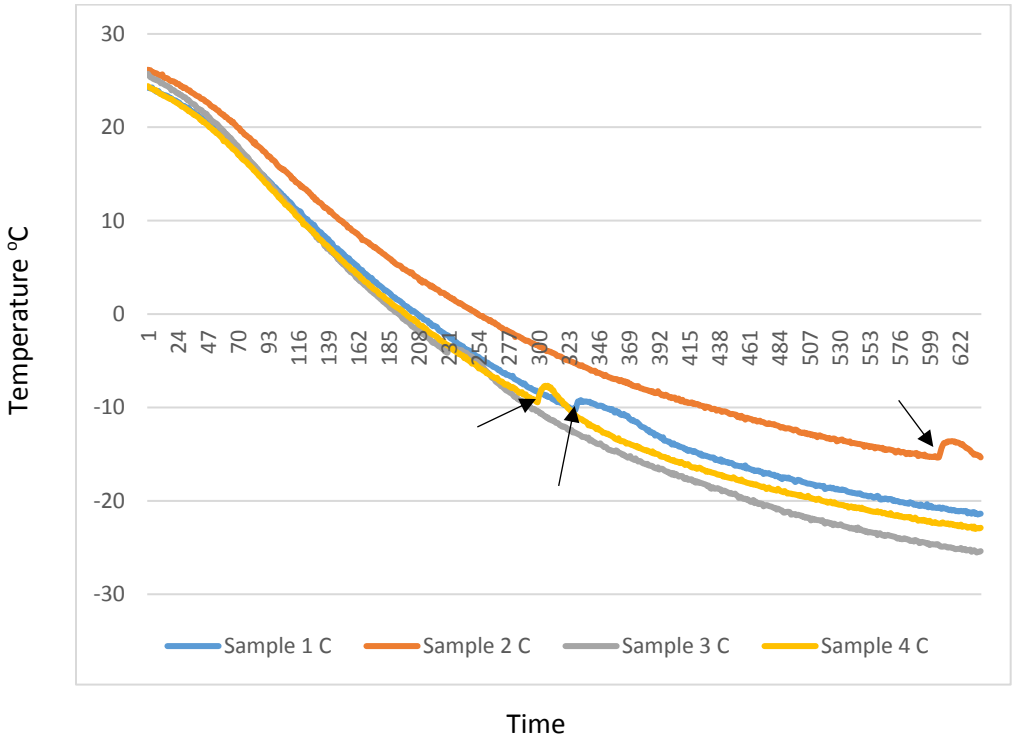

Figure 3.3: An example of the supercooling point testing on the northern population of *L. osakensis* in January 2017. Sample 1: SCP =-10.9 ºC, Sample 2: SCP =-15.4 ºC, Sample 3: no measured SCP was detected, Sample 4: SCP =-9.5 ºC. The arrows point to the supercooling point on the thermal curve.

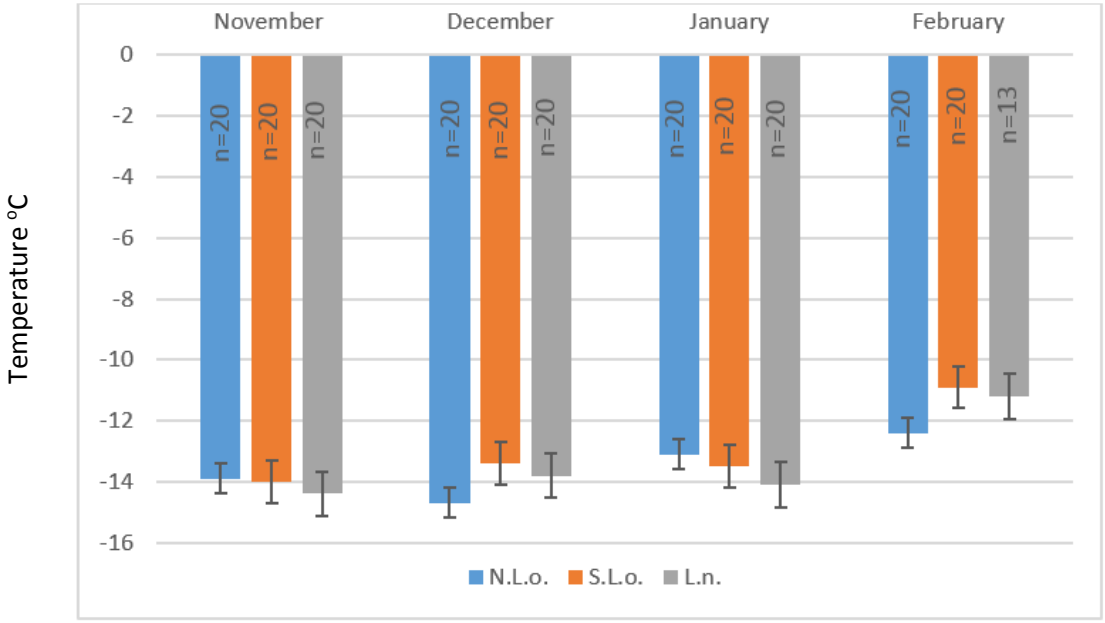

Figure 3.4: Comparison of the average supercooling points (± S.E.) of *L. osakensis* populations (N.L.o = northern; S.L.o = southern) and *L. nigrinus* (L.n.) from November 2016 to February 2017. Using a standard least squares statistical test, no significant differences were found between the different species/ populations.

3.4 Discussion:

There was a high level of beetle mortality in the fine mesh cages. Access to food should not have been an issue, since HWA were kept at high densities in the mesh cages. The high mortality rate could be a result of the small area of the mesh cages reducing the ability of the beetles to locate preferred, protected areas when weather conditions were not ideal. The mesh cages may have created microclimates and with decreased temperatures of otherwise increased moisture, less than ideal conditions for the beetles. Additionally, some of the southern population beetles used for this study emerged prematurely, which may have contributed to their poor health and high

mortality in the first few weeks of the study. Ideally, this study would have continued through March if there had been sufficient numbers of beetles that would have provided more information on these populations to the environmental conditions.

The warmest SCPs were observed in February. This was most likely due to a high monthly average temperature, along with three consecutive days of >10 ºC before beetles were collected for SCP analysis. The physiological changes in cold hardiness change quite rapidly and may have impacted the SCPs. The SCPs appear to correlate with the most recent daily temperatures before collection (Figure 3.5). November and December had the lowest temperatures before collection, and also had the lowest SCP this observation is consistent with the fact that drastic changes in temperature can lead to greater cold hardiness (Sinclair, 2015).

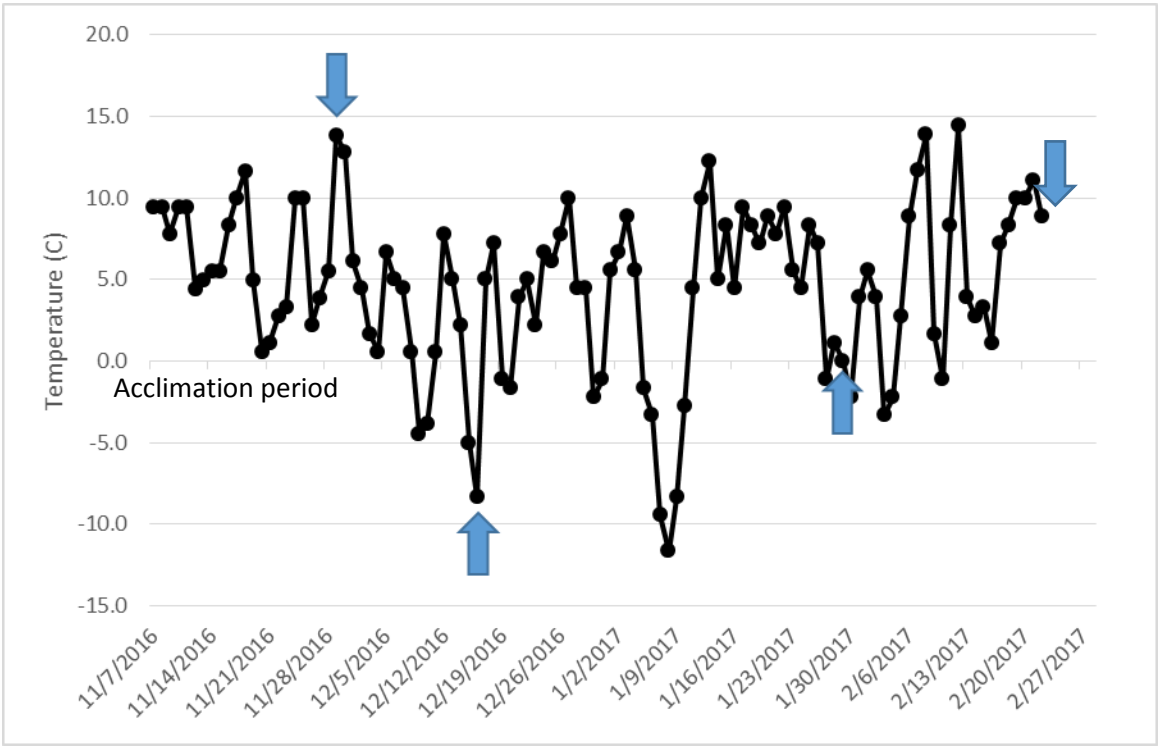

Figure 3.5: The daily average temperatures in Blacksburg, VA recorded during the cold hardiness study. The temperatures were plotted as four sampling time intervals: November

(11/7/16-11/28/16); December (11/29/16-12/16/16); January (12/17/16-1/29/17); and February (1/30/17-2/22/17), dates beetles were removed from the field for the SCP determination are indicated by arrows.

The supercooling points of individual beetles in this study were highly variable. Individual SCPs ranged from -5 to -23 °C. It is not uncommon to see high variability in supercooling point studies. There are natural variations of an insect's cold hardiness from one individual to the next, depending on the time of day, and the season (Overgaard and Sorensen, 2008). There is also a physiological phenomenon known as rapid cold hardening, which is a short time scale plastic response (Teets and Delinger, 2013). In as little as a few minutes, an insect can rapidly adapt to cold temperatures, and this has been shown to impact the supercooling point (Worland and Convey, 2001). This most often occurs when the cooling rate is slower than the recommended rate of about -0.5 centigrade per minute, and with the rate of cooling was slower than anticipated several times in the study, this could be one explanation for some of the variability found in this study. Another possible contributing factor is that the supercooling points could have been impacted by the 2 day waiting period, as described by Mausel et al., 2011, in the lab before the supercooling determination. This could potentially be enough time for the insects physiology adapt to the conditions of 0 ^{o}C when on ice.

The supercooling point of the northern and southern population were expected to differ from each other because of where they were collected from in Japan, where the average temperatures from the collection site are different. From November to February in the Nagano region of Japan where the northern population was collected is between -1 to 7 ^{o}C. The average temperature from

November to February in the Osaka region of Japan where the southern population was collected is between 6 to 14 ºC. Though there was a small numerical difference with the species average thus the expected difference was not demonstrated by the results since there were no significant differences in the supercooling points. The supercooling points of the *Laricobius* spp. would be expected to be similar to what Mausel et al. (2011) found, when they determined the SCPs of the interior and coastal strains of *L. nigrinus* in January of 2008. The average supercooling point from the Mausel et al. (2011) study was -16.9 °C (± 0.3) for the coastal strain, and between -18.6 (± 0.6)and -19.2 °C (± 0.7) for the inland strain. The beetles from that study were kept in mesh cages on hemlock trees in Leverett, MA for four weeks before the supercooling lab work was conducted (Mausel et al., 2011). These supercooling points are lower than what we found in our study, which was -14.1 (± 0.9) for *L. nigrinus* in January. Many factors impact the supercooling point of an insect from year to year, like beetle health, the locations the beetles were acclimated to, time of year, and extreme weather conditions, which could all be other explanations for the differences between this study and the Mausel et al. (2011) study.

Since the first *L. osakensis* releases beginning in 2012 (Mooneyham et al., 2016), the northern population has been released principally in the northern states impacted by HWA (VA, PA, OH, WV, MD), while the southern population has been released principally in the southern states HWA (NC, TN, SC). While supercooling points are of limited ecological value, this study suggests that the different species and populations of *Laricobius* are equally equipped to withstand cold temperatures. Future work on cold hardiness should focus on the insect's ability to withstand sustained cold temperatures and the insect's lower lethal temperature to fully understand the cold hardiness of an insect. Elkinton et al. (2016) found that in February 2015,

December 2015, and February 2016 that the average supercooling point of HWA acclimated at Kentland Farm in Blacksburg, VA ranged from approximately -16 to -18 °C. Despite the year-to-year variation of supercooling points, data suggest that HWA is more cold-tolerant than its predators. This implies that the lack of recoveries for *Laricobius* species after the polar vortex was not only due to the fact that there was a lack of prey to feed on, but also because some of the beetles themselves could not withstand the extreme cold weather. Based on our data, it may not be necessary to link releases of norther collected *L. osakensis* to northern location releases and southern location releases.

3.5 References:

Andersen, J. L., T. Manenti, J. G. Sorensen, H. A. MacMillan, V. Loeschcke, and J. Overgaard. 2015. How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. Funct. Ecol. 29: 55-65.

Block, W., 1990. Cold tolerance of insects and other arthropods. Philos. Transact. Royal Soc. London B. 326: 613–633.

Cira, T. M., R. C. Venette, J. Aigner, T. Kuhar, D. E. Mullins, S. E. Gabbert. 2015. Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales. Environ. Entomol. 45: 484-491.

Crosthwaite, J. C., S. Sobek, D. B. Lyons, M. A. Bernards, and B. J. Sinclair. 2011. The overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). J. Insect Physiol. 57: 166–173.

Danks, H. V. 2005. Key themes in the study of seasonal adaptations in insects I. Patterns of cold hardiness. Appl. Entomol. Zool. 40: 199–211.

Duman, J.G., 2001. Antifreeze and ice nucleator proteins in terrestrial arthropods. Ann. Review Physiol. 63, 327–357.

Elkinton, J. S., J.A. Lombardo, A.D. Roehrig, T.J. McAvoy, A. Mayfield, and M. Whitmore. 2016. Induction of cold hardiness in an invasive herbivore: the case of hemlock woolly adelgid (Hemiptera: Adelgidae). Environmental entomology. 46: 118-124.

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

Holmstrup, M., K. Hedlund, and H. Boriss. 2002. Drought acclimation and lipid composition in *Folsomia candida*: implications for cold shock, heat shock and acute desiccation stress. J. Insect Physiol. 48: 961–970.

Hopper, K. R., R. T. Roush, and W. Powell., 1993. Management of genetics of biological control introductions. Annu. Rev. Entomol. 38: 27–51.

McAvoy, T. J., J. Régnière, R. St-Amant, N.F. Schneeberger, and S. M. Salom. 2017. Mortality and recovery of hemlock woolly adelgid (*Adelges tsugae*) in response to winter temperatures and predictions for the future. Forests. 8: 497.

Mooneyham Loke T. Kok, K. S. M. S. 2016. Release and Colonization of *Laricobius osakensis* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid, Adelges tsugae. N.E. Naturalist. 23: 141–150.

Mausel, D. L., R. G. Van Driesche, and J. S. Elkinton. 2011. Comparative cold tolerance and climate matching of coastal and inland *Laricobius nigrinus* (Coleoptera: Derodontidae), a biological control agent of hemlock woolly adelgid. Biol. Control 58.2: 96-102.

Overgaard, J., J. G. Sørensen. 2008. Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. Cryobiology. 56: 159-162

Preisser, E. L., K. L. F. Oten, and F. P. Hain. 2014. Hemlock woolly adelgid in the eastern United States: What have we learned? S.E. Naturalist 13: 1-15.

Ramlov, H., and R. E. Lee. 2000. Extreme resistance to desiccation in overwintering larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). J. Exp. Biol. 203: 783–789.

Salt, R.W.1961. Principles of insect cold hardiness. Ann Rev Entomol. 6: 55-74.

Sinclair, B. J., P. Vernon, C. J. Klok, and S. L. Chown. 2003. Insects at low temperatures: an ecological perspective. Trends Ecol. Evol. 18: 257–262.

Sinclair, B. J., L. E. C. Alvarado, and L.V. Ferguson. 2015. An invitation to measure insect cold tolerance: methods, approaches, and workflow. J. Therm Biol. *53*: 180-197.

Teets, N.M., D.L. Denlinger. 2013. Physiological mechanisms of seasonal and rapid coldhardening in insects. Physiol Entomol. 38: 105-116.

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

Worland, M. R., P. Convey. 2001. Rapid cold hardening in Antarctic microarthropods. Funct Ecol. 15: 515-524.

Zachariassen, K. E. 1985. Physiology of cold tolerance in insects. Physiol. Rev. 65: 799–832.

Zachariassen, K. E., and E. Kristiansen. 2000. Ice nucleation and antinucleation in nature. Cryobiology. 41: 257–279.

Chapter 4: Summary

Hemlock trees are a climax species in eastern United States forests. The loss of this species is causing drastic changes in ecosystem function. The spread of the invasive species, hemlock woolly adelgid (HWA), in the eastern United States has caused extensive hemlock tree mortality. Efforts to manage HWA rely on a variety of methods. Biological control is the most promising long-term solution in forest settings. *Laricobius* beetles (Coleoptera: Derodontidae) are ideal candidates for biological control because they are adelgid specialists. *Laricobius osakensis*, native to Japan, could be an effective biological control agent as it and the strain of HWA in the eastern United States are native to the same area of Japan. *Laricobius osakensis* has several characteristics that make it an effective predator of HWA including, it being host specific, having a synchronous life cycle, and a high feeding rate. Once established, *L. osakensis* may play an important role in the suppression of HWA populations.

In Chapter 2, field work was conducted to monitor previous *L. osakensis* release sites to see if any sites indicated establishment. Tree health and HWA density were also monitored. The first year of monitoring yielded low *L. osakensis* recoveries, with 3 adults and 14 larvae collected throughout the year. This was attributed to low winter temperatures caused by the polar vortex in 2014, which led to a decrease in HWA populations. The cold temperatures and a lack of prey, created difficult conditions for *L. osakensis* to establish. The second year of field work yielded 57 adults and 90 larvae. The rebound in *L. osakensis* populations suggests that *L. osakensis* is establishing, and this is the first report of that. These results indicated there was a significant statistical correlation between the amount of *Laricobius* spp. recovered at each field site and

HWA density. There was also a negative correlation between average tree health and the number of *L. osakensis* and *Laricobius* spp., and a significant correlation between HWA density and plant hardiness zone. We conclude that *L. osakensis* is establishing at some release sites, and with increased HWA populations, *L. osakensis* populations will increase when given sufficient time to colonize.

In Chapter 3, supercooling point temperatures (SCP) were analyzed for the northern and southern populations of *Laricobius osakensis,* and a mixed population of *Laricobius nigrinus*. Observing the SCP of each population and species monthly provided insight on the insect's physiology and ability to adapt as the temperatures changed throughout the winter. These results indicated a no significant differences between the SCPs between species, but a significant difference between February and other months in the study. This work provided insight as to why there was a lack beetle recovery after the polar vortex in 2014 and informs the selection of the natural enemy population best suited for different regions. We concluded that the *Laricobius* species and populations that we analyzed appear to have similar SCPs, are likely equally equipped to withstand cold weather and that the source population for a release need not be a consideration.

Future work

Continued long-term monitoring of current and future release sites will help indicate how well these beetles are establishing. Once the beetles have higher recovery numbers, their impact on HWA density and dispersal should be evaluated. Continued monitoring of tree health and HWA densities will help show how the HWA densities and the hemlock trees are impacted by

increasing *Laricobius osakensis* densities. Determining a site of a successful field insectary is important to reduce reliance on lab reared beetles.

Since *L. osakensis* releases of the northern strain have been contaminated since 2015, this creates an unexpected research opportunity. Researchers will now be able to study the interaction of *L. osakensis*, *L. nigrinus*, and *L. rubidus* populations at one release site. This could provide insight into the competition among species for establishment at these sites.

Continued work on determining the supercooling point, ability to withstand sustained cold temperatures, and the lower lethal temperature of the *Laricobius* spp. is of interest. Since the physiology of an insect changes drastically as temperature changes, more data are needed to understand *Laricobius* spp. cold tolerance.