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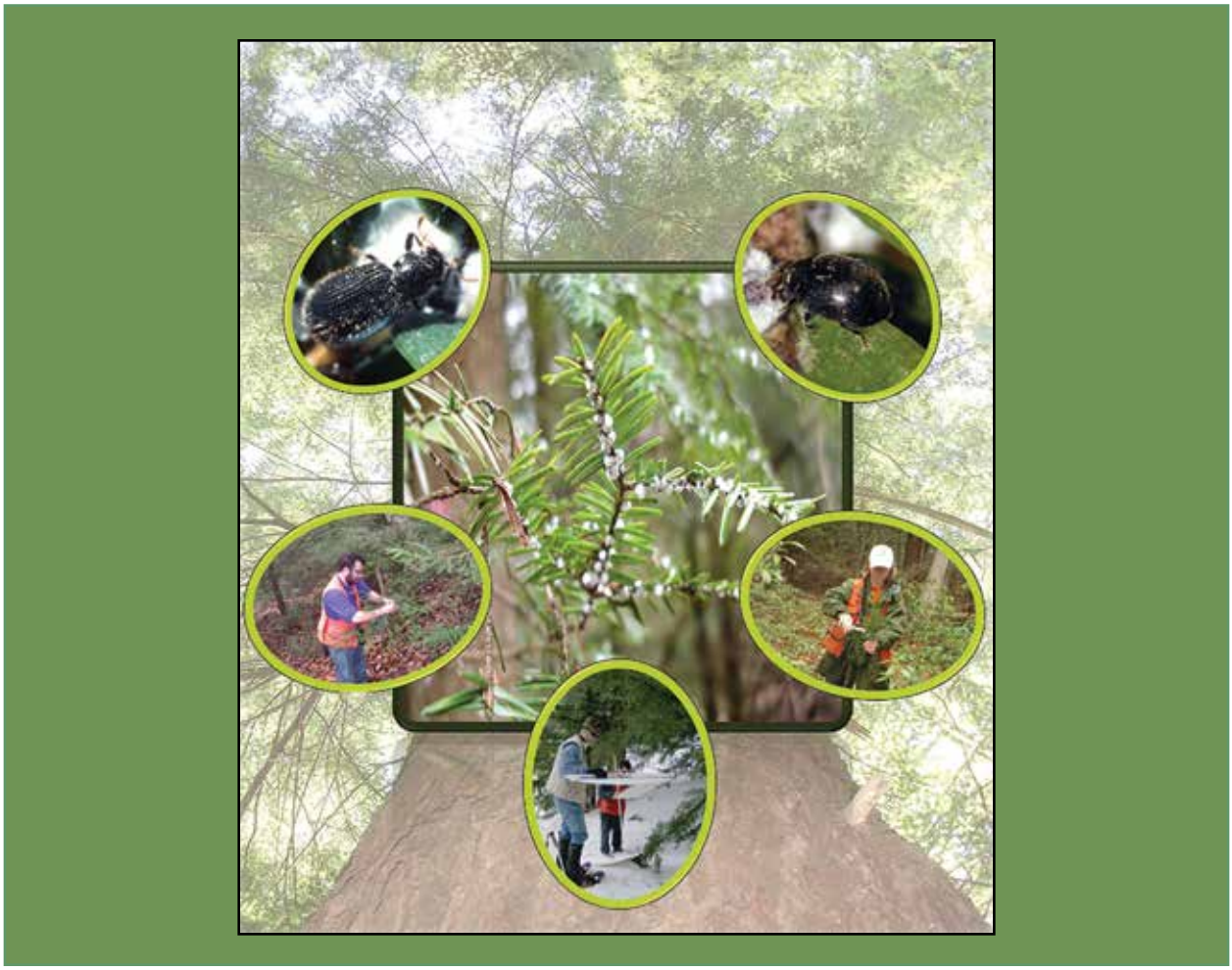
Implementation and Status of Biological Control of the Hemlock Woolly Adelgid



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On the cover:

Center photo: Hemlock woolly adelgid white woolly masses on a hemlock branch (USDA Forest Service, Karen Felton)

Top right: *Sasajiscymnus tsugae* predatory beetle (USDA Forest Service, Lynn Jones);

Middle right: Collecting and checking hemlock branch samples for *Laricobius nigrinus* predatory beetle larvae (USDA Forest Service, Brad Onken);

Bottom center: Collecting *Laricobius nigrinus* predatory beetles in Idaho (USDA Forest Service, Brad Onken);

Middle left: Releasing *Laricobius nigrinus* predatory beetles (USDA Forest Service, Brad Onken);

Top left: *Laricobius nigrinus* predatory beetle (USDA Forest Service, Lynn Jones)

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Preface

Hemlock woolly adelgid (HWA), *Adelges tsugae* (Annand), remains the single greatest threat to the health and sustainability of hemlock as a forest resource in the eastern United States. It is an exotic pest native to Asia and western North America. First discovered in the eastern United States in 1951 near Richmond, VA, the pathway and source of the introduction are believed to have originated from southern Japan on nursery stock. In the late 1980s, HWA began to spread rapidly from its point of introduction. It has since spread to 17 eastern states threatening two species of hemlock: the eastern hemlock, *Tsuga canadensis* (L.) Carr., and Carolina hemlock, *Tsuga caroliniana* Engelm. On these hemlocks, HWA has two generations per year and is parthogenic; that is, all individuals are female, capable of reproducing.

The Hemlock Woolly Adelgid Initiative began in 2003 with the development of a 5-year program (2003-2007) in cooperation with the U.S. Department of Agriculture Forest Service and the Animal Plant Health Inspection Service (APHIS), the National Association of State Foresters, and the National Plant Board for the development and implementation of management options to reduce the spread and impact of HWA. A second 5-year program (2008-2012) was funded to continue and to accelerate the development of research and technology, management, and information transfer program components.

Although the earliest investigations into HWA biological control date back to the early 1990s, the biological control effort has been greatly expanded with the onset of the Hemlock Woolly Adelgid Initiative and has involved 28 federal and state agencies, 24 universities, 7 institutions in China and Japan, and numerous private industries. Biological control offers a potential long-term solution to suppression of HWA and needs to become the focal component of an integrated management program.

The purpose of this book is to provide a reference guide for field workers and land managers on the historical and current status of the biological control of hemlock woolly adelgid. This book is a substantial revision of FHTET-2004-04, *Biological Control of Hemlock Woolly Adelgid*.

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SECTION I INTRODUCTION

Chapter 1: Hemlock Woolly Adelgid and its Hemlock Hosts: A Global Perspective

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INTRODUCTION

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hempitera: Adelgidae), threatens the health and sustainability of the native eastern North American hemlocks, *Tsuga canadensis* (L.) Carrière and *T. caroliniana* Engelman. The lineage of HWA that was introduced to the eastern United States came from Japan sometime prior to 1951, and did not co-evolve with eastern North American forest ecosystems (Havill et al. 2006). As a result, eastern hemlock species cannot adequately resist or tolerate the impacts of adelgid feeding, and the native community of natural enemies in eastern North America is not capable of maintaining HWA populations below damaging levels. This results in widespread death of hemlock trees, which is having serious consequences for biodiversity, ecosystem functions, and ornamental and urban resources.

There are quite a few studies that have examined the effects of HWA on hemlock ecosystems. Hemlocks are the most shade tolerant of conifers (Farjon 1990) and hemlock dominated forests provide a uniquely cool and densely shaded micro-environment. Loss of hemlock because of HWA is changing forest composition and structure (Orwig and Foster 1998, Spaulding and Rieske 2010), nutrient cycling (Kizlinski et al. 2002, Stadler et al. 2006; Nuckolls et al. 2009, Albani et al. 2010, Cobb 2010), and the composition of wildlife communities (Becker et al. 2008, Allen et al. 2009).

Since the late 1980's, there has been a concerted effort to understand HWA biology, evolutionary history, host effects, ecological impacts, and natural enemies, with the goal of finding ways to control this devastating pest. Although progress has been made on all of these fronts, trees continue to die at an alarming rate. Individual trees can be protected with repeated application of insecticidal soap, horticultural oil, or systemic insecticides (Ward et al. 2004), and silvicultural thinning is being evaluated as a way to prolong the health of hemlock stands (Fajvan and Wood 2008), but these interventions are expensive and not sustainable at the landscape scale.

Manipulating hemlock resistance to HWA is another approach with potential to control adelgid populations. Researchers are searching for naturally resistant trees (Ingwell and Preisser 2011), developing resistant crosses between North American and Asian species (Montgomery et al. 2009), and establishing protected plantings to conserve hemlock genetic diversity (Jetton et al. 2011). Unfortunately, restoring forest and urban ecosystems with these trees would take many decades and there is no guarantee that this can be completed in time to safeguard hemlock's unique role in eastern forests. As a consequence, the establishment of effective biological control agents is a critical component of efforts to maintain hemlock resources in eastern North America. Predicting the safety and success of biological control is challenging because natural enemies function within a complex system of multi-species, multi-trophic interactions. In this chapter, we summarize the evolutionary history of the interaction among hemlocks and adelgids. At the end of the chapter, we discuss how this information can help in the selection and establishment

of biological control agents to maximize their potential to control HWA populations and minimize undesirable non-target effects.

DIVERSITY AND DISTRIBUTION OF HEMLOCK

There are nine species of hemlock currently accepted (Farjon 1990), five are found in Asia and four in North America (Fig. 1). Hemlock trees grow naturally in cool, humid areas from sea-level to the subalpine zone, depending on the species and region. All species have a strict requirement of adequate soil-moisture throughout the growing season.

Both species of hemlock native to eastern North American are susceptible to HWA. Eastern hemlock, *T. canadensis*, has a broad distribution, spanning from New Brunswick in the north, to Alabama in the south, and west to Minnesota, with isolated disjunct populations to the south and west of its main range. Eastern hemlock is also highly valued as an ornamental, thus HWA is impacting property values throughout the eastern United States (Holmes et al. 2010). The other hemlock species in eastern North America, Carolina hemlock, *Tsuga caroliniana*, has a very limited distribution in the southern United States, and is not commonly planted as an ornamental. It is typically found in small, isolated populations in the southern Appalachians on exposed ridges and rocky outcroppings where it can escape competition from hardwoods (Jetton et al. 2008). Because of its restricted range, Carolina hemlock is at even higher risk from HWA than eastern hemlock.

A recent molecular phylogeny of *Tsuga* provided new information about the diversity, evolutionary relationships, and historical biogeography of hemlock (Havill et al. 2008). The results suggest that in addition to the nine species typically recognized, there are two endemic island species of hemlock that should probably be given species status. Hemlock from Taiwan is often treated as a variety of *T. chinensis* (Franchet) Pritzel in Diels (e.g. Farjon 1990); however, phylogenetic analyses show that this variety is not closely related to hemlocks from mainland China. It appears that Taiwanese hemlock was correctly described as a separate species, *T. formosana*, by Hayata in 1908. Hemlocks on Ullung Island, Korea, a small volcanic island in the Sea of Japan, were previously thought to be *T. sieboldii* Carrière, the southern Japanese hemlock. The Ullung Island hemlocks are actually more closely related to, but distinct from, *T. diversifolia* (Maximowicz) Masters, the northern Japanese hemlock. Work is underway to determine whether the Ullung Island hemlock is distinct enough to be considered a new species.

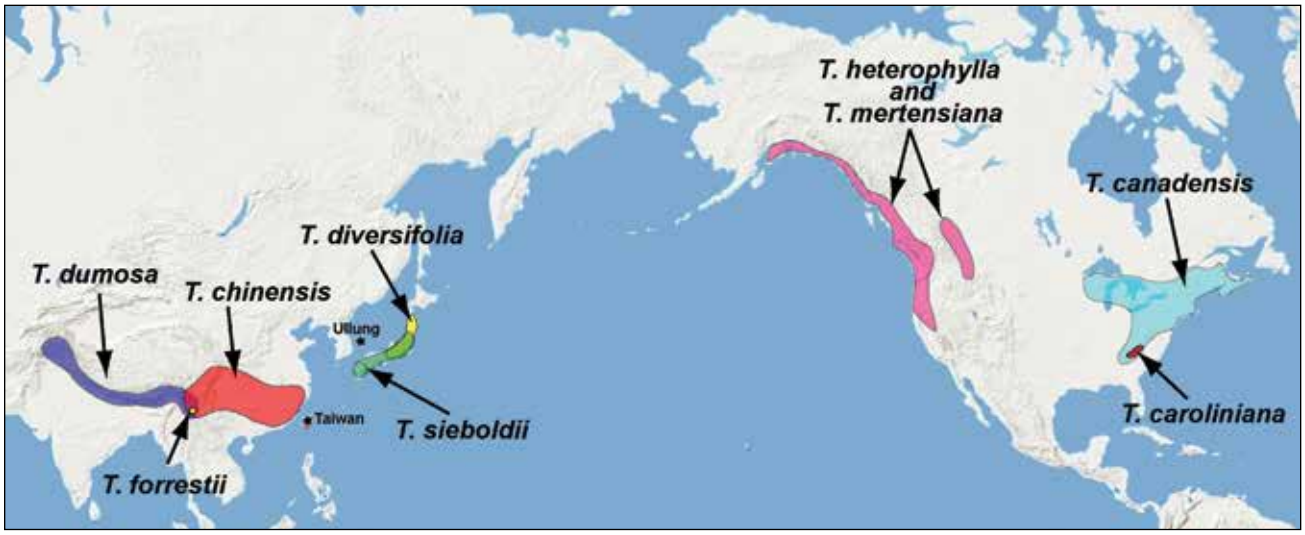

Figure 1. Map showing the distribution of hemlock species worldwide (reprinted with permission from Havill et al. 2008).

Havill et al. (2008) also showed that the two eastern North American hemlocks are not close relatives. *Tsuga canadensis* diverged from all the other hemlocks very early in the evolutionary history of the genus, but *T. caroliniana* diverged more recently and is more closely related to the Asian species. The observation that *T. caroliniana* is closely related to the Asian hemlocks is consistent with its ability to successfully hybridize with the HWA resistant Asian species, whereas attempts to cross *T. canadensis* with other species have failed (Bentz et al. 2002). Hybrids between *T. caroliniana* and *T. chinensis* are resistant to HWA and could be good replacements for *T. canadensis* in the urban environment (Montgomery et al. 2009). *Tsuga chinensis* by itself is also highly resistant to HWA and grows well in the northeast (Del Tredici and Kitajima 2004; Evans 2008; Weston and Harper 2009). Although the two western North American species appear to be resistant or tolerant to HWA, they do not survive well in the east.

Tsuga canadensis has low genetic variation compared to other hemlock species and other eastern North American conifers (Zabinsky 1992; Potter et al. 2007). This could have implications for HWA control if this pattern translates into less natural variation in resistance to HWA. According to the pollen record, there were two periods of rapid decline in eastern hemlock that occurred approximately 9,800 and 5,300 years ago (Zhao et al. 2010). The more recent decline has been attributed to insect feeding (e.g. Bhiry and Filion 1996), but it is more likely that both periods of decline were due to increased variation in temperature and drought that occurred during the early- to mid-Holocene (Foster et al. 2006, Shuman et al. 2009, Zhao et al. 2010). Among conifers, hemlocks are the most susceptible to drought (Farjon 1990), which may explain why the decline of eastern hemlock was more severe than other tree species experiencing the same environmental changes. The pattern of genetic diversity in *T. canadensis* in the southern part of its range suggests that when its range contracted, there was a refuge southeast of the Appalachians out of which the species eventually spread to re-occupy its current distribution (Potter et al. 2007).

Carolina hemlock was found to have moderate levels of genetic diversity and the genetic signature of a similar glacial refuge southeast of the Appalachians (Potter et al. 2011). Interestingly, Havill et al. (2008) hypothesized that *T. caroliniana* was closely related to European hemlocks based on an analysis that took into account hemlock phylogeny, molecular dating, the fossil record, and the timing of ancient connections among the continents. Hemlock eventually recovered in eastern North America, but this was not the case in Europe. Hemlock was common throughout Europe until approximately 750,000 years ago when it went extinct due to drier climate and repeated glaciations (LePage 2003; Follieri 2010).

The combination of eastern hemlock's low genetic diversity and relatively narrow site requirements may make the search for resistant trees difficult. With this in mind, Camcore (International Tree Conservation and Domestication, N.C. State University) is collecting seeds from eastern and Carolina hemlocks throughout their ranges, placing them in long-term storage, and growing them in protected plantations to conserve their genetic diversity for future restoration (Jetton et al. 2011). If biological control of HWA is successful, these trees could be used as a source to restore hemlock to eastern forests.

DIVERSITY AND BIOGEOGRAPHY OF HEMLOCK ADELGIDS

HWA has been documented on all hemlock species including those present on Taiwan and Ullung Island (Annand 1924, Takahashi 1937, Inouye 1953, Ghosh 1975, Montgomery et al. 2000). The earliest reports of HWA in North America are from the west coast. The earliest North American specimens were collected in 1907 from South Bend, Washington (U.S. National Collection of Insects, Beltsville, Maryland). Other early records from the west include a report of damage to western hemlocks in Vancouver, British Columbia (Chrystal 1916), and specimens collected in Oregon and California used to formally describe *A. tsugae* as a new species (Annand 1924). In eastern North America, the earliest specimens were collected decades later in

Richmond, Virginia in 1951, and damage on eastern hemlocks was reported in Pennsylvania starting in 1969 (Gouger 1971). Widespread mortality of eastern hemlocks began in the Mid-Atlantic States then spread to southern New England in the mid 1980's. HWA is currently established in more than half of the range of eastern hemlock, occupying 18 states from Maine to Georgia (Fig. 2)

This sequence of records led to the incorrect assumption that HWA was first introduced into western North America in the early 20th century, and then brought to eastern North America from the west some time after that. This is understandable since HWA collected from different regions do not show any obvious morphological differences. It was only after a series of genetic analyses that *A. tsugae* was recognized as a diverse group of related insect lineages with a complex evolutionary history (Havill et al. 2006, Havill et al. 2007, Havill et al. 2009). Molecular dating methods estimated that the diversification of hemlock adelgids began approximately 30 million years ago, which corresponds to when much of the genus *Tsuga* was also diversifying. There are at least six distinct lineages of hemlock adelgids endemic to different parts of the world: one each in China, Taiwan, and western North America, and two in Japan. HWA is also found in India and Nepal, but it is not yet known how these populations relate to the others. We now know that HWA was introduced to the eastern United States directly from Japan, and that the lineage in western North America is native.

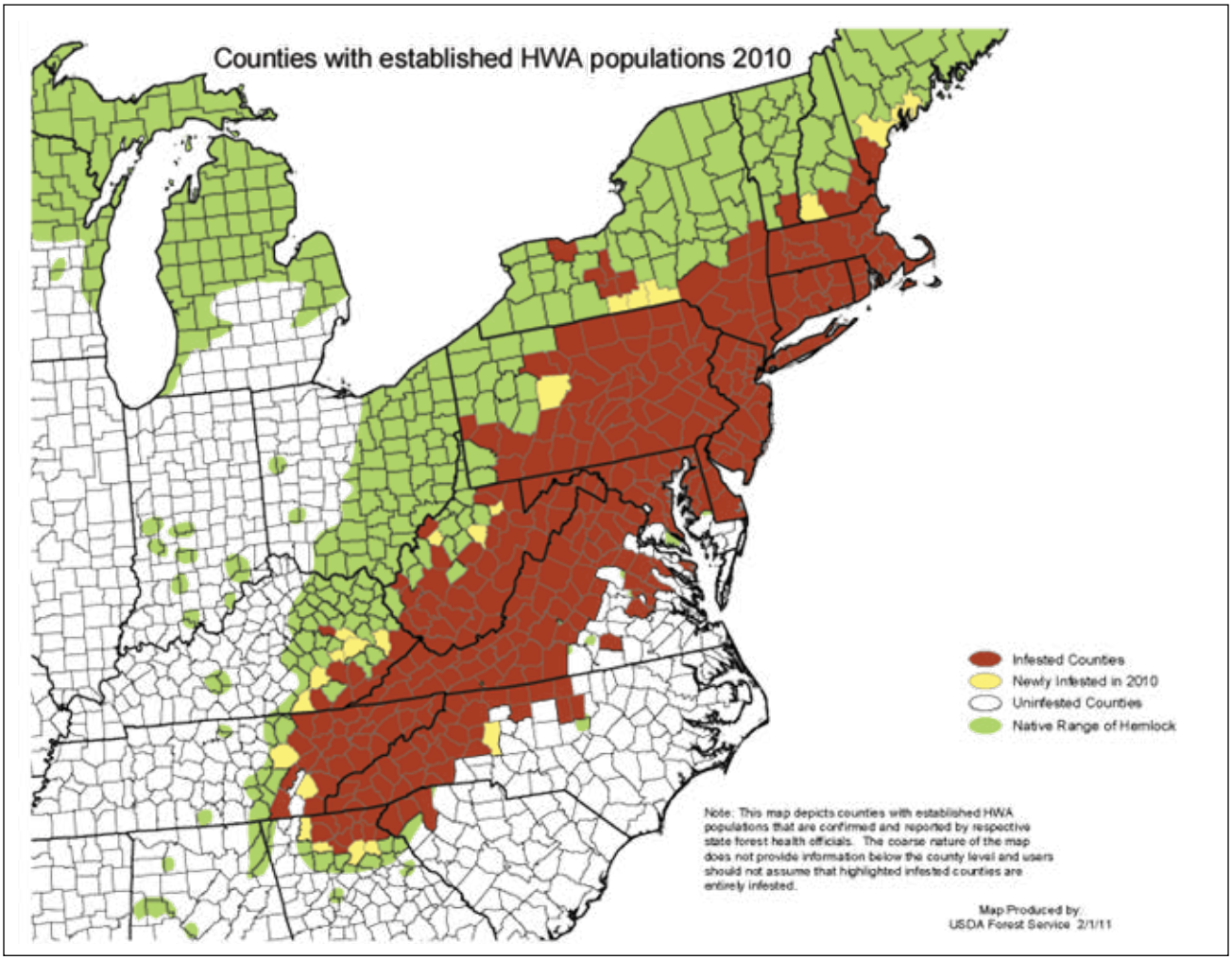

Figure 2. Native range of hemlock in the eastern United States (green) and range of hemlock woolly adelgid (brown) in 2010.

In Japan, there are two lineages of HWA that specialize on each of the Japanese hemlock species, *T. sieboldii* and *T. diversifolia*. *Tsuga sieboldii* grows at lower elevations and further south while *T. diversifolia* grows to the north at higher elevations. The geographic ranges of the two species overlap in central Honshu, but it is rare for them to grow naturally in the same stands. For example, they could be found on the same mountain, but *T. sieboldii* will grow at the base, and *T. diversifolia* will grow at the top. Extensive sampling throughout Japan has confirmed that the two Japanese HWA lineages are not exchanging genes despite the existence of areas where the two species grow in close proximity (Havill, unpublished data).

The adelgids that were introduced to the eastern United States are from the lineage that lives on *T. sieboldii*, the southern Japanese hemlock. We know this because DNA sequences from adelgids in the eastern United States are an exact match to adelgids living on *T. sieboldii* in Japan. In the eastern United States, we observe only a fraction of the genetic variation found naturally in Japan (Havill et al. 2009). This is characteristic of a recently introduced species with a single introduction. In contrast, HWA in western North America is much more genetically diverse than in the east and their DNA does not match any of the Asian lineages (Havill et al. 2009). Hemlock adelgids from China and Taiwan are conspicuously different from those in Japan and North America; perhaps enough to consider them separate species.

ADELGID BIOLOGY

Adelgids have multi-generation, complex life-cycles with many different morphological forms within a single species (Havill and Foottit 2007). The typical adelgid life-cycle involves alternation between spruce (*Picea*) primary hosts where they form galls and where there is a sexual generation, and other conifer secondary hosts where reproduction is strictly asexual.

The lineage of HWA that was introduced to the eastern United States alternates between *T. sieboldii* and tigertail spruce, *Picea torano* (K. Koch) Koehne, in Japan. The HWA gall is morphologically different than the typical "pineapple" adelgid gall. It is nearly spherical and can be quite large, up to 4 cm in diameter (Fig. 3). Tigertail spruce is a protected species in Japan where it is uncommon and patchily distributed on the Japanese landscape. Like many other adelgid species, HWA can maintain continuous asexual generations on its secondary hosts in areas where there are no suitable spruce primary hosts. In Japan, this has resulted in a patchwork of sexual and asexual populations of HWA, depending the proximity and availability of primary and secondary host trees. If both host species are present, a proportion of the HWA population migrates from hemlock to spruce where there is a sexual generation. When tigertail spruce is absent, winged migrants do not survive to reproduce and the population is limited to asexual generations on hemlock. In southwestern China, HWA alternates between *T. chinensis* and *Picea likiangensis* (Franchet) Pritzel, and *P. brachytyla* (Franchet) Pritzel (Montgomery and Havill, unpublished data). In western North America, HWA feeds on both *T. heterophylla* and *T. mertensiana* but winged migrants have not been observed and it does not alternate to spruce.

Figure 3. Hemlock woolly adelgid gall on tigertail spruce in Japan.

The ability of HWA to continue reproducing asexually on hemlock probably contributed to its successful invasion of eastern North America where there are no suitable spruce species to support the sexual generation (McClure 1989). HWA has two generations per year on hemlock in eastern North America. One generation consists of wingless sistens individuals that hatch in late spring to early summer, and quickly enter a diapause which continues through late summer and into the fall. Sistentes then overwinter as nymphs, becoming adults in early spring when they lay a large clutch of eggs. The next generation consists of both wingless progrediens and winged sexuparae. Most individuals develop as wingless progredientes in early spring, progress very quickly to the adult stage, and lay eggs in late spring and early summer. The remaining individuals of this generation develop into the winged sexuparae that would give rise to the sexual generation, though these individuals do not reproduce because suitable spruce species are not available. The phenology of the life cycle is somewhat more accelerated in the southern versus the northern areas of the introduced range because of the warmer climate (Mausel et al. 2008), and overwintering mortality is much higher in the north than in the south (Trotter and Shields 2009). The overwintering sistentes are generally more fecund than the progredientes (Fig. 4).

Population genetic analyses indicated that HWA has very little genetic variation in the eastern United States, and since it only reproduces asexually, new genotypes can only arise from mutation, not from recombination. However, its extremely high population sizes are likely to harbor enough mutations to allow for adaptation to local environmental conditions as it spreads. For example, there is evidence that it is evolving increased cold tolerance as it moves north (Butin et al. 2005).

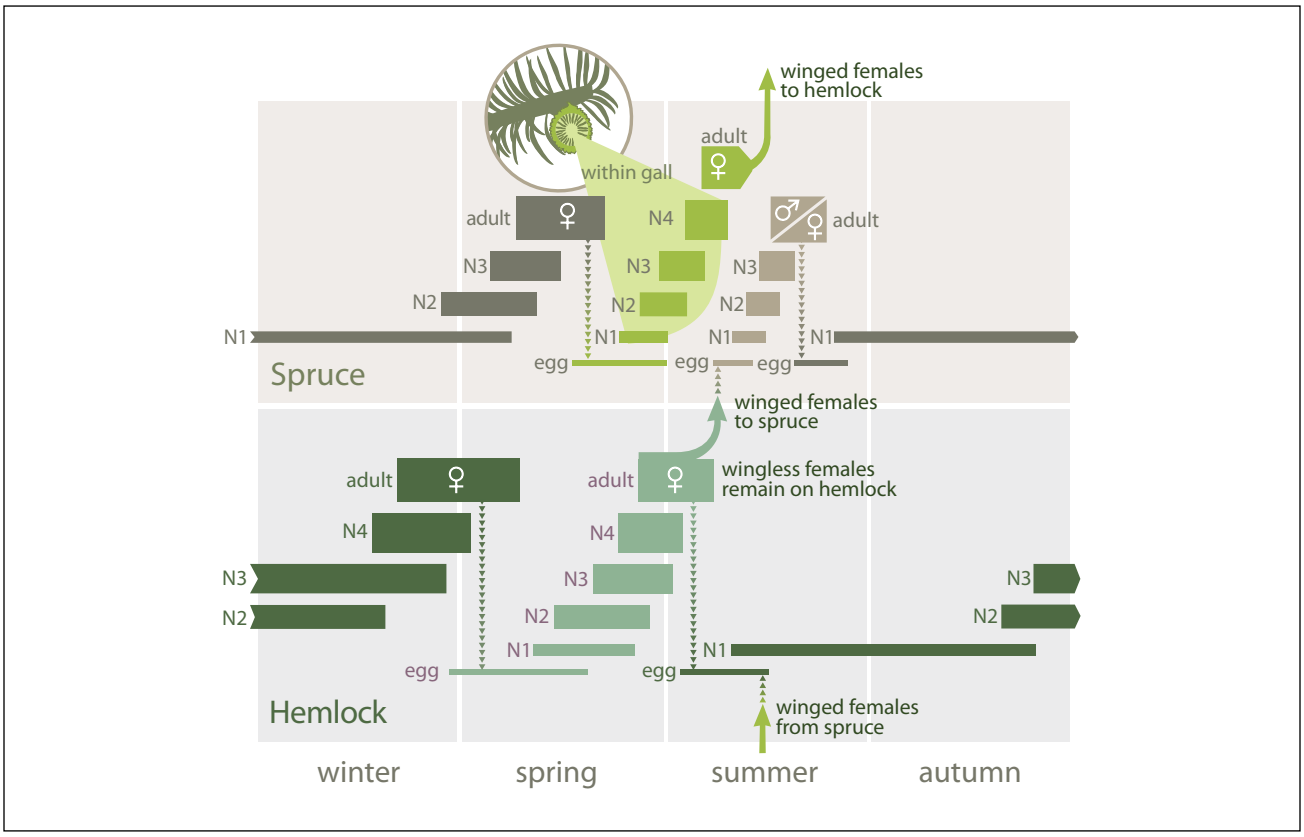

Figure 4. Hemlock woolly adelgid life cycle. In Japan, the adelgid alternates between hemlock and tigertail spruce. Tigertail spruce supports a sexual generation and gall formation. In the eastern United States there are only two generations on hemlock, because winged migrants do not find suitable spruce species on which to complete the entire life cycle. (Vince D'Amico and Nathan Havill created the artwork for this figure.)

HOST IMPACTS

HWA settles at the base of hemlock needles, primarily on the underside of branches. HWA has piercing-sucking mouth parts that extend through the leaf cushion into the ray parenchyma cells where it extracts stored plant nutrients (Young et al. 1995). Feeding causes the loss of hemlock needles, and the mortality of buds and branch tips. Loss of foliage and dieback can become apparent 2 to 4 years after infestation in many locations, and trees can die within a few years, or can survive in a weakened state for many years (Paradis et al. 2008).

Recent work suggests the damage caused by HWA may be more complicated than simple depletion of nutrients. We know that adelgids feeding on spruce induce dramatic changes in the host, as evidenced by the formation of galls. Adelgids settled at the base of spruce buds cause the developing needles to be stunted, to expand laterally, and merge together into a gall rather than form a normal shoot. Gall tissue is high in lipids and starch and low in phenolic compounds making them more suitable for adelgid feeding (reviewed in Havill and Foottit 2007). Secondary hosts such as hemlock do not respond to adelgid feeding by producing galls, but they may have similarly complex reactions to adelgid feeding. Another introduced adelgid species, *Adelges piceae* (Ratzeburg) for example, induces abnormal growth of bark and wood tissue in susceptible *Abies* species called "gout disease" or "rotholtz" (Balch 1952). HWA feeding appears to induce similar responses in hemlock including: 1) formation of abnormal xylem that limits the tree's ability to transport water (Rivera et al. 2010); 2) increased foliar nitrogen (Stadler et al. 2005); 3) dramatic changes in amino acid concentration and composition (Gómez et al. 2011); and 4) the maintenance of high levels of starch at the feeding site (Schwartzburg and Montgomery 2011). Thus, HWA may be able to induce localized changes in both its primary and secondary hosts that favor its survival and reproduction. In eastern North America, where hemlocks did not co-evolve with adelgids, tree response to feeding appears to be hypersensitive, which coupled with the lack of population suppression by natural enemies, can result in tree death.

The terpenoid chemistry of the two eastern North American hemlock species is an intriguing example of herbivore/host co-evolution. Although *T. canadensis* and *T. caroliniana* are not phylogenetically closely related, both species have relatively high levels of isobornyl acetate (about 40%), which is twice the percentage of total terpenoids found in other hemlock species, and low levels of alpha-humulene (2-4%), which is less than half the percentage found in all other species, except *T. mertensiana* (Lagalante et al. 2003). In terms of the chemical signature of all 40 terpenoids detected, the terpenoid composition of *T. caroliniana* is more similar to the Asian species than the North American species. Since eastern North American hemlocks have evolved with more chewing insects such as the hemlock looper, *Lambdina fiscellaria* (Guenée), and fewer piercing-sucking insects, their defenses may not be effective against sucking insects such as HWA (Lagalante et al. 2007, Montgomery and Lagalante 2008). HWA infestation is also known to increase the release rate of volatile monoterpenes in eastern hemlock branches (Broeckerling and Salom 2003), but the role of terpenes in defense against HWA is not known, nor is their role in attracting natural enemies to HWA infested trees.

IMPLICATIONS FOR BIOLOGICAL CONTROL

The evolutionary history of the interaction among hemlocks, adelgids, and their natural enemies should be considered when developing a successful biological control program for HWA. We know of eleven distinct hemlock taxa that support six different HWA lineages. Each population represents a multitrophic community that is a potential source of HWA biological control agents. These communities share fundamental traits that can be traced to shared ancestry within each trophic level (i.e. within *Tsuga*, *Adelges tsugae*, *Laricobius*, Chamaemyiidae, etc.). Each community of predators, herbivores, and hosts has unique adaptations to local climatic conditions and to the community of specific species present. For example, each endemic adelgid lineage co-evolved with different host species, has adapted

different variations on the life cycle to fit local ecological conditions, and contends with a different community of natural enemies. Knowledge of these systems can inform and guide the development of biological control agents. For example, because HWA was introduced from southern Japan, this region could yield biological control agents that are well adapted to feed on this specific lineage of HWA. *Laricobius osakensis* Montgomery and Shiyake, a beetle recently described from populations of HWA in Japan is therefore a promising biological control of HWA (Montgomery et al. 2011). In western North America, there is an additional assemblage of adelgid-specific natural enemies (Kohler et al. 2008) on a different lineage of HWA which, like the one in the eastern United States, does not alternate hosts. This lineage is also closely related to the one in southern Japan, and so predators of the western North American lineage of HWA may also be effective biological controls in the eastern United States. One such western predator, the beetle *Laricobius nigrinus* Fender, collected from western hemlock has been widely established as a biological control of HWA in the east (Mausel et al. 2010). Predaceous flies in the family Chamaemyiidae from the west also show potential as biological controls. In southwest China, there is a remarkably diverse assemblage of adelgid predators, especially in the lady beetle *Scymnus* (Montgomery et al. 2000). This diverse community of natural enemies could yield effective biological controls, especially if the native and non-native climates are similar, but careful attention should be paid to the prominent differences between the Chinese and Japanese lineage of HWA.

Predators of specific herbivores often use characteristic volatile chemicals released by specific plants as cues to locate their prey. The interaction between HWA and different host species could influence the ability of predators to control HWA. To examine this, Wallin et al. (2011) tested whether *L. nigrinus* was attracted to conifer hosts of different adelgid species. *Laricobius nigrinus* collected from western hemlock infested with western HWA was more attracted to western hemlock volatiles than to Ponderosa pine, Douglas fir, white spruce, or eastern hemlock volatiles. They were also attracted to western white pine. Beetles that were lab reared on eastern hemlock infested with Japanese HWA did not respond well to volatiles from either hemlock species. Put in the context of what we know about HWA in western North America, this lends further evidence that *L. nigrinus* is adapted to locating HWA in the west, and raises questions about the roles of learning and pre-conditioning in prey location.

Tsuga sieboldii, the host species with which the introduced HWA lineage co-evolved, is genetically and chemically different than the eastern North American hemlock species. The importance of a shared evolutionary history is highlighted when we recognize that *Tsuga canadensis* and *T. caroliniana* are not closely related to each other, yet both have independently evolved similar chemical signatures, perhaps because of the absence of pressure from sucking insects. For HWA control to be effective, it may be necessary to combine biological control with more resistant hemlocks. This may be difficult because *T. canadensis* has low genetic variation and does not readily hybridize with other hemlock species.

These studies are examples of why it is useful to consider adelgid biology and host interactions in the context of its evolutionary history when evaluating biological control agents. Different natural enemy species will behave differently in the context of the complex interaction between HWA and its hosts in different environments. An understanding of the diversity of hemlock species and HWA lineages in different parts of the world, and careful consideration of how natural enemies perform in their native and introduced ranges can be used to optimize the impact of biological control.

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Chapter 2: Simulations of Population Dynamics of Hemlock Woolly Adelgid and Potential Impact of Biological Control Agents

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ABSTRACT

The hemlock woolly adelgid (*Adelges tsugae*) is a small invasive Hemipteran herbivore that threatens the continued presence and abundance of hemlock in eastern North America. Efforts to control the adelgid have focused on the introduction of classical biological control agents. These biological controls include six different species of predatory beetles, two of which have established. Of these predatory beetle species, *Laricobius nigrinus* has the most potential to alter the dynamics of hemlock woolly adelgid, because it has been recovered in large numbers at multiple release sites in eastern North America. However, establishment does not guarantee the predators will maintain the density of the adelgid at levels sufficiently low to prevent hemlock damage and death. Here we present results of a simulation model in which the potential impact of the introduced predator *L. nigrinus* is explored in the context of what is known about the dynamics of hemlock woolly adelgid in the absence of predators.

INTRODUCTION

The hemlock woolly adelgid (*Adelges tsugae* Annand) was first documented in eastern North America near Richmond, Virginia in 1951 (Gouger 1971). Since then, the adelgid has moved through natural stands of hemlock and now infests at least 17 states from Georgia to Maine (http://na.fs.fed.us/fhp/hwa/maps/distribution.shtm). The rapid growth and expansion of adelgid populations, and the resulting decline in hemlock health and abundance has prompted research into the biology, ecology, and management of this forest pest over the last 30 years.

Research on the biology of the hemlock woolly adelgid in North America has shown the annual life-cycle of hemlock woolly adelgid (hereafter HWA) to consist of two generations, both of which reproduce asexually (McClure 1989) on members of the genus *Tsuga*. The overwintering sistentes generation begins development in the fall after a summer aestivation period. Development continues through the winter, with adults maturing and laying eggs in early spring. These eggs hatch after a short development period to produce progredientes crawlers. These crawlers then settle on the previous year's growth and mature in June. Some individuals in this generation become sexuparae that disperse and settle on spruce (*Picea* spp.). Sexuparae in the native range of China and Japan initiate a sexually reproducing generation on spruce, but McClure (1989) showed that none of the North American species of spruce trees are suitable hosts. The progredientes which do not develop into sexuparae continue to develop into adults, and lay eggs that hatch in June. The crawlers of the new sistentes generation settle primarily on new (current year) hemlock growth and enter a summer aestivation phase that lasts until October, whereupon they resume development and feeding. Development is completed the following March.

In eastern North America, HWA has very few known natural enemies, and those that have been observed are not believed to have any significant impact on HWA populations (Montgomery and Lyon 1996; Wallace and Hain 2000). There are no known parasitoids that attack any species in the family Adelgidae. Little is known about the potential or actual impact of pathogens, and the dynamics of the invaded system are largely driven by the interaction of HWA and its hemlock host as shown by McClure (1991) who provided the first comprehensive account of the population dynamics of HWA. Using artificially established populations on uninfested plantation trees and recently infested forest trees in Connecticut in 1986-1989, McClure showed that the HWA populations built to densities as high as 25 per cm of hemlock twigs in as little as one year after the initial infestation (Fig. 1a). As a result, trees produced little or no new growth the following year. This reduction in new growth forced the crawlers to colonize 1-2 year old growth where survival of nymphs was much lower relative to new growth. Additionally, the proportion of nymphs which became sexuparae instead of progredientes nymphs increased. Because sexuparae are unable to reproduce in North America, population densities declined dramatically in the second year (Fig. 1). The lower HWA densities allowed the trees to partially recover, leading in turn to an increase in HWA densities. This caused a second precipitous decline in hemlock growth that ended in the death of all the trees and HWA.

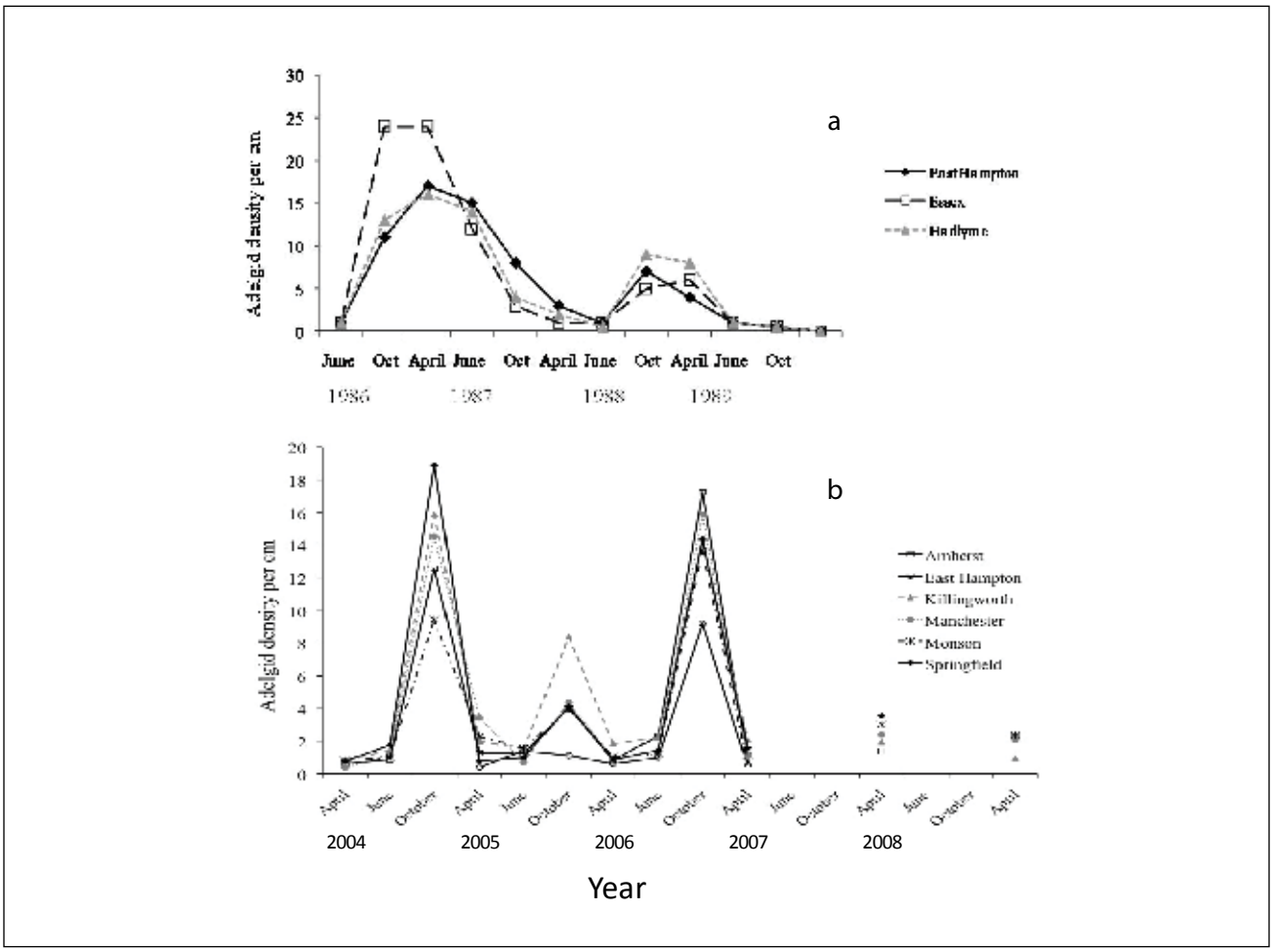

Figure 1. (a) Fluctuations in density of HWA at three naturally infested forest sites studied by McClure (1991) in Connecticut from 1986-1989 (figure redrawn from McClure 1991); (b) Six sites studied by Paradis (2011) in Massachusetts and Connecticut 2004-2008 using methods designed to be comparable with McClure's original studies. In 2008 and 2009 only the April counts of adult sistentes were made.

The pattern of hemlock death four years (or even two years) after infestation has been described by many observers in the southern Appalachians, an area which has recently been invaded by HWA. In northern New England, however, hemlock trees have been known to host infestations of HWA for ten years and continue to survive (Orwig and Foster 1998, Orwig et al. 2002, Paradis 2011). Several investigators have shown that overwintering mortality is much higher in northern compared to southern states (e.g. Trotter and Shields 2009) due to colder winter temperatures (Paradis 2008).

Paradis (2011) (Fig. 1b) conducted a study of naturally established HWA populations in Massachusetts and Connecticut between 2004 and 2008 that was designed to be comparable with previous work by McClure (Fig. 1a). Her purpose was to gain insight as to why very few hemlock trees were dying from HWA at these northern sites. Like McClure, Paradis (2011) (Fig. 1b) recorded declines in the production of new growth by infested hemlocks at high HWA densities. In contrast to McClure's results, however, some new growth was produced even on highly infested trees, and none of the 60 trees in her study were dead after six years. Paradis also investigated various factors that might explain the apparent stability of HWA densities in her study sites, and found that higher sistentes densities increased mortality and decreased fecundity in the progredientes generation (Fig. 2). Overwintering mortality varied from year to year depending primarily on winter temperature, and was not consistently density dependent (Paradis et al. 2008).

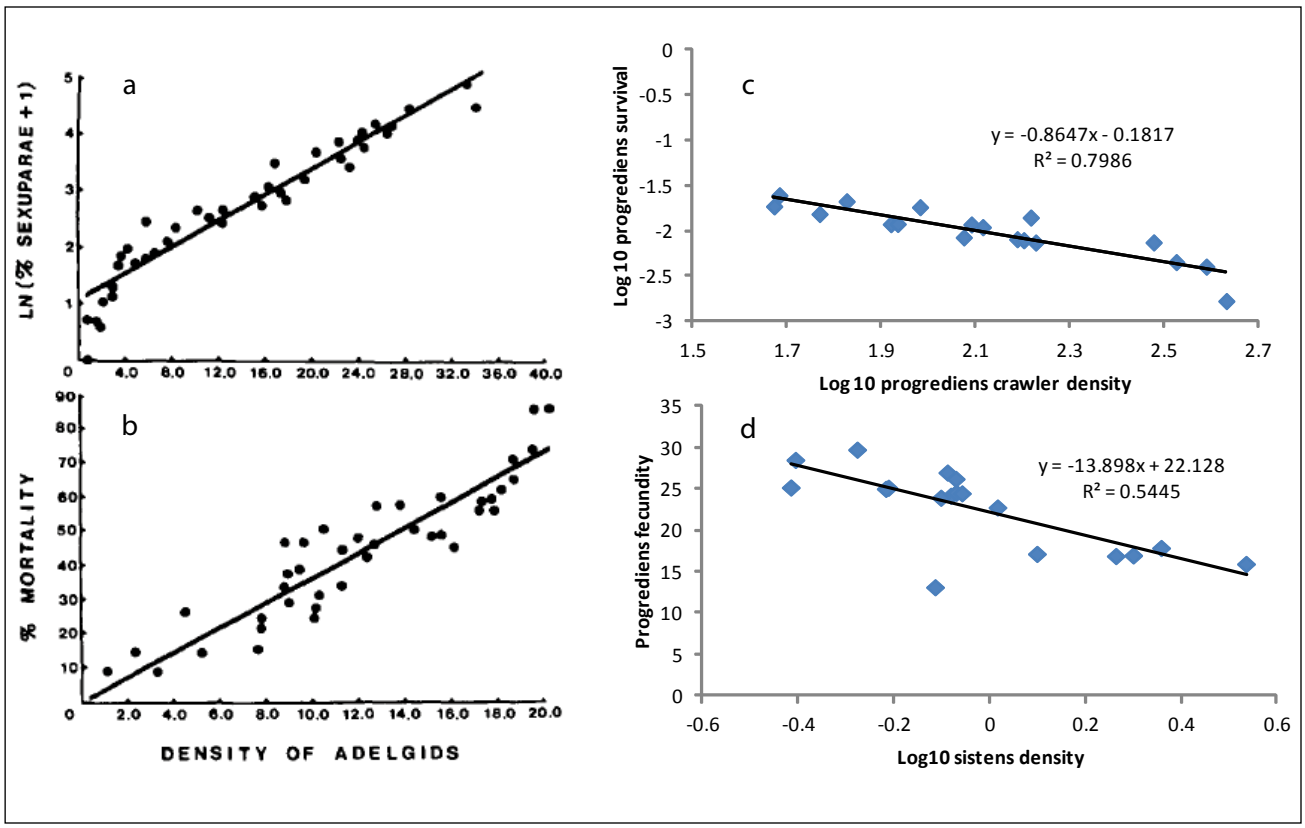

Figure 2. Density dependent a) production of sexuparae; b) mortality in the progredientes stage from McClure 1991; c) density dependent survival of progredientes; and d) progredientes fecundity recorded by Paradis (2011). Figure 2b plots mortality versus density of progredientes whereas Figure 2c plots survival versus density. They presumably represent the same process in the two different studies.

It is clear from the yearly variation in HWA density (Fig. 1) that some factor(s) limit the growth of HWA populations; this limitation is presumably caused by interaction of HWA with its hemlock host (McClure 1991). Both McClure (1991) and Paradis (2011) found density-dependent mortality among immature progredientes (Fig. 2). McClure attributed at least part of this mortality to density-dependent production of sexuparae (Fig. 2a), which disperse to settle on spruce. However, because none of the North American spruce species are suitable for HWA, none of these adelgids survive. Paradis (2011) also showed that sistentes density is negatively correlated with the fecundity of the following progredientes generation (Fig. 2d). Paradis (2011) found no evidence for density-dependent mortality in any other life stage, or for density-dependent changes in sistentes fecundity.

In the absence of native predators and parasites, the natural enemies of HWA in the invaded range appear to be the two predatory beetle species *Sasajiscymnus tsugae* and *Laricobius nigrinus* Fender. Both have been successfully introduced in the eastern United States as part of the ongoing biological control effort against HWA. The long-term impact of these releases is not yet known, though it appears unlikely that *S. tsugae* has a measurable impact on HWA population densities. Although the beetle was released in the mid 1990s (Cheah et al. 2004) at three of the sites that Paradis (2011) studied, intensive sampling for the beetles by Paradis (2011) did not recover any *S. tsugae* at these sites. Although *S. tsugae* has been recovered at sites many years after release, in the northeastern states it is rarely if ever abundant enough to significantly affect HWA densities.

In contrast, the predatory beetle *Laricobius nigrinus* (Derodontidae) was imported from the Pacific Northwest and has been recovered in large numbers at many release sites throughout the eastern United States (Mausel 2007, Mausel et al. 2008). It is by far the most promising biological control agent released against HWA so far, yet we still do not know how much of an impact it is having on HWA densities or dynamics. For this reason we concentrate our simulation efforts on this species.

SIMULATION

Data collected by Paradis (2011) (Fig. 1b), was used to parameterize simulations of HWA population densities. We began by constructing a simulation of the system in the absence of any predators based on generalized (averaged) values of sistentes and progridientes survival and fecundity. The data consist of information collected from eight locations, with 3-5 years of data for each location (Table 1).

These data provided the basic values needed to generate an empirically-based simulation of HWA dynamics. We constructed our model using two parallel approaches. In the first, a flow model was built to describe the population dynamics of the adelgid over the course of multiple life-stages and generations using Stella (V9.1 IE systems).For verification, a similar model was constructed in MS Excel®. In Stella we built a flow model using the following basic structure:

$$SA_{(t+1)} = (PA_{(t)} * PF_{(t)}) * SS_{(t+1)}$$

$$PA_{(t+2)} = (SA_{(t+1)} * SF_{(t+1)}) * PS_{(t+2)}$$

in which SA = Density of Sistentes Adults,
PA = Density of Progredientes Adults,

Table 1. Average values of density, mortality and fecundity of HWA life stages recorded by Paradis (2011) (Fig. 1b).

	Density: HWA per cm			Mortality: proportion dying				Fecundity	
	OW sistens	Progred adults	Immature sistentes	OW sistens	Progredientes	Sistens crawlers	Prewinter sistentes	Sistentes	Progredientes
Mean	2.21	1.33	10.60	0.52	0.99	0.64	0.75	146.97	22.54
Std. dev	0.90	0.43	5.41	0.27	0.01	0.13	0.15	29.77	4.85

SF = Sistentes Fecundity, PF = Progredientes Fecundity, SS = Sistentes Survival Rate, and PS = Progredientes Survival Rate. Based on this set of relationships, and using the above parameters, our simulated HWA populations grow exponentially. This behavior was expected, since any population with constant rates of mortality and fecundity will either grow or decline exponentially, and no doubt this behavior describes the growth of HWA populations when they first colonize a tree or stand. Such populations soon reach the carrying capacity represented by the available hemlock foliage and the densities are subsequently governed by the density-dependent processes described above. We thus incorporated density-dependent progredientes survival (Fig. 2c) and fecundity (Fig. 2d) into our simulation. The result is a population system that stabilizes at around two adult sistentes per cm of hemlock twig, but that exhibits the expected variation in density of different life stages evident in Figs. 1a and 1b. If we add a modest amount of annual variation in overwintering mortality, drawn at random from a normal distribution with a mean and standard deviation equal to that recorded by Paradis (2011) (Table 1), we generate fluctuations in simulated densities (Fig. 3a) similar to those observed by McClure (1991) (Fig. 2a) and Paradis (2011) (Fig. 1b). These results give us confidence that our simulation has captured the essence of HWA dynamics in the absence of predators, at least in the northeastern US where McClure and Paradis conducted their studies.

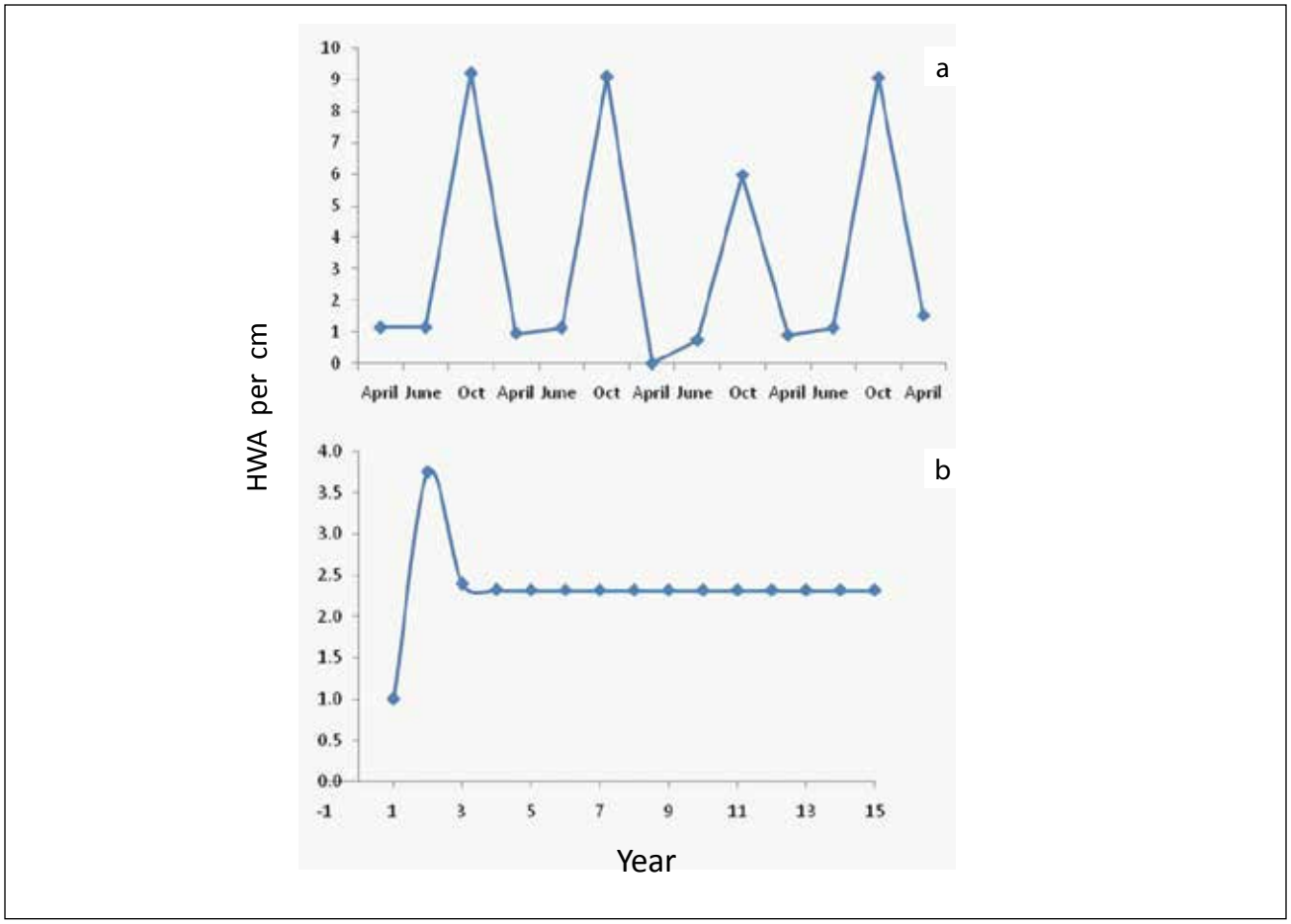

Figure 3. Our simulated time series of HWA density incorporating mean values of fecundity and survival of HWA life stages recorded by Paradis (2011) (Table 1) and density-dependence in progredientes survival (Fig. 2c) and fecundity (Fig. 2d). In Figure 3a we plot HWA densities at 3 times per year as in Figure 1 and we add variation in overwintering mortality recorded by Paradis (2011). In Figure 3b, we plot only one life stage per year (the overwintering adult sistentes) and we remove the variation in overwintering mortality. Otherwise they are the same simulation.

Although the population shown in Figure 3a demonstrates the fundamental stability evident in this system caused by the density dependence in mortality and fecundity discussed above, the stability is partially obscured by the inclusion of variable HWA overwintering mortality. This is highlighted if we remove this variation and plot only one life stage per year (overwintering sistentes, Fig. 3b) rather than the three life stages plotted in Figures 1 and 3a. As Figure 3b shows, the system stabilizes at an equilibrium value of approximately 2.1 sistentes per cm. Although instructive with regards to evaluating the fundamental parameters responsible for long-term population dynamics, it is important to remember that this simulation represents an idealized system. In natural settings, annual fluctuations still occur, and sometimes result in tree death (McClure 1991).

With these results in hand, we simulated the impact of predation by *L. nigrinus* on the eggs produced in early spring by the overwintering sistentes generation. This was accomplished by reducing the survival of HWA progredientes eggs (eggs laid by sistentes). We were surprised to learn that the removal of even 80 or 90 percent of these eggs had negligible effects on the subsequent density of the next generation in our simulation (Fig. 4a). This result occurred because the density-dependent mortality in the progredientes crawler stage (Fig. 2b,c) decreased as egg predation increased, offsetting the population decreases that would otherwise have occurred.

On further reflection, we realized that this result depends on the actual cause and timing of the mortality evident in this stage. If most or all of it is due to density dependent production of sexuparae (Fig. 2a) as McClure (1991) suggests, then the density dependence presumably occurs when the eggs are laid, rather than after the progredientes hatch. If that is true, then egg predation occurs after sexuparae production. If we incorporate this into our simulation, then egg predation has a profound effect on the equilibrium density (Fig. 4b), because it is now preceded instead of followed by the compensating density-dependent

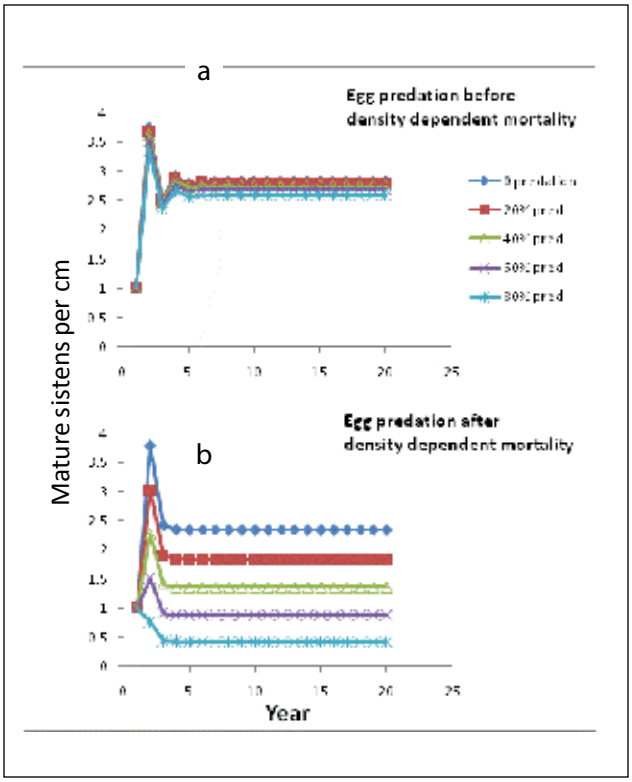

Figure 4. Simulated effect of predation on HWA eggs produced by overwintering sistentes a) after or b) before density dependent mortality occurring in the progrediens generation as in Figure 2a,b,c.

mortality. Alternatively, it seems likely that the response of the hemlock host is involved in some way that is not well understood in causing the density dependent mortality evident in Figure 2, as McClure (1991) suggested. Recent work by Radville et al. (2011) shows that HWA attack induces a systemic hypersensitive response in hemlocks. If the host response causes the mortality documented in Figure 2 and it occurs in response to HWA density during the overwintering sistentes stage, then the mortality may occur whether or not the egg densities are reduced by predation. The resulting effect would be like Figure 4b. If, on the other hand, the host response occurs when the immature progredientes settle, then the effect of egg predation would be compensated as in Figure 4a. The experiments by Radville et al. involved inoculating hemlock foliage with progredientes crawlers and their results thus suggest the latter

conclusion (Fig. 4a). These considerations make it clear that understanding the exact cause and timing of the mortality evident in Figure 2 should be a high research priority. We have initiated experiments designed to accomplish this need.

Thus far we have only considered the effects of constant rates of egg predation on HWA densities. In nature, egg predation rates presumably vary with density of *L. nigrinus* and we know from Mausel et al. (2008) that density of *L. nigrinus* increases with the density of HWA (Fig. 5). Furthermore, we know that adult *L. nigrinus* feed on late-instar sistentes in autumn. Incorporating these effects into our simulation also has profound effects on the equilibrium density, including the version of the simulation where egg predation precedes (Fig. 4a) rather than follows (Fig. 4b) progredientes mortality. In this version of our simulation, we make assumptions about the number of eggs and late instar sistentes consumed per beetle and allow beetle density to vary according to Figure 5. In Figure 6, we show the effects of varying the number of late-instar sistentes eaten per beetle. The resulting dynamics are complex. As the numbers of sistentes eaten per beetle increases, the mean HWA density declines, but the amplitude of the density fluctuations increases. As the number of HWA eaten per beetle increases, the HWA density progresses from equilibrium (10 sistentes per beetle), to damped oscillations, to evident two- and four-year cycles, and eventually extinction, which occurs when the fluctuating densities overlap zero. This behavior is reminiscent of the progression from equilibrium, to damped oscillations, to cycles with periods of 2n, and finally to chaos first explored by May (1974) with the discrete logistic model. This behavior was demonstrated subsequently in many other models. Whether our system is chaotic under particular parameter values is difficult to answer and of little practical importance. We present these results, not because we believe it to be an accurate description of the impact of *L. nigrinus* on HWA dynamics, but because it illustrates the complex dynamics that even simple models such as ours can exhibit.

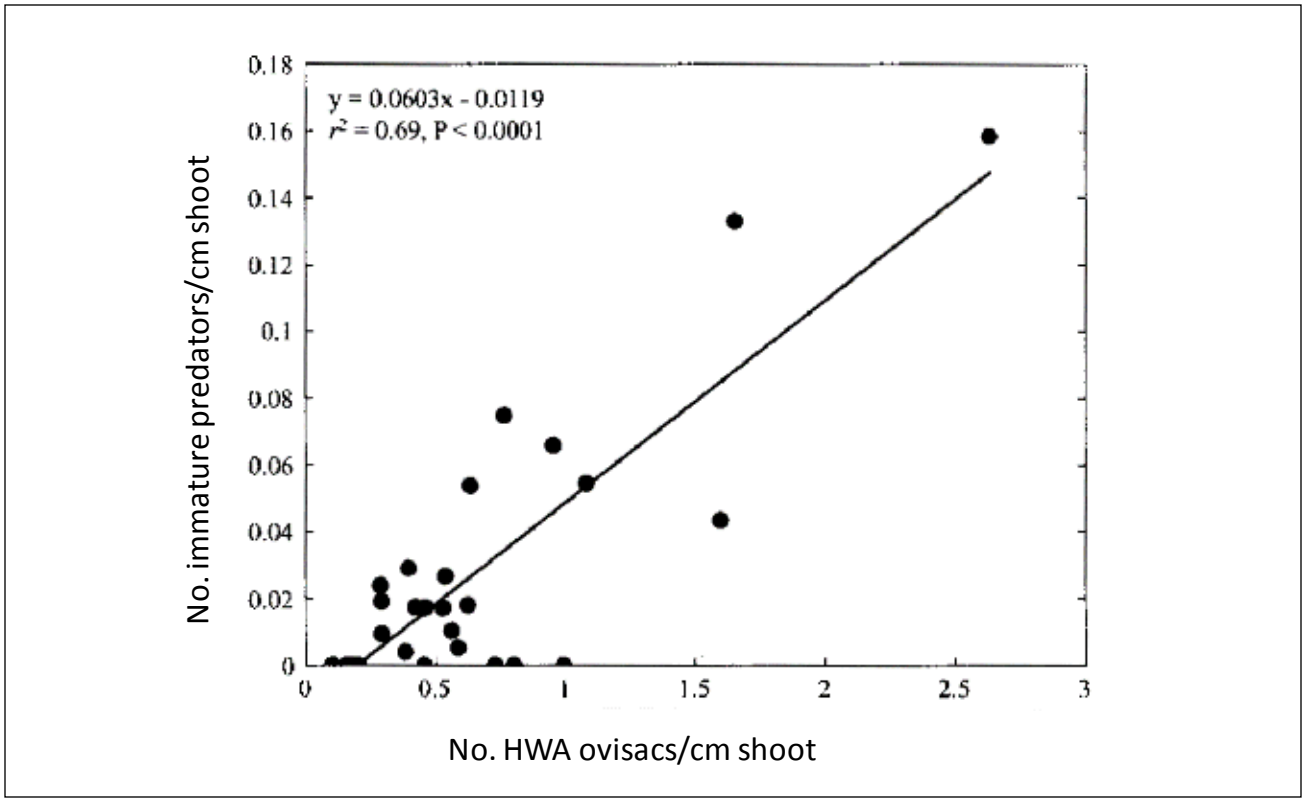

Figure 5. Increases in density of L. nigrinus as a function of HWA density recorded by Mausel et al. (2008).

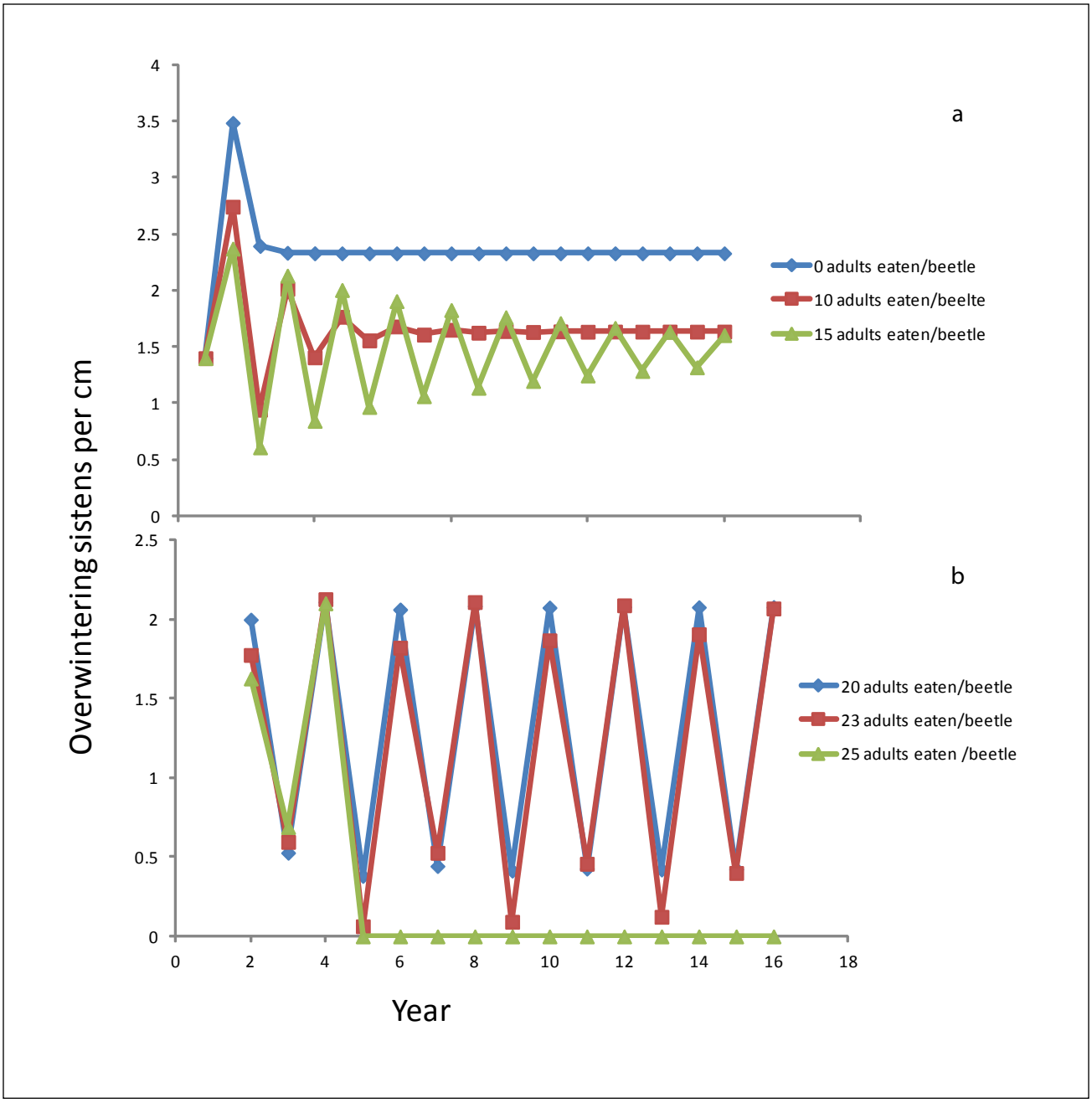

Figure 6. Variation in simulated HWA density as a function of numbers of late-instar HWA consumed by each individual adult L. nigrinus in the autumn.

DISCUSSION AND CONCLUSION

We are tempted to add more and more details to our model, incorporating various other factors that we already know affect HWA population dynamics. We might include host tree effects and factors influencing survival and fecundity of other life stages. We might also include the impact of elongate hemlock scale, *Fiorinia externa*, another invasive species co-infesting hemlock in the eastern US that Preisser and Elkinton (2008) have shown has a significant impact on HWA. We are mindful however, of the rather sorry history of complex simulations in forest entomology. The Gypsy Moth Life System Model (Sharov and Colbert 1994) for example, was constructed in the 1980s and 1990s at great expense and incorporated much of what was known about gypsy moth population dynamics. It was one of several such models, all of which were soon abandoned, because they were too complex to understand and they yielded little insight into the dynamics of the system (Liebhold 1994, Sharov 1996). Rather, models that incorporate minimal to intermediate complexity (Liebhold 1994, Logan 1994, Sharov 1996) have been shown to yield the most insight. The art of building such models is to know what to put in and what to leave out. It is our belief that any useful model of HWA dynamics must include the strong density dependent mortality in the absence of predators (Fig. 2) documented by McClure (1991) and Paradis (2011). These effects involve the interaction of HWA with its hemlock host, as McClure (1991) described, but they continue to govern the system even in the absence of tree mortality, as Paradis (2011) has shown.

Our simulation suggests that the impact of biological control agents on HWA can be counter-intuitive and can only be understood in the context of the rest of the population dynamics of the system. For example, our simulations revealed that very high rates of predation can have almost no effect on mean HWA densities, if they are followed by other compensatory density-dependent mortalities. These findings underscore the importance of introducing biocontrol agents that prey on multiple life stages, and the importance of the timing of predation.

Finally, we believe our simulation shows the importance of understanding the various factors that affect mortality and fecundity of different life stages of a pest population system undergoing biological control and can suggest critical experiments that need to be done to understand these effects. As additional information on the biology of the controls becomes available, our ability to estimate their potential impact should continue to improve.

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Chapter 3: Understanding Federal Regulations as Guidelines for Classical Biological Control Programs

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INTRODUCTION

This chapter reviews the legislation and rules that provide the foundation for federal regulation of the introduction of natural enemies of insects as biological control agents. It also outlines the steps for complying with regulatory requirements, using biological control of *Adelges tsugae* Annand, the hemlock woolly adelgid (HWA), as an example. The program to establish biological control agents for HWA in eastern North America dates from 1993 to present and involves importation from other continents, from other countries in the North American continent, as well as the interstate movement of biological control agents. Thus, biological control of HWA provides examples of regulation under old and new federal regulations and rules for foreign importation and interstate movement. With these regulations in mind, the several steps involved in putting a biological control program into practice will be reviewed—finding and importing agents, rearing and studying the agents in a containment facility (aka quarantine), acquiring data to show that the agent will be effective and safe, and release into a new environment. Monitoring the biological control agent's establishment and efficacy is also part of a biological control program, but this is not discussed here.

In its broadest meaning, biological control is the action of an organism that maintains the population of another organism at a lower average density than would occur if it was absent. From an applied viewpoint, biological control is the use of natural enemies (predators, parasites, pathogens) to reduce a pest population and thus the damage it causes. Classical biological control is the introduction and permanent establishment of natural enemies in order to reduce populations of a non-indigenous pest. It is the movement (importation from a foreign country, release from containment, and interstate transport) of biological control organisms that is federally regulated.

The federal government recognizes that biological control is often a desirable, low-risk means to reduce pests of crops and other plants; however, the unregulated movement of certain biological control organisms may present an unacceptable risk. Thus, the government has a dual role to facilitate biological control and also to assess the risks and benefits of releasing specific organisms for biological control of pests. Regulations regarding the movement of entomophagous (insect-eating) biological control organisms have not been promulgated, although federal legislation was passed in 2000 authorizing regulation of all biological control organisms. Because regulatory policies now in place are not transparent and widely available, many practitioners may not be adequately informed. This review of the underlying legal framework and the steps in importing and releasing biological control organisms will hopefully fill some of the information gaps practitioners may have.

[1]Disclaimer: Views and statements are those of the author and should not be interpreted as official policy of any federal agency. Anyone considering releases of biological control organisms must follow official regulations and should not rely only on the information in this chapter.

DEFINITIONS

The terminology associated with biological control varies with disciplines and can have uncertain and conflicting meanings. Even familiar terms when used by government agencies can have meanings that are narrower or broader than when used scientifically. Therefore, the following definitions are provided for terms used in this chapter.

Act means a public law passed by the United States Congress. These acts are listed sequentially by Congressional session (e.g., Public Law 106-224 is the Agricultural Risk Protection Act of 2000). These are organized by topic in the United States Code (USC).

Biological Control has various meanings, but the definition of DeBach (1964) is appropriate for this chapter—"the action of parasites, predators, or pathogens (disease-causing organisms) in maintaining another organism's population density at a lower average than would occur in its absence." DeBach recognized three approaches to achieve biological control: *classical biological control* is the purposeful introduction and permanent establishment of natural enemies to suppress populations of a pest; *augmentation* is the supplemental or inundative release of natural enemies in areas where they are missing, too scarce to provide adequate control, or arrive too late in the season to be effective; and *conservation of natural enemies* is management to enhance the survival and impact of established natural enemies.

Biological Control Organism, as defined by public law, means "any enemy, antagonist, or competitor used to control a plant pest or noxious weed" (7 USC 7702).

Indirect Damage is when an organism adversely affects another organism that is beneficial to plants, and those adverse effects cause losses in yields of crops or forage plants or a reduction in the viability or vigor of ornamental or native plants (*cf.* definition proposed but not adopted (USDA 2001)).

Movement, **Move**, and **Moving** includes release into the environment as well as the transport or facilitating the transport, by any means, into the country or between states.

Nonindigenous refers to a plant or animal that is not native to a place. Introduced, adventive, alien, exotic, nonnative, and nonindigenous (non-indigenous) species are used rather interchangeably to indicate a species living outside its native distributional range and that arrived there by human activity, either accidental or deliberate.

Regulations are general and permanent rules developed by executive branch agencies to administer and enforce the Acts passed by the U.S. Congress. These rules (also called administrative laws) are published in the Federal Register and codified under 50 titles in the Code of Federal Regulations (CFR), which is updated annually.

LEGAL FRAMEWORK

Legislation

In the United States, there are many acts that may pertain to biological control organisms, but three are especially important. The most significant is the Plant Protection Act (PPA) passed by Congress on June 20, 2000 (U.S. Congress 2000). This Act consolidated all or part of ten existing plant health laws into one law that gives the Secretary of the United States Department of Agriculture (USDA) broad authority to regulate movement of any plant, plant product, biological control organism, noxious weed, and plant pest. This authority has been delegated to the Department's Animal and Plant Health Inspection Service (APHIS) and the regulation of biological control organisms has been assigned to APHIS's Plant Protection and Quarantine (PPQ) program. Most of the provisions of the Federal Plant Pest Act (FPPA) of 1957, which regulated the importation and interstate movement of plant pests, were retained with the new act providing additional authority for regulation of noxious weeds and biological control organisms.

The PPA recognizes the need to prevent the dissemination of plant pests and to facilitate the use of biological control to protect plants from their pests, including noxious weeds. Excerpts from the Act (Table 1) illustrate how regulation of the movement of plant pests (Section 411) is simple and direct—movement of plant pests is prohibited without a permit; whereas Section 412, regulating the movement of biological control organisms, is much more complex. For example, Section 412 (a) states that any biological control organism would be regulated only if it is determined that this is necessary to prevent the introduction or dissemination of a plant pest; and Section 412 (l) indicates that certain biological control organisms may be listed as exempt from restrictions on movement in interstate commerce. The following section on regulations illustrates the difficulty of developing administrative rules to both promote and ensure the safety of biological control organisms.

The second important piece of legislation affecting biological control programs in the United States is the National Environmental Policy Act (NEPA). This law, effective January 1, 1970, applies to all federal activities, including projects receiving federal funds and permits issued by federal agencies. Non-federal applicants are not responsible for compliance; rather, the federal agency that proposes the action, provides the funds, manages the land where the activity will occur, or issues the permit is responsible for compliance. To fulfill NEPA provisions, the agency first determines which of three levels of analysis is required. The simplest level of analysis is for an activity that has been predetermined to be Categorically Excluded (CE). These are specific activities listed by the agency that it has determined have limited environmental effects. Examples of CE activities listed by APHIS include (a) interstate movement of nonindigenous species between containment facilities; (b) importation of nonindigenous species into containment facilities; and (c) releases into a State's environment of pure cultures of organisms that are either native or are established introductions (see 7 CFR Ch. III 372.5). If the activity is characterized

Table 1.—Excerpts from the Plant Protection Act.

Section 411. REGULATION OF MOVEMENT OF PLANT PESTS. (7 USC 7711)

(a) PROHIBITION OF UNAUTHORIZED MOVEMENT OF PLANT PESTS.—No person shall import, enter, export, or move in interstate commerce any plant pest, unless the importation, entry, exportation, or movement is authorized under general or specific permit.

Section 412. REGULATION OF MOVEMENT OF PLANTS, PLANT PRODUCTS, BIOLOGICAL CONTROL ORGANISMS, NOXIOUS WEEDS, ARTICLES, AND MEANS OF CONVEYANCE. (7 USC 7712)

(a) IN GENERAL—The Secretary may prohibit or restrict the importation, entry, exportation, or movement in interstate commerce of any plant, plant product, biological control organism, noxious weed, article, or means of conveyance, if the Secretary determines that the prohibition or restriction is necessary to prevent the introduction into the United States or the dissemination of a plant pest or noxious weed within the United States.

(b) POLICY.—The Secretary shall ensure that processes used in developing regulations under this section governing consideration of import requests are based on sound science and are transparent and accessible.

(c) REGULATIONS.—The Secretary may issue regulations to implement subsection (a), including regulations requiring that any plant, plant product, biological control organism, noxious weed, article, or means of conveyance imported, entered, to be exported, or moved in interstate commerce.

(e) STUDY AND REPORT ON SYSTEMS APPROACH [for plant pathogens]

(e) NOXIOUS WEEDS.—

(g) BIOLOGICAL CONTROL ORGANISMS.—

(1) REGULATIONS.—In the case of biological control organisms, the Secretary may publish, by regulation, a list of organisms whose movement in interstate commerce is not prohibited or restricted. Any listing may take into account distinctions between organisms such as indigenous, nonindigenous, newly introduced, or commercially raised.

(2) PETITION TO ADD OR REMOVE BIOLOGICAL CONTROL ORGANISMS FROM THE REGULATIONS.—Any person may petition the Secretary to add a biological control organism to, or remove a biological control organism from, the regulations issued by the Secretary under this subsection.

(3) DUTIES OF THE SECRETARY.—In the case of a petition submitted under paragraph (2), the Secretary shall act on the petition within a reasonable time and notify the petitioner of the final action the Secretary takes on the petition. The Secretary's determination on the petition shall be based on sound science.

as being limited in scope to specific sites, specific species, or activities that potentially would impact few environmental values or systems, then the agency would prepare an Environmental Assessment (EA). The purpose of the EA is to determine the significance of the environmental effects and to examine alternative means to achieve the objective. The EA includes a brief discussion of: (1) the need for the proposal, (2) alternative courses of action, (3) the environmental impacts of the proposed action and alternatives, and (4) a listing of agencies, institutions, and persons consulted. The applicant, the public, and other agencies may be involved in preparing or commenting on the draft EA. The process concludes with either a Finding of No Significant Impact (FONSI), or the application is denied. The third NEPA category, the Environmental Impact Statement (EIS), is used for changes in policy, such as changes in agency regulations, and for activities that do not qualify for a FONSI.

The third important piece of federal legislation is The Endangered Species Act of 1973. This act requires federal agencies, in consultation with the U.S. Fish and Wildlife Service (F&WS) or the NOAA Fisheries Service, to ensure that any action that a federal agency authorizes, funds, or carries out is unlikely to jeopardize the continued existence of a listed species, or result in the destruction or adverse modification of designated critical habitat of such species. Proposals to release a nonindigenous species into a new environment would be reviewed by the F&WS for potential impacts on endangered species. The federal agency, such as APHIS, conducting an EA usually asks the F&WS for its opinion regarding threats to federally-listed endangered or threatened species. The F&WS is also responsible for monitoring the movement of wildlife, which includes insects (50 CFR 14—Importation, Exportation and Transportation of Wildlife). The F&WS is particularly concerned about species that are listed according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Import permits from F&WS are required for species that are on the CITES list. Currently there are no entomophagous insects on the list; however, shipments of all biological control organisms should be accompanied by a Declaration for Importation or Exportation of Fish or Wildlife (USFWS Form 3-177).

Regulations

Implementation of the Acts of Congress is done usually by agencies in the Executive Branch by a process of "rule-making." The proposed regulations are published in the Federal Register for public comment and as final regulations. These are then compiled in the Code of Federal Regulations (CFR), which is available on the internet.

Regulations for movement of biological control organisms are provided in 7 CFR Part 330–Federal Plant Pest Regulations; General; Plant Pests; Soil, Stone, and Quarry Products; Garbage. In response to passage of the PPA of 2000, Section 330.102 was revised in April, 2001 to include biological control organisms among the items that "the Secretary of Agriculture may prohibit or restrict the importation, entry, exportation, or movement in interstate commerce, . . . if the Secretary determines that the prohibition or restriction is necessary to prevent the introduction into or the dissemination within the United States of a plant pest or noxious weed." Although the CFR was updated to reflect USDA's current authority to regulate the importation and movement of entomophagous biological control organisms, new rules to exercise this authority have not been incorporated into the Regulations. Extensive revisions were proposed in the Federal Register of October 9, 2011, but public concerns about the proposed rules and reorganization of security following events of September 11, 2001 sidetracked their adoption. Nonetheless, these proposed rules (USDA 2001) provide insight into the complexity of developing detailed rules for the regulations for the many aspects of biological control of arthropods and noxious weeds.

In 2009, APHIS-PPQ asked for public comment to help it determine which alternative it should examine in preparing an Environmental Impact Statement for Movement of Plant Pests, Biological Control Organisms, and Associated Articles (USDA 2009). The alternatives proposed were:

(1) Take no action—leave current rules unchanged

(2) Revise requirements for movement of plant pests to cover biological control organisms consistent with the scope of the PPA (preferred alternative)

(3) Implement a comprehensive risk reduction program (more expansive regulations to address specific risk categories)

The statement "Establishment of clear, coherent, and streamlined regulations at the national level will be important to ensuring objective assessment of the risks and benefits of biological control in the U.S." (Mason et al. 2005) still applies today. Although new regulations of biological control organisms have not yet been issued, APHIS has new policies and procedures for regulation of entomophagous biological control organisms that are largely unpublished or available on web pages; thus, it is important to check with the agency regarding compliance if you wish to import, release, or move any biological control organism.

HISTORICAL REGULATION OF BIOLOGICAL CONTROLS FOR HWA

Regulation of the importation of entomophagous biological control agents from foreign countries to approved containment facilities has changed little in the last 50 years. What has changed is regulation of the first-time release of an entomophagous biological control agents into the environment. The hemlock woolly adelgid (HWA) provides a good example of these changes (Table 2), which fall into three groups:

Table 2.—Imported arthropods released from containment for biological control of the hemlock woolly adelgid and procedures used to assess potential risk of release.

Species, author, year described	Origin	Released from containment	Evaluation process used by APHIS	NEPA compliance[5]
Diapterobates humeralis (Hermann) 1804	Japan[1]	1992	OPRA[3], Limited Review	?
Sasajiscymnus tsugae (Sasaji & McClure) 1997	Japan	1995	OPRA, Limited Review	APHIS, FS, NPS
Scymnus sinuanodulus Yu & Yao 1997	China	1998	First-tier Risks (Not Regulated)	FS, NPS
Laricobius nigrinus Fender 1945	Canada[2]	2000	First-tier Risks (Not Regulated)	FS, NPS
Scymnus ningshanensis Yu & Yao 2000	China	2000	First-tier Risks (Not Regulated)	FS
Scymnus camptodromus Yu & Liu 1997	China	2000	First-tier Risks (Not Regulated)	—[6]
Laricobius osakensis Montgomery & Shiyake 2011	Japan	2010	NAPPO[4], Independent Review, Public Comment	APHIS

[1]Widespread, including North America

[2]Endemic to western North America; permits not issued for subsequent movement from western States to eastern States

[3]OPRA=Organism Permitting and Risk Analysis conducted by the Biological Assessment and Support Team (BATS) of APHIS-PPQ

[4]Standards to release entomological biological control agents adopted by the North American Plant Protection Organization

[5]Compliance with the National Environmental Policy Act by Federal Agencies, Animal & Plant Health Inspection Service, Forest Service or National Park Service

[6]Has not been released into the environment

(1) Following the passage of NEPA in 1970, APHIS prepared an EA for the first-time release of all biological control organisms into the environment. The process for obtaining a release permit was not much different from the process currently used, except that it was less rigorous in the information required and the scope of the review. The permit granted, however, was usually restricted to a single State and of limited duration.

(2) Sometime between 1995 and 1997, Federal lawyers interpreted that the FPPA of 1957 did not apply to the release of entomophagous insects, but only to their importation and holding in containment. When an application to release an entomophagous insect from containment was received, APHIS would decline jurisdiction if it determined that the organism met First-Tier Risk criteria (Table 3). Because a permit was not issued, the need for an EA was not triggered. After APHIS declined jurisdiction, biological control practitioners could move entomophagous organisms to the laboratory for further research or mass rearing; however, if they were federal employees, received federal funds for the project, or the organisms would be released on federal lands, then NEPA applied and an EA was needed prior to a release into the environment. Biological control agents for weeds (e.g., herbivorous insects) still required an EA for release from containment and into the environment. For these "weed-eaters", APHIS has had in place, for more than fifty-years, published guidelines and a Technical Advisory Group (TAG) to evaluate applications for release (http://www.aphis.usda.gov/plant_health/permits/tag/index.shtml).

(3) In early 2006, under the authority of the PPA of 2000, APHIS resumed issuing permits for the release of entomophagous organisms from containment and preparing EAs for their first-time release into the environment. The current procedure is similar to that used before 1995, except that the EA provides a more thorough analysis of risks and benefits, and public comment on the EA is solicited. Although it can take a year or more for final approval, the permit is usually comprehensive, with few restrictions, and is often a key to quickly obtaining subsequent approvals from States and other federal agencies, if needed.

Although APHIS's policies and procedures for the regulation of entomophagous biological control organisms are not formally established, my understanding of these is incorporated in the following procedures. It is strongly advised to first check with APHIS before attempting the first-time introduction of any biological control organism in any State, regardless of whether the source of the organism is domestic or foreign.

Table 3.—First-tier risk assessment of nonindigenous invertebrates and micro-organisms proposed as candidates for release from containment.

1) This organism has been identified to species /strain /biotype by a recognized authority.
2) All reasonable efforts have been made to exclude undesirable plant pests and other contaminants.
3) This organism does not feed on or infect living plant tissues.
4) This organism does not feed on, infect or contaminate plant products.
5) This organism does not transmit plant pathogens.
6) No life stage or sex of this organism develops as a parasite or pathogen of a primary parasite.
7) Release of this organism is not expected to cause significant losses in yields of crop or forage plants by causing major, population-level damage to commercially important pollinator or important natural enemies of plant pests or weeds.
8) This organism is not expected to feed upon, attack, infect or otherwise adversely impact endangered or threatened plants or animals in the United States.

THE PROCESS FROM EXPLORATION TO RELEASE

The process for introduction of biological control agents is often presented as a series of sequential steps; however, the path usually is neither linear nor uniform. In reality an outline of procedures is analogous to a roadmap that shows a fairly straight mountain road, which upon travelling is found to have ups and downs, switch-backs, and wrong turns, but also the excitement of new discoveries. Table 4 is an example of a "roadmap" intended to help with planning. Chapters in other books give a broader, more general explanation for each step of a program to introduce new natural enemies (Van Driesche and Bellows 1996, Bellows and Fisher 1999). The emphasis in this chapter is on compliance with federal regulations and aspects of the process that usually are not provided in generalized descriptions.

Initial Surveys

The literature on the target pest and its natural enemies should be compiled for both where the pest is indigenous and where the natural enemies will be introduced. This literature survey should also include relatives of the target pest and their natural enemies. Besides taxonomic information, this compilation should include the distribution and host records of the natural enemies, if available. This information not only forms the basis to define suitable areas to explore and what groups of natural enemies to search for in these areas, but also what taxonomic expertise may be needed. The ability to identify natural enemies is critical for biological control programs as the potential candidates often are undescribed species—five of the seven natural enemies imported for control of HWA (Table 2) were species previously unknown to science.

Table 4.—Synopsis of steps and activities for obtaining and introducing biological control organisms.

Step	Partial List of Activities
Survey potential release areas	For potential release areas, define the climate and existing natural enemies attacking the target pest; start monitoring target population and potential non-target hosts.
Initial planning/preparation (Where, What, When)	Define search area and targets; review rules for export from search area; establish support team and funding; obtain use of approved containment facility; obtain import permit (PPQ-526)
Exploration/collection	Have suitable equipment for collecting, studying and keeping agents alive; do preliminary host range study; make arrangements for additional collections and study of natural enemies in collection area(s)
Shipment	Have proper forms for export (if needed) and import (PPQ-526); have suitable shipping materials; alert APHIS and your quarantine officer of shipment
Rearing, and evaluation in containment	Free agents of contaminants, obtain positive identification of candidate agents, develop rearing methods, study biology and potential host range in target release area(s)
Biology in indigenous habitat	Obtain information on biology and feeding range of selected natural enemies in indigenous area
Release from containment	Prepare release petition using NAPPO guidelines; apply for release permit (PPQ-526); APHIS-PPQ prepares an EA and solicits comments
Field release/establishment	Mass rear, as needed; decide where, when, how many/location for releases; obtain State and local permission for release; initial assessment of establishment and efficacy

A field survey for natural enemies of the target pest, and its relatives, in the areas where it has become established should be made prior to making plans to collect and import natural enemies of the target pest. What native natural enemies may interact with the prospective natural enemy (e.g., its parasites and competitors) should also be identified. This background information about existing fauna in potential release areas helps to define the missing components (e.g., natural enemies) and to identify potential nontarget or alternative hosts of the prospective natural enemy. Potential nontarget or alternate hosts and possible interactions with native natural enemies are very important considerations in risk analysis.

Another purpose of the pre-introduction surveys is to establish a reference collection of positively identified natural enemies in the prospective introduction areas. The biological control program for the balsam woolly adelgid, *Adelges piceae* (Ratzeburg), failed to recognize native congeners of some of the introduced species resulting in false reports of the establishment of introduced species (Montgomery et al. 2011). Pre-introduction surveys of HWA natural enemies made in Connecticut and North Carolina (Montgomery and Lyon 1995, Wallace and Hain 2000) provided some background information for the HWA biological control program.

Where, What, When

Although the literature on the distribution of the target pest may indicate where to search for its natural enemies, its biology and natural enemies may not have been reported, since introduced insects are often not pests in their indigenous regions. For HWA, we know where it originated—Japan (Havill et al. 2006)—and that HWA is also indigenous in western North America and in China, but these populations differ genetically from the population in the eastern United States (Havill et al. 2007). Other areas where HWA is indigenous include Taiwan, Nepal, Vietnam, and India, but these areas have climates that are less similar to target release areas in the eastern United States than areas already explored. Because of concerns about climate matching, the populations of *Laricobius nigrinus*, from moderate, coastal climates that were released for biological control of HWA in the eastern U.S. were supplemented with populations from cold, mountainous areas (Mausel et al. 2011).

Unlike most insects, adelgids have no parasites and no specific pathogens; thus, the search for natural enemies is limited to predators. Past introductions of biological controls for HWA have been limited to predatory beetles—lady beetles and derodontids (Cheah et al. 2004). Surveys in western North America have identified *Leucopis* flies (Diptera: Chamaemyiidae) that are part of the predator complex that feeds on HWA (Kohler et al. 2008), but their introduction is hampered by their abundant parasites, difficulty in rearing them, and taxonomic problems.

Ideally, what to import would be based on the knowledge that the natural enemy actually regulates indigenous HWA populations. Life table analysis is a robust method, introduced by Varley et al. (1973), for describing the sources of and the quantifying mortality of a population in order to provide insight into the regulation of insect populations. Unfortunately, life tables are difficult to construct for field populations, especially when predators, rather than parasites, are the source of mortality. Additional information on construction of a life table can be found in Morris (1957), Royama (1981), Buonaccorsi and Elkinton (1990), and Bellows and Van Driesche (1999). A study of white fly mortality is a good example of application of a life table (Naranjo and Ellsworth 2005). McClure's (1995, 1997) analysis of HWA mortality in Japan is one of the few efforts to collect mortality caused by prospective biological controls in their indigenous environment. He concluded that both density-dependent negative feedback (host resistance) and natural enemies played important roles in keeping populations of HWA low in Japan (McClure 1997), but it is unclear what natural enemies were responsible. For example, one article points to an orbatid mite, which dislodged HWA eggs (McClure 1995), another to four insect predators (McClure 1997), and another to a lady beetle (Sasaji and

McClure 1997). He also noted that HWA mortality was high (>99%) in forests, where HWA density was low, but that mortality from predators was very low (<9%) on cultivated hemlocks with high HWA populations. He concluded that "the best biological control agents of introduced pests may not be those that help maintain pest populations at non-outbreak levels in natural habitats, but rather those that are most responsive to pest outbreaks in cultivated and disturbed habitats" (McClure 1997).

In considering if natural enemies of relatives of the target species should be introduced, the life history and habitat requirements of the relatives and targets should be considered. The natural enemies released to control balsam woolly adelgid in North America were not successful, partly because they were collected from other adelgids and were not adapted to the climate (Schooley et al. 1984). It seems unlikely that reintroduction of these species for biological control of HWA would be successful.

The best time to observe natural enemies in their indigenous habitat may not be the best time to collect them to establish colonies in containment. The greatest diversity and abundance of natural enemies of HWA seems to be in the spring, when the eggs of the overwintering and spring generations of HWA are present. Most discovery and first-time importations of natural enemies of HWA were made in the spring, but successful establishment of breeding colonies was done with fall collections. This is because most HWA predators are univoltine and lay eggs in the spring; thus spring imports may have already produced most of their eggs. Predators that feed on HWA during the fall can be collected then and stored in the containment facility until spring, enabling oviposition by the predator to be synchronized with the life history of HWA in the target region.

The Containment Facility

Organisms imported for classical biological control are usually brought first into a containment facility (aka quarantine). Thus, well before a permit is requested, the applicant must have access to a containment facility that has been inspected and approved by APHIS-PPQ. The process of certifying a facility for containment of arthropods takes 1-4 months and APHIS should be consulted prior to its construction. APHIS consults with state officials about the construction of the containment facility and before issuing permits to import or release organisms into the state. It is good protocol to inform the state official about your program in advance. An approved containment facility must have a Standard Operating Procedure (SOP) and this should be reviewed in advance of importations to make sure that your activity fits within the SOP. The Quarantine Officer should also be provided with an outline of your proposed activities and a copy of the approved permit so that he or she can ensure all protocols and restrictions are followed. Additional information regarding containment can be found at http://www.aphis.usda.gov/plant_health/permits/organism/containment_facility_inspections.shtml.

Import Permits

The form PPQ-526, titled "Application for Permit Move Live Plant Pests or Noxious Weeds," is used to obtain a permit for importing biological control agents. (As the title suggests, this form is also used for movement of plant pests and noxious weeds.) It has existed for more than 25 years, although it has been modified several times and adapted for electronic filing. On the form, the word "pest" means the organism(s) for which you are seeking a permit, thus organisms intended for use as biological control agents are to be listed on the form as "pests to be moved." Use scientific names, but species group names—genus, family, order—may be acceptable since APHIS recognizes that little may be known about the natural enemy complex of the target organism in its indigenous habitat. If hosts of the natural enemy or foliage will be included in shipments, the scientific names of these are listed in a separate category. The countries where the collections will be made must be listed. Methods of containment and final disposition are required; thus, an approved containment facility and standard operating procedure usually is a prerequisite for importing biological control candidates. Application for permits should be made by the

research scientist or other leader of the project rather than the quarantine officer. A minimum of eight weeks should be allowed to receive the permit.

The application (PPQ-526) can be filed electronically or using a paper form (APHIS recommends the former). Electronic filing requires that the applicant receive a USDA eAuthentication Account with Level 2 Access. Obtaining an eAuthentication account involves filling out a simple form online and then going to the nearest USDA Service Center to show a driver's license or other government-issued photo ID. There are several advantages in using eAuthentication to apply for permits: (1) there is helpful guidance in filling out the permit, such as pull down menus for countries and organism names, (2) processing is much faster—initial review takes one week whereas a paper application takes one month, (3) progress of the approval can be tracked, (4) your template is saved for renewal or application for another permit, (5) tasks, such as ordering shipping labels and filing annual reports, can be done by email, and (6) you will receive advance notice of permit expiration.

Shipping

After receiving the permit, the permit holder will need to request the PPQ Form 599 Red/White labels to enable foreign shipments to enter the United States (the labels are not issued or used for domestic, interstate shipments). Each of these distinctive labels has an individual number and an address of a USDA Plant Inspection Station (PIS). The red and white labels are not reusable or transferable and records of each use are tracked electronically using a barcode on each label. When shipping natural enemies, a red and white label is affixed to the outside of the package and supplemental information is placed inside (minimum is the permittee name, permit number and label number). Legally, the package should include the USFWS Form 3-177 on the outside (see http://www.fws.gov/le/ImpExp/faqs.htm). To expedite the shipment, I make an invoice (Table 5) with all the information that may be needed to clear the package and place this invoice in a clear pouch on the outside of the container addressed to Inspectors with a copy between the inner and outer layer of the shipment packaging.

The permit includes detailed information on packaging the shipment, conditions regarding what may be shipped, as well as detailed step by step instructions regarding clearance for U.S. Customs and Border Protection (CBP) and APHIS PPQ Agriculture Plant Inspection Station (PIS). Shipments brought to the U.S. via commercial bonded-carriers or hand carried go first to CBP for clearance, with PIS and F&WS helping as needed. After clearance, the shipment may be transported to the containment facility by the same bonded carrier or it may be reshipped to the containment facility by APHIS-PPQ using the permittee's designated carrier, billed to the permittee's account. My experience is that the inspectors from these three Departments work closely, shipments are cleared very quickly, and delays can be traced to necessary information not being provided with the shipment, or to the carrier.

Table 5.—Outline of invoice letter.

1) Permit number and label number, permittee name.
2) The species (genus or family, if species unknown), both plants and animals, in the shipment
3) Statement that the shipment does not include CITES species
4) Statement that no venomous animals are in the shipment.
5) Statement that the contents have no commercial value
6) Address to forward the shipment to its final destination
7) Your carrier and billing account number for reshipment (APHIS will not pay shipping costs)
8) Name and phone number of a contact (quarantine officer) at the final destination.

Although hand-carrying live natural enemies from a foreign country directly to the containment facility can be arranged, this may not be safer or more expedient than shipping by bonded carrier. This privilege must be requested when applying for the permit and can be done only by the permittee or others designated on the permit. At least 20 days before the entry, each hand-carry event must be submitted and pre-authorized by the PPQ Permit Compliance Officer, who will notify CBP and provide you with a red and white label specifically prepared for the hand-carry event. The request should include details about who, when and where (i.e., the person who will carry, specific date, flight, and scheduled arrival), as well as details about what the package will contain, including foliage or other host material. Any deviations from what was pre-authorized, or changes in the airline or the travel date, will create the risk that the CPB officer will seize the package and send it to the nearest PIS or have it destroyed. Flight delays typical of airline travel should not create problems. After the package is released by CBP, it must be taken directly to the containment facility, and the quarantine officer (not the person who carried the package) must notify the PPQ Compliance Officer of the organisms received within 24 hours of their arrival.

Biology and Host Specificity Research

Information on the agent's biology and host specificity should be conducted in its indigenous habitat as well as in the containment facility. This is listed as two steps in Table 4, but one does not necessarily precede the other and they may occur simultaneously. Frequently, it is not until the species is imported and in the containment facility that its identity and potential for biological control is recognized. It often is necessary to return to where the species was collected to examine its biology and host range more thoroughly.

The biological information for a petition to release an entomophagous biological control agent not only includes its identity but also methods to distinguish it from its relatives. Additional information should be provided about closely related species so that potential interactions with these can be assessed. For example, *Laricobius nigrinus* Fender, which was introduced in the eastern United States, can hybridize in nature with the indigenous *L. rubidus* LeConte (Davis et al. 2011). The likelihood that the agent may compete for food resources with native predators and be attacked by predators, parasites, and pathogens currently established in the proposed release area should be discussed. Information should also be provided on the agent's dispersal capability and potential to thrive in the climate of the proposed release area.

Predictions of host range should be based on observations in the putative agent's indigenous environment as well as host specificity testing conducted in the laboratory. When a potential biological control is first discovered, the collectors should also search for relatives of the target pest and determine if the putative agent also attacks it. This can be done using simple tests—for parasitoids, potential hosts can be recovered and reared to see if the putative agent emerges, and with predators simple feeding tests in small dishes may be done overnight. Notes should be made of the flora and fauna in the putative agent's indigenous environment. Once the identity of the putative agent is confirmed, then literature searches may reveal other potential hosts.

Laboratory evaluation of host specificity begins with compiling a list of potential non-target species for testing that includes species with phylogenetic and ecological similarities, and species that may be endangered or of special ecological significance (Kuhlmann et al. 2005). Often a hierarchical framework is used that starts with no-choice tests done in small arenas followed by choice tests, to determine prey that are attacked and prey preferences, and then rearing trials to determine prey suitability for development (van Lenteren et al. 2006). A good example of hierarchical testing is the evaluation of *L. nigrinus* (Zilahi-Balogh 2005a,b), although Simberloff (2011) has questioned the adequacy of this. Natural enemies in the laboratory may utilize hosts that they would not utilize in nature; thus it is important to validate host specificity testing with knowledge

of the host range in the candidate's native areas. Anomalies in host specificity and biology in the laboratory can often be clarified with information about the natural enemy in its native range.

Movement from Containment and Environmental Release

In terms of federal regulation, the removal of entomophagous agents from containment facilities is regarded as a release into the environment. The same extensive information and thorough review as done for a "full" release into the environment would be needed for a "partial" release to rearing laboratory or for caged field studies. The process to remove a biological control agent from a containment facility begins with the same form used to import the organism into containment—Form PPQ-526. However, this time the permit application should include a separate report with the information (Table 6) requested in the North American Plant Protection Organization (NAPPO) "Guidelines for Petition for First Release of Non-indigenous Entomophagous Biological Control Agents" (NAPPO, 2008). The United States does not have a committee to review these petitions, as it has for biological controls for weeds; therefore, APHIS asks the Biological Control Review Committee, which has members from Mexico, the United States, and Canada, and is coordinated by Agriculture & Agri-Food Canada, to review petitions for release of entomophagous agents.

Preparing a draft environmental assessment (EA) is the next step if the above review is favorable and APHIS concurs. This draft is prepared by APHIS based on information supplied by the applicant in the petition, and other resources the agency may have. Native Tribes and states in affected areas are contacted for comments on the draft. Then, a notice is published in the Federal Register of the availability of the draft EA and that anyone can comment on it for a 30-day period. Public involvement is required by NEPA and APHIS implementing regulations (7 CFR 372.5). If warranted, a finding of no significant impacts to the environment (FONSI) is issued along with a final EA. Only then may the permit to release the organism from containment be issued. Although not up to date, Hunt et al. (2008) provides a review of the procedure in the United States and other countries.

The EA for the field release of *L. osakensis* in the continental United States is an example of this aspect of the regulatory process (APHIS 2009). The petition for its release was submitted October 30, 2008 and on April 9, 2009, the Biological

Table 6.—NAAPO guidelines for petitions for first release of nonindigenous entomophagous agents.*

(1) **Proposed action**, with the purpose, need, and reasons for the release as well as specific location, timing, and method for the initial release.

(2) **Target pest information**, including its taxonomy, economic impact, life history, and distribution as well as knowledge of other natural enemies (native and introduced) that attack the pest, and potential non-target species related phylogenetically or ecologically to the target pest.

(3) **Biological control agent information**, consisting of its taxonomy and recognition characters; depository of voucher specimens (some must be deposited in the U. S. National Collection); other closely related species or genera in North America; its current and potential geographic, habitat, and climatic range; source of the agent; its life history; its known host range; and its natural enemies and that it will be free of these when released.

(4) **Environmental and economic impacts of the proposed release**, based on known impact on vertebrates, direct impact on target and non-target species, indirect effects including competition with resident natural enemies, and any potential effects on threatened and endangered species.

(5) **Post-release monitoring**, including its establishment and spread and affect on target population densities, and, when sufficient data are available, the economic and environmental impact of the program.

*Abbreviated from North American Plant Protection Organization, Regional Standards for Phytosanitary Measures, No. 12 (NAPPO 2008)

Control Review Committee issued a favorable evaluation. The notice of the availability of the EA was published a year later on 20 May in Federal Register (USDA 2010), and the FONSI was issued June 22, 2010. The process from receipt of the petition to issue of the permit took 18 months, which is within published timelines.

Movement within the Continental United States

The PPA uses the level of a State in defining the area where an organism is considered to be established or native. Although this definition may not have scientific basis, it reflects the importance of States in federal laws. The procedure to obtain a federal permit to move an entomophagous biological control organism from a State where it is native to another State where it is not established is the same as for the release of an organism imported from a foreign country, except that a petition following NAPPO guidelines is not prepared since NAPPO addresses only species not established in the North American continent. The policy of regulating movement of entomophagous insects between States began sometime after 2006—a new edition of PPQ Form 526 (Dec 2011) has deleted the statement on its reverse side that it does not apply to interstate shipment of entomophagous insects (http://www.aphis.usda.gov/plant_health/permits/downloads/forms/ppqform526.pdf).

Many States also regulate the importation of biological control organisms into their State from other States. These states generally "piggy-back" on the federal permit and the purpose of the State permit is usually to provide a notification of specific release information, such as release date, place, and number to be released. Since new federal regulations regarding interstate movement of entomophagous biological control organisms have not been issued, it is best to consult with APHIS-PPQ and affected states prior to interstate movement of any biological control organism. It is anticipated that the pending proposal of new regulations will nominate more than 150 phytophagous and entomophagous biological control organisms for interstate movement without a permit and have procedures for nominating additional species. This will provide for dissemination of biological controls still expanding their range and not established in all states where the target pest occurs.

IMPLICATIONS FOR BIOLOGICAL CONTROL

Federal regulations are often viewed as an inconvenience or obstacle to biological control programs; however, understanding the regulations can not only facilitate compliance but also guide the development of biological control programs. The information required for a permit aligns with the information a conscientious scientist would obtain prior to releasing a new biological control organism. The regulations reflect the need to assure the safety of biological controls and to facilitate their dissemination. The NAPPO guidelines not only provide a framework for scientifically based risk assessment by regulatory agencies, but also can serve as guidelines in planning a biological control program.

The hemlock woolly adelgid illustrates the need to regulate the movement of biological controls between ecological regions, whether the biological controls are native or imported from another country. This adelgid has regional populations in the United States, one that is indigenous to western North America, and another that is nonindigenous to eastern U.S., which originated in Japan (Havill et al. 2006, Havill et al. 2007). The permit for the release of *Laricobius osakensis*, a HWA predator from Japan, is valid for the continental United States. While it seems unlikely that someone would deliberately introduce *L. osakensis* to the western U.S., the consequences of its establishment there were not considered by the EA. *Laricobius nigrinus*, from the western U.S. and western Canada, was released in the eastern U.S. at a time when APHIS did not regulate the environmental release of entomophagous insects. It has since been found that *L. nigrinus* hybridizes with *Laricobius rubidus*, a related beetle native to eastern U.S. (Havill et al. 2010). It is only through the use of molecular

genetics that this problem was identified, and this issue highlights the need for both classical morphological identification, and information on the phylogeny of pest populations and their natural enemies in their native and introduced habitats.

In summary, under existing regulatory authority and current policies, APHIS requires a permit for all importations and for any movement that crosses the "border" of the containment facility, or the border of a state. Federal regulatory authority is necessarily grounded in political (State) boundaries, and often fails to incorporate concepts such as ecological zones. While the example of biological control of HWA illustrates the need to regulate the movement of entomophagous insects, it remains unclear how to do this in a manner that facilitates biological control programs while protecting the environment from adverse impacts. Public response to new, proposed regulations will likely reflect the complex interactions of ecological and political boundaries, and variation between the intended use and the behavior of an organism.

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SECTION II AGENTS FOR BIOLOGICAL CONTROL

Chapter 4: Sasajiscymnus (=Pseudoscymnus) tsugae, a Ladybeetle from Japan

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ORIGIN, DESCRIPTION, AND HISTORY OF INTRODUCTION

The first exploration for native natural enemies of hemlock woolly adelgid, *Adelges tsugae* (HWA) in Japan, conducted by The Connecticut Agricultural Experiment Station, began in 1992. Several predators were collected from adelgid-infested Japanese hemlocks, but it was a tiny ladybeetle which proved to have the most potential for biological control in subsequent laboratory and field evaluations. This ladybeetle was collected from adelgid-infested Japanese hemlocks, *Tsuga sieboldii* and *Tsuga diversifolia*, between mid-May and late June 1992, in 13 of 37 forests and at 11 of 37 ornamental sites in 12 prefectures throughout Honshu, Japan from sea-level to 1,980 m elevation (Sasaji and McClure 1997). In Japan, McClure determined that *S. tsugae* was responsible for 86% adelgid mortality in landscaped sites and 99% adelgid mortality in forest sites. In 1997, Sasaji and McClure named this new cocinellid species *Pseudoscymnus tsugae* (Coleoptera: Coccinellidae). In 2004, the genus was renamed *Sasajiscymnus* as a replacement for a name already in use for a genus of shark, and *P. tsugae* is now known as *Sasajiscymnus tsugae* (Vandenberg 2004). North American colonies of *S. tsugae* originated from field collections made in 1994 and 1995 from Takatsuki, Osaka prefecture, Japan (approximately 34° N), and were much later diversified with more recent collections from the Kansai district, central Honshu, Japan (Shiyake et al. 2008). *Sasajiscymnus tsugae* belongs to the Tribe Scymnini, a group of small coccinellids, less than 3mm in length, which are specialist predators of aphids, scales, mealybugs, and adelgids. The adult is entirely jet black, on average 2 (1.5-2.5) mm in length, with dorsal pubescence and 9-segmented antennae (Sasaji & McClure 1997) (Fig. 1a). Amber-colored eggs are laid singly or in small clusters in concealed locales on hemlock foliage, buds, cones and stem crevices. Eggs, measuring 0.48 mm on average (Fig. 1b), hatch in 6-10 days, and development from egg to adult takes 24 and 40 days at 25 °C and 20 °C, respectively (Cheah and McClure 1998). There are four larval instars and a pupal stage, with the mature fourth instar (Fig. 1c) consuming > 70% of the total adelgid stages required for completion of development to adult (Cheah and McClure 1996). The mature fourth instar larva is on average 2.25-3.30 mm in length and dark grey or reddish brown in color while the pupa is a reddish brown (Fig. 1d) (Cheah and McClure 1998). *Sasajiscymnus tsugae* highly prefers adelgids to aphids and can also complete development on other adelgid species such as balsam woolly adelgid, *Adelges piceae*, pine bark adelgid, *Pineus strobi*, and Cooley spruce gall adelgid, *Adelges cooleyi* (McClure and Cheah 1998, Cheah and Donahue 2003). Host range tests showed that *S. tsugae* preferred *A. tsugae* to *Pineus strobi* on *Pinus strobus*, *Adelges laricis* on *Larix deciduas*, *Adelges cooleyi* on *Pseudotsuga menziesii* and the woolly alder aphid, *Paraprociphilus tessellatus* on *Alnus serrulata* (Butin et al. 2004). This species has no reproductive diapause and is, therefore, amenable

a. Adult

b. Egg

c. Mature larva

d. Pupa

Figure 1. Stages of Sasajiscymnus tsugae (photos by C. Cheah).

to laboratory mass rearing on field collections of *A. tsugae* infested foliage from fall to mid-summer. Mass rearing magnitude is primarily limited by healthy adelgid prey availability. A federal permit for the release of *S. tsugae* in Connecticut was issued in April 1995 (Hennesy 1995). The first field release of *S. tsugae* was made in a town park in Windsor, Connecticut in May 1995. A starter colony of *S. tsugae* was transferred to the Philip Alampi Beneficial Insects Laboratory (PABIL), New Jersey Department of Agriculture in 1997, where mass rearing for the release in multiple states was initiated. Releases of *S. tsugae* were then expanded to other states in 1999, largely through the early mass rearing efforts of PABIL and EcoScientific Solutions LLC (Scranton, PA). More insectaries rearing *S. tsugae* and other HWA predators were later established in the southern states, greatly expanding releases in the southern range of HWA. Since 1995, over two million *S. tsugae* have been released in more than 400 sites on federal and non-federal lands to combat hemlock woolly adelgid in 16 eastern states from South Carolina to Maine.

BIOLOGY AND SYNCHRONY OF LIFE CYCLE WITH PREY

Scientists at The Connecticut Agricultural Experiment Station investigated the biology, life cycle and potential of *S. tsugae* for biological control of HWA. *Sasajiscymnus tsugae* has a high lifetime fecundity, and females lay an average of 280 (64-513) eggs over 14 (5-30) weeks (Cheah and McClure 1998). The adult has a long life span (> 1 year with overwintering), and exhibits excellent field synchrony with the both sistens and progrediens generations of *A. tsugae*. This species is the only multivoltine introduced HWA predator, producing two generations in the northeast (Cheah and McClure 2000). One hypothesis examined the relative developmental time of the predator in relation to its prey (Kindlmann and Dixon 1999). Kindlmann and Dixon predicted that predators that have a longer developmental time than their prey are unlikely to be successful biological control agents. Generation time ratio is defined as the ratio of predator to prey developmental times (Kindlmann and Dixon 1999). Three year field studies in Connecticut indicated that temperature regulated *S. tsugae* F1 generation time is about 5 weeks in late spring and early summer and is similar for the F2 generation in mid to late summer (Cheah and McClure 2000). In contrast, generation time for the sistens *A. tsugae* is about 32 weeks, and 10 weeks for the summer progrediens generation (McClure, 1987). Generation time ratios for the *S. tsugae-A. tsugae* relationship are very favorable and between 0.16 and 0.5, conferring an advantage on *S. tsugae*. Comparison of these relative development times indicates the effective predatory impact of *S. tsugae*. In addition, successive, overlapping F1 cohorts of *S. tsugae* also span the second progrediens generation of *A. tsugae* and *S. tsugae* is the primary introduced predator with impact on this second adelgid generation. Furthermore, adults continue to feed and survive on aestivating first instar adelgid nymphs throughout the summer, augmenting the predation impact on its adelgid prey. Adults and larvae of *S. tsugae* are highly mobile and voraciously feed on all life stages of *A. tsugae*, from eggs and first instars to adults. Each beetle larva consumes about 500 adelgid eggs or 50 to 100 adelgid nymphs, depending upon their size, to complete development to adult. Adults can live for more than one year and may consume about 50 adelgid nymphs each week during times of peak reproductive and feeding activities (Cheah and McClure 1996).

The synchrony between life cycles of *S. tsugae* and *A. tsugae* studied in Connecticut is shown in Fig. 2 (Cheah and McClure 2000). Adult beetles emerge from overwintering sites in hemlock forests in March and April. Females generally mate before the onset of winter and begin oviposition on egg masses of the *A. tsugae* sistens generation in April, when daytime temperatures average 15 °C. Eggs of *S. tsugae* are laid throughout the spring into mid-summer during periods of both adelgid generations. Incubation periods and larval developmental times are variable and dependent on seasonal spring temperatures. The first field generation of adults generally emerges in June and July, but the timing is seasonally dependent on ambient temperatures.

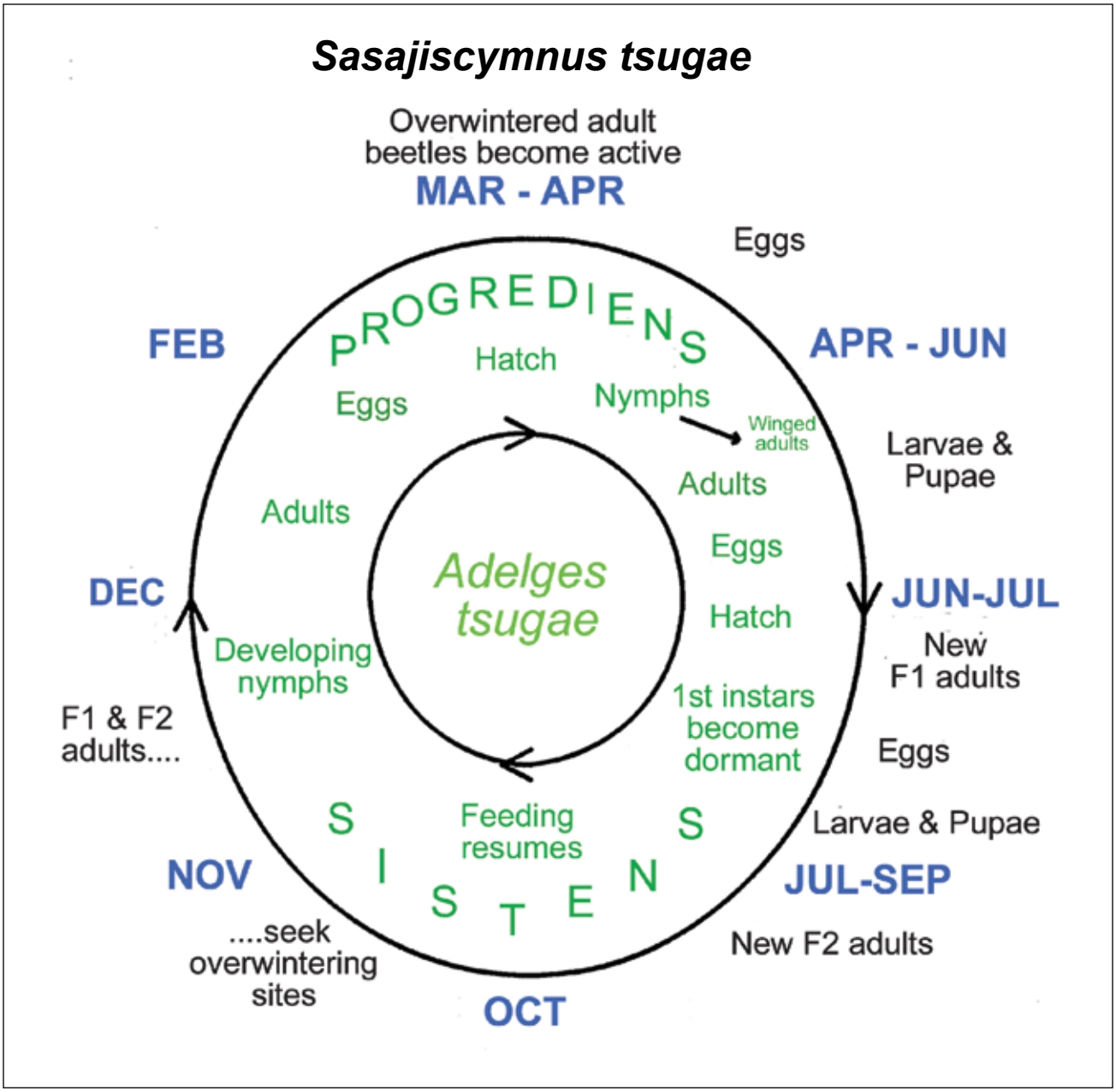

Figure 2. Seasonal synchrony between Sasajiscymnus tsugae, and its prey, the hemlock woolly adelgid, Adelges tsugae in Connecticut (adapted from Cheah and McClure 2000).

A smaller second generation of *S. tsugae* is also produced on the progrediens generation of *A. tsugae*, with new adults emerging in mid-August into September. Adult *S. tsugae* can survive the late summer period of dormant settled adelgid nymphs and were found in hemlock forests during the late summer and early fall. Sampling at various different sites in Connecticut and Virginia over 4 years showed that *S. tsugae* remained with its adelgid prey year round. In Connecticut, in the northern end of the adelgid distribution, *S. tsugae* adults were detected throughout the year during warm winter years, with larvae occurring from May to September in the field. Parallel sampling at two forest sites in Virginia, in the southern range of adelgid distribution, showed that *S. tsugae* adults were present from April to November (Cheah and McClure 2000). During milder winters in the northeast, adults overwinter on hemlock foliage (Cheah and McClure 2000). *Sasajiscymnus tsugae* has been documented in field cages in 2003 to survive minimum daily winter lows of -7 °F in northern Connecticut and -5.8 °F in 2002 in north central Maine during studies of adaptation to balsam woolly adelgid (Cheah & Donahue 2003).

FIELD RECOVERIES

Field studies and recoveries of the beetle have documented the ability of *S. tsugae* to reproduce after release, locally disperse, survive heat waves, survive mild and severe Connecticut winters, and establish in a variety of different hemlock habitats in Connecticut between 1995-2005 (Cheah and McClure 2000, 2002; Cheah et al. 2005). Recoveries of overwintered beetles (larvae and adults) were made in Connecticut release sites in years following severe Connecticut winters in 1996, 2000, 2003, and 2004 (McClure et al. 1999, Cheah and McClure 2002, Cheah et al. 2005). All stages from larvae, pupae, to adults of *S. tsugae* could be found through intensive forest ground sampling after the year of release in 65% of Connecticut release sites from 1996-2001 (Cheah et al. 2005). However, the persistent challenge in predator field sampling efforts continues to be the use of the limiting beat sampling technique, which only samples accessible lower canopy foliage of hemlocks and results in the consequent misinterpretation of the establishment of *S. tsugae*. Bucket tree sampling of mature hemlocks in Connecticut and New Jersey has shown that the beetle is highly mobile and dispersed to upper canopies after release. Adults and larvae have been retrieved at 5-20 m heights on the outer hemlock branches in the forest canopy while concurrent ground sampling yielded no recoveries, indicating that the beetle was not evenly distributed in the hemlock canopy (Cheah et al. 2005). However, bucket truck sampling is expensive and not suitable for many release sites. In Japan, sweep net sampling up to 5 m in the canopy in a landscape setting in Osaka was effective in recovery of *S. tsugae* every month from March to December (Shiyake et al. 2008). Shiyake and colleagues recovered 27 predacious species from adelgid-infested *T. sieboldii* during 2007 sampling and found that *S. tsugae* was observed on Japanese hemlocks for more months than any other predator; only absent in samples during January and February. Recoveries of *S. tsugae* have also been documented in western North Carolina, including in areas where *S. tsugae* was not known to have been released (McDonald et al. 2008). More recent detailed studies in the Great Smoky Mountains National Park using pole pruners and beat sampling techniques recovered adults and larvae of *S. tsugae* in 21.2% of sites sampled in 2008 and 2009 and these recoveries were significantly associated with older release sites (5-7 years after release) (Hakeem et al. 2010).

FIELD IMPACT AND HEMLOCK RECOVERY IN CONNECTICUT

In Connecticut, *S. tsugae* releases from 1995-2007 have occurred in hemlock forests statewide over three climatic divisions and in a wide variety of soil types and habitats. This was also a period in which severe droughts occurred in 1995, 1999, and 2002. Initial hemlock mortality was recorded on marginal hemlock sites on ridge-tops, resulting from hemlock borer infestations and on the heels of devastating outbreaks of hemlock looper in the

early 1990s. Concurrent with the expansion of HWA infestations in Connecticut in the 1990s, hemlocks were also stressed by the elongate hemlock scale, *Fiorinia externa*. Populations of this scale have significantly increased in density and range in Connecticut during the past 5 years, resulting in the decline of hemlock stands. *Sasajiscymnus tsugae* is the major introduced predator for biological control in Connecticut and efficacy of these releases on hemlock crown health has been monitored annually in a comparative approach since 2003. In the decade of the 1990s, severe winters punctuated the climate of Connecticut, and while the adelgid suffered major population reductions, populations tended to rebound to damaging levels in subsequent milder years. Ten years after the first release of *S. tsugae*, a period in which around 170,000 adult beetles were released in Connecticut for biological control of HWA, dramatic recovery of adelgid-impacted, declining hemlocks was recorded in many of the older established release sites, beginning in 2005, after successive extreme winters in 2003 and 2004 significantly reduced adelgid populations (Cheah 2006, Cheah 2011 in press). Recent winters in 2006-2008 and 2010 have been mild in comparison, but adelgid populations in the forests of Connecticut have not rebounded to original widespread damaging levels. Annual foliage transparency trends and other hemlock crown indicators such as new shoot production and tip dieback in *S. tsugae* release sites showed that even declining hemlocks recovered in one year when there was ample precipitation and a reduction in adelgid densities (Fig. 3). A comparative survey in Connecticut of 14 non-release sites, which were matched to *S. tsugae* release sites climatically, topographically and in HWA infestation history, was performed in 2005 to compare hemlock crown conditions in release

Figure 3. Recovery of an adelgid-infested hemlock in one year in Connecticut where S. tsugae was released in 1999 (photos by C. Cheah).

and non-release stands. Hemlock health assessments were also made in eight baseline hemlock sites which had no infestations of either adelgid or elongate hemlock scale. Crown health of hemlocks were rated (n = 15 trees/site) using standard U.S. Forest Service Forest Inventory Analysis (FIA) crown health assessment procedures, which has also been the method used to assess the conditions of hemlocks in release sites. A total of 287 hemlocks in release sites, 210 in non-release sites, and 90 hemlocks in baseline sites were compared statistically. In 2005, hemlock foliage transparency was significantly lower in the 14 annually monitored *S. tsugae* sites, as compared to that in paired non-release matches (Cheah 2011 in press). Mean foliage transparency in 6-11 year release sites was also similar to that in the baseline sites, located at high elevation in the colder northwest corner of the state, indicating that recovery of hemlocks had approached that in non-infested sites. An increase or stabilization of foliage transparency readings is interpreted to be due to the concurrent abundant new shoot production on previously infested hemlocks, an indication in itself that adelgid populations in the whole crown have been depressed. In addition, HWA crown levels in 2006 and 2007, measured in classes of <10%, 11-50%, 51-75% and >75% showed that in the majority of release sites, average levels of adelgid in the site have been reduced from the initial pre-release levels. Annual hemlock crown health ratings from 2006-2010, show that this overall recovery has persisted to the present in *S. tsugae* release sites (Fig. 4; Cheah 2011 in press). This recovery has continued even in southern most sites, which have not had significant recent winter mortality of HWA until the winter of 2010-2011. With the reduction of adelgid populations during severe winters, abundant precipitation and cool growing seasons,

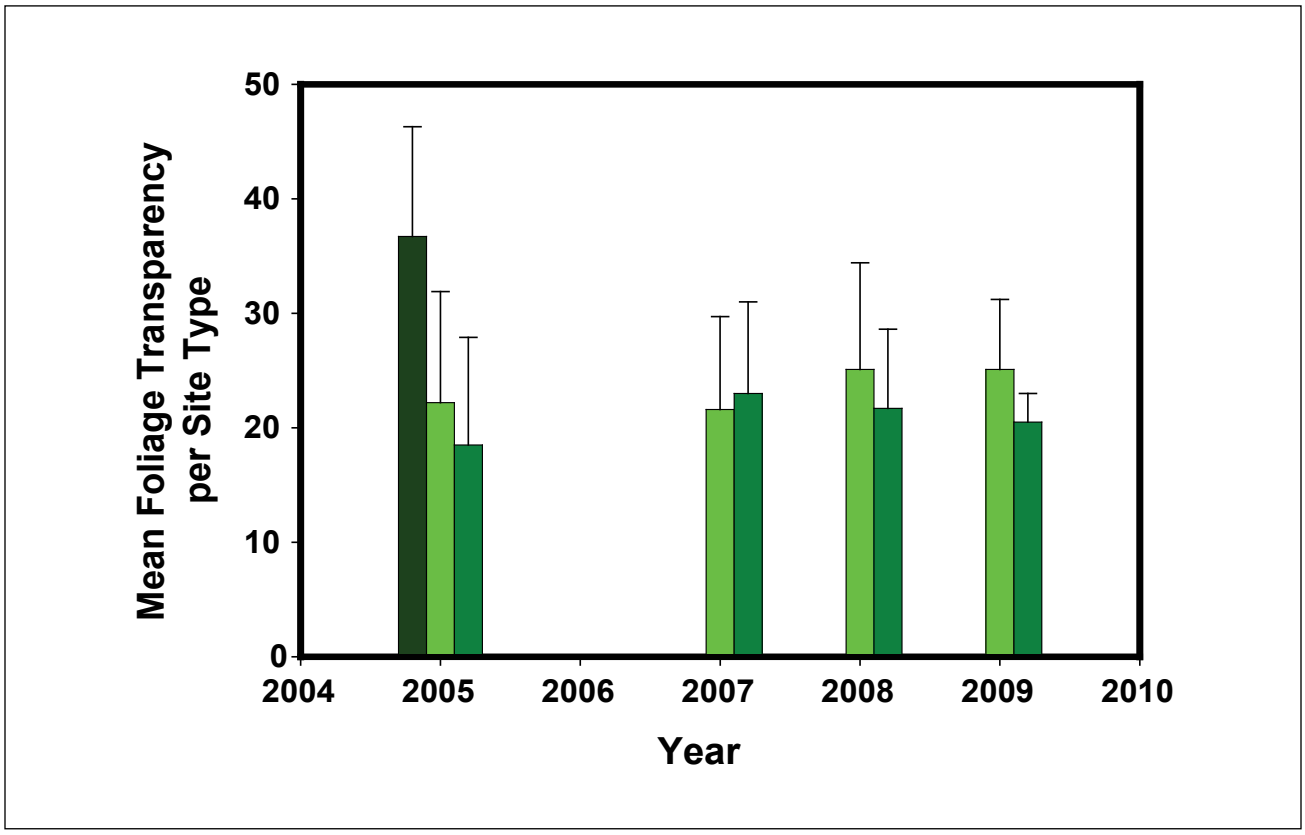

Figure 4. Comparison between S. tsugae release on-release d uninfested baseline hemlock forest sites in Connecticut (Cheah 2011 in press).

hemlock recovery in monitored release sites has been recorded in Connecticut in all types of soil types and sites, from rocky ridge tops to riparian, ravine to level habitats since 2005 to date (Fig. 5; Cheah unpublished). There has been negligible hemlock mortality in release sites since 2001. But in 2010, Connecticut sites which have had concurrently high infestations of elongate hemlock scale, had thinner crowns and higher foliage transparencies than sites with low or negligible scale infestations, indicating the deleterious impact of uncontrolled elongate hemlock scale populations (Cheah unpublished).

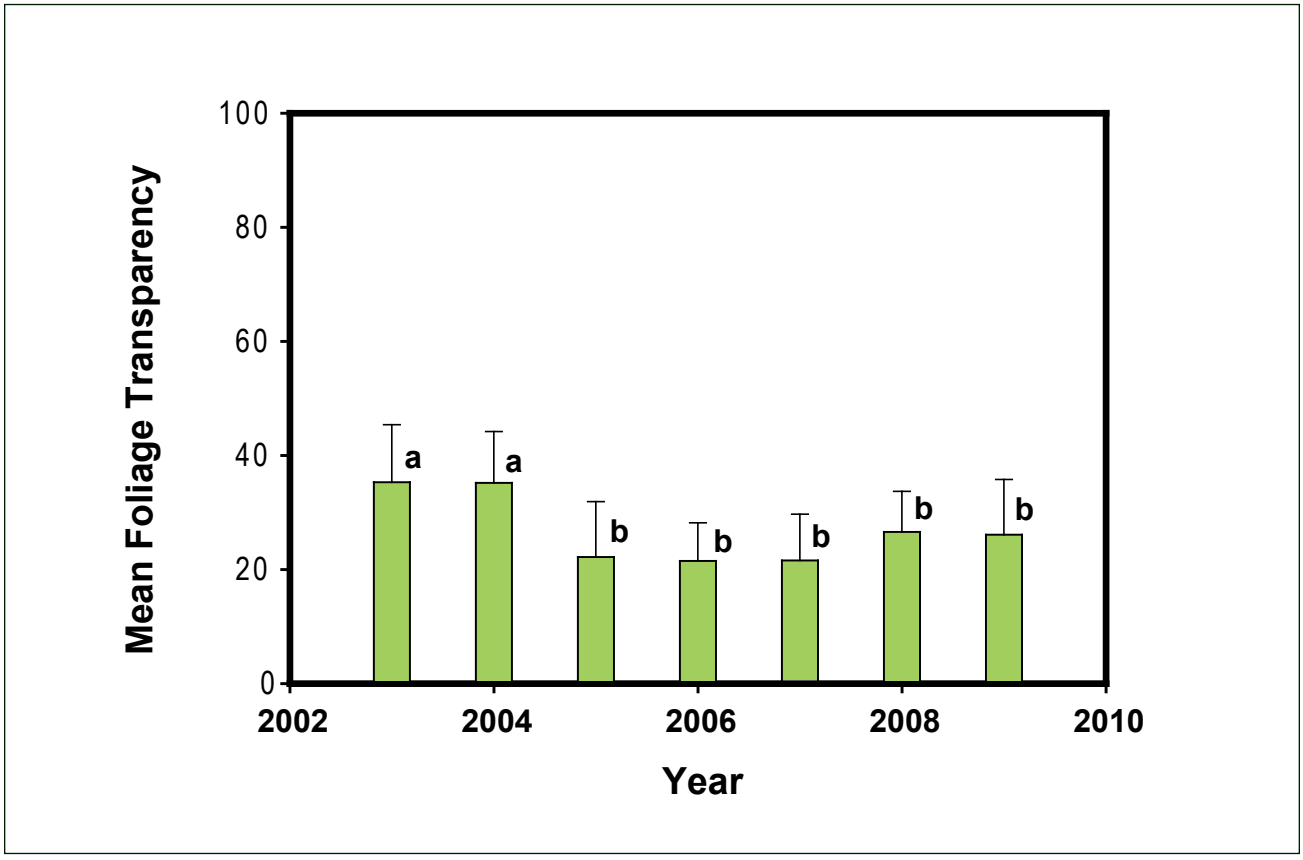

Figure 5. Mean annual foliage transparencies of previously adelgid-damaged hemlocks in 16 Connecticut S. tsugae release sites (9-15 years from year of first release), showing sustained crown recovery from 2005-2009. Bars are followed by different letters showing significant differences at the $p < 0.05$ level (Cheah unpublished).

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Chapter 5: Scymnus (Neopullus) Lady Beetles from China

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INTRODUCTION

In 1995, we found our first *Scymnus* (*Neopullus*) lady beetle in China (*Neopullus* is a subgenus of *Scymnus*, the largest genus in the family Coccinellidae). At that time there were just a few known species in the subgenus and very little was known of their biology. By the end of the project, 14 years later, we had doubled the number of known *Scymnus* (*Neopullus*) species and thoroughly studied the biology of the three species that seemed the most promising candidates for biological control of *Adelges tsugae* Annand, the hemlock woolly adelgid (HWA) in the eastern United States. This discussion of the research on the three *Scymnus* (*Neopullus*) species is a good example of the process of classical biological control—(1) the discovery of potential agents and determination of their biology and host range in their indigenous environment; (2) importation and further study in quarantine of their biology and potential host range in the areas targeted for their release; (3) using sleeve cages to evaluate the response of natural enemies to their new environment and their impact on the pest; and (4) the free release of the biological control agents into the environment.

The "we" refers to the several teams that participated in the project. The context of the research can be better understood by recognizing the principals of the China team, with a few remarks about how and where we worked. The project's good luck was to have started with Yao Defu of the Chinese Academy of Forestry who was skilled in natural enemy research and forming partnerships. Yu Guoyue of the Beijing Academy of Agriculture and Forestry Science was indispensible in identifying or providing new descriptions for the more than 70 species of lady beetles we found on hemlock in China. In Sichuan Province, Jianhua Zhou provided excellent leadership as well as hands-on help in the lab and field. In Yunnan, Li Li proved to be a most proficient collector and became so involved that he earned an engineering degree for his research on HWA natural enemies. Wenhua Lu of the University of Rhode Island did the initial studies in quarantine and acted as a liaison with the team in China.

It is not possible to describe here the adventures and hardships this team experienced. Hemlock in China occurs in remote, rugged mountains; hence, access was a challenge (Havill and Montgomery 2008). These forests have high diversity and both the Chinese and "Lao-wei" (foreigners) were excited about exploring these areas. Our working relationship was exemplified by the fun the Chinese had in speaking of "Lao-Mike" ("lao" literally means "old," but depending on context it shows respect as an old friend or teacher or can be derogatory as a silly-old-fool) and the custom of sharing a meal and drink with local officials and forest workers who helped us with our research.

OVERVIEW OF THE GENUS SCYMNUS

Scymnus is the largest genus of lady beetles (Coccinellidae) with more than 600 described species. Adults are relatively small, less than 3 mm, compact, and pubescent. Larvae are characterized by a white waxy covering. Most of the species in the genus are aphidophagous, feeding on either aphids or adelgids. The genus *Scymnus* is divided into seven subgenera: *Scymnus* (*Scymnus*) Kugelann 1794, *Scymnus* (*Pullus*) Mulsant 1846, *Scymnus* (*Didion*) Casey 1899, *Scymnus* (*Neopullus*)

Sasaji 1971, *Scymnus* (*Parapullus*) Yang 1978, *Scymnus* (*Mimopullus*) Fursch 1987, and *Scymnus* (*Orthoscymnus*) Canepari 1997 (Kovar 2007). The subgenera *Pullus* and *Scymnus* are widespread, with the former having three-fourths of the species and the latter about 50 species. The other subgenera have less than 10 known species, except for the subgenus *Neopullus*, which has 22 known species (Kovar 2007) that are Palaeartic, except for *Parapullus* and *Didion*, which also occur in North America.

The subgenera can be separated by a combination of the number of antennal segments (10 or 11), the length of the postcoxal line (complete or incomplete) and the presence or absence of distinct carinae on the intercoxal projection of the prosternum. Species in *Scymnus* (*Neopullus*) can be distinguished from the *Scymnus* species indigenous to North American by a combination of 10-antennal segments, a complete postcoxal line, and distinct carinae on the intercoxal process. Using a key to North American Coccinellidae (Gordon 1985), *Scymnus* (*Neopullus*) would key to *Didion*, except that the latter is distinguished by its feeble intercoxal carina. (Note: Gordon placed *Didion* in a separate genus rather than a subgenus of *Scymnus*.) *Sasajiscymnus tsugae* (Sasaji & McClure), imported from Japan to the United States for biological control of the hemlock woolly adelgid, has 9-segmented antennae and an incomplete postcoxal line.

There have been 17 species of *Scymnus* lady beetles introduced to the United States, but only two are known to have established (Hagen et al. 1999). Both of the species that established are in the subgenus *Pullus* and attack adelgids (Gordon 1985). *Scymnus* (*Pullus*) *impexus* Mulsant, native to Europe, was introduced in the United States and Canada during 1959-1963 to control the balsam woolly adelgid, *Adelges piceae* Ratzeburg. Although *S. impexus* was initially promising, it may have died out since its last recovery was 1978 in British Columbia (Harris and Dawsen 1979). *Scymnus* (*Pullus*) *suturalis* Thunberg, also native to Europe, was introduced to Michigan in 1961 to control adelgids on pine and is now established in several northeastern States (Gordon 1985). It attacks *Pineus strobi* (Hartig), *P. pini* (Macquart) and *A. tsugae* in Connecticut (Lyon and Montgomery 1995). The abundance and seasonality of *S. suturalis* were examined on white pine and eastern hemlock, which had up to one larvae/branch (Montgomery and Lyon 1996). This lady beetle is now seldom collected in Connecticut (Montgomery, pers. obs.). There also is a *Pullus* lady beetle, *Scymnus* (*Pullus*) *coniferarum* Crotch, indigenous to the western United States, that feeds on both pine and hemlock adelgids (Whitehead 1967, Montgomery and McDonald 2010, chapter 10).

Worldwide there are 22 described species in the subgenus *Scymnus* (*Neopullus*) and the hosts are known for only a few of these. *Scymnus* (*Neopullus*) *hoffmanii* Weise is an important predator of aphids on crops in China and Japan (Yang and Zheng 1991, Kawauchi 1997). Seven *Scymnus* (*Neopullus*) species have been collected from hemlock in China (Yu et al. 2000) and three of these (Fig. 1) are the focus of this chapter: *Scymnus* (*Neopullus*) *camptodromus* Yu & Liu (*Sc*), *S.* (*N.*) *sinuanodulus* Yu &Yao (*Ss*) and *S.* (*N.*) *ningshanensis* Yu & Yao (*Sn*). Although many species of Coccinellidae and other families of predators were collected, these three species seemed the most promising for biological control of HWA.

DISTRIBUTION AND BIOLOGY IN NATIVE RANGE

Distribution

The search for natural enemies of the hemlock woolly adelgid in China focused on three provinces with extensive, widely distributed stands of hemlock—Yunnan and Sichuan in Southwestern China and Shaanxi in Central China (Fig. 2). These collection locations occur between 26.3°N and 33.3°N latitude and 1900 and 3200 meters elevation.

Habitat

The Sino-Himalayan region has three species of hemlock: *Tsuga chinensis*, which is widespread, occurring in 15 China provinces; *T. dumosa*, which occurs in a narrow zone across the southern Himalayan Range belt from Sichuan and Yunnan

Figure 1. Adults of three species of Scymnus (Neopullus): left to right, S. camptodromus (Sc), S. ningshanensis (Sn), and S. sinuanodulus (Ss). The beetles are about 2 mm in length.

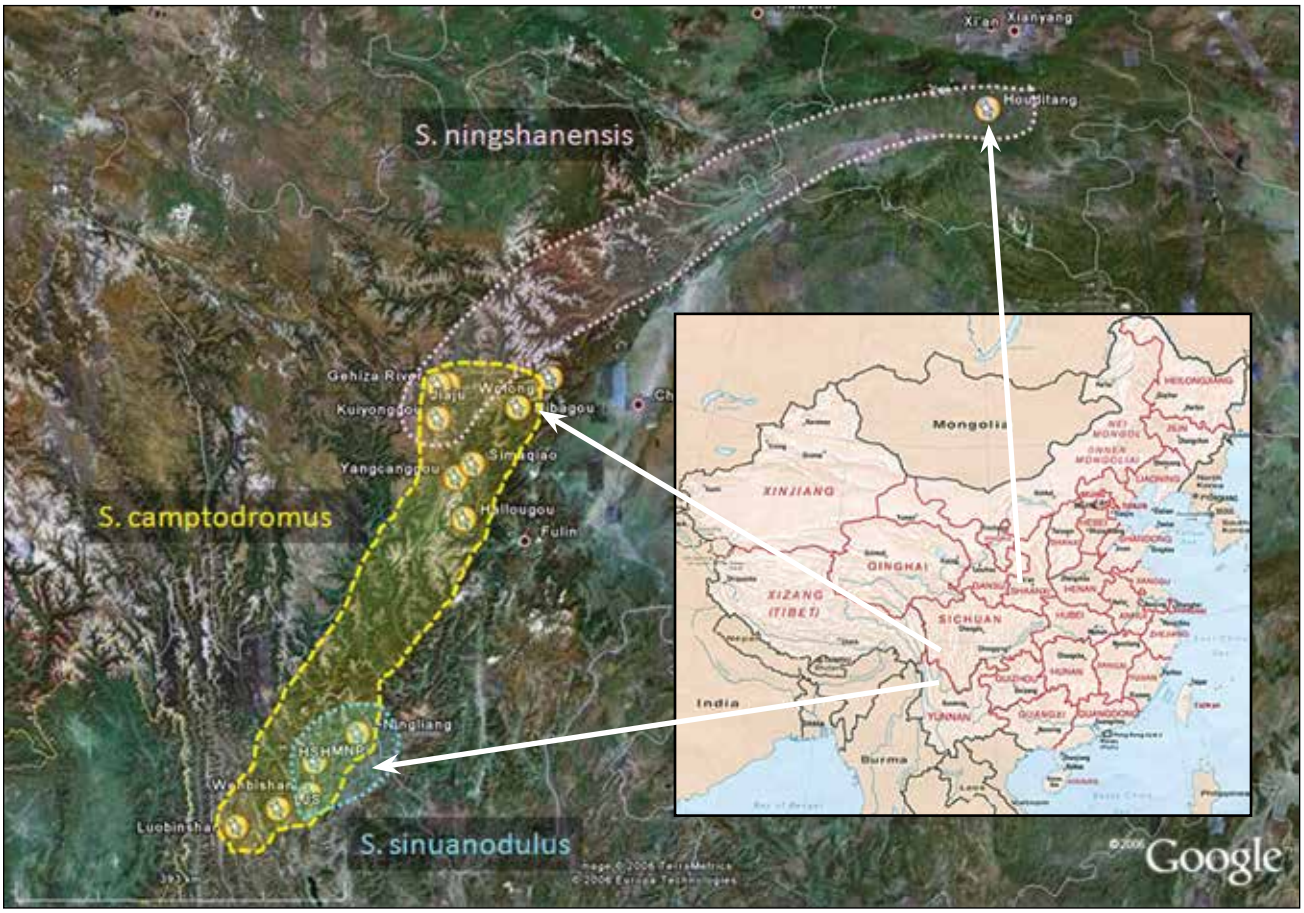

Figure 2. Map showing primary collecting area in China, with known distribution of three species of Scymnus (Neopullus) lady beetles outlined.

Provinces to Pakistan; and *T. forrestii*, which is limited to northwest Yunnan and southwest Sichuan (Fargon 1990). Like their eastern United States congeners, the hemlocks in China are shade tolerant and drought intolerant. Montgomery et al. (1999) provides additional information about the nomenclature and distribution of hemlock in China.

In China, hemlock occurs only in mountainous regions, especially fog-belts at elevations between 1,800 to 3,500 meters where moisture is plentiful during the growing season. Because the hemlock occurs in a limited elevation zone and the mountains are very steep and isolated, the hemlock often occurs in isolated "islands" (Wang 1961). This is a transitional area between broad-leaved deciduous forest and montane coniferous forest. These stands are extraordinarily diverse and have many of the same genera found in forests in the eastern United States. Besides *Tsuga*, there are species in the genera *Abies*, *Aesculus*, *Carpinus*, *Cercis*, *Chamaecyparis*, *Clethra*, *Corylus*, *Ilex*, *Juglans*, *Juniperus*, *Lindera*, *Magnolia*, *Malus*, *Prunus*, *Picea*, *Pinus*, *Pseudotsuga*, *Sorbus*, *Taxus*, *Tilia*, and *Ulmus*. There are also species in genera not present in eastern North America such as *Castanopsis*, *Cercidiphyllum*, *Schima*, *Cunninghamia*, *Keteleeria*, and *Lithocarpus*. This assemblage of a large number of tree species with the crown layer shared by several species is a characteristic of the mixed mesophytic forest type. This area of southwest China and the Southern Appalachian region of eastern North America are the only areas in the world where this forest type occurs (Wang 1961).

In China, hemlock occurs only as a forest tree—it is not grown as an ornamental or landscape tree. Often it is the tallest tree in the forest with characteristic flat tops above the canopy. Although HWA and their lady beetle predators occur in the crowns of these trees, the crowns of big trees are not accessible. The distribution of HWA in the crown of *T. dumosa* is uniform vertically and by quadrant (Zhou et al. 2007, Li and Lu 2008). Most collecting is done in the lower crown, reachable from the ground, and on small trees growing near wet areas. The forests where collecting was done are protected and managed forest farms or preserves in which livestock are sometimes allowed to graze.

Climate

The areas where *Scymnus* (*Neopullus*) lady beetles were collected in China generally are more southerly in latitude and higher in altitude than the target areas for biological control in the eastern United States. Seasonal temperatures can be similar, however, because a 1000 m increase in altitude has roughly the same effect on air temperature as a 7.5 degree increase in latitude (http://en.wikipedia.org/wiki/Alpine_climate). A comparison of average monthly temperatures for two of the collecting areas in China and two potential release areas in the United States provide examples of seasonal temperatures (Fig. 3). This shows that these four areas have similar winter temperatures, between -21 °C and -18 °C, but the areas in China have much lower summer temperatures. The lower summer temperatures are associated with the seasonal monsoon that occurs as warm tropical winds from the southeast collide with the rise of the Tibetan Plateau. Rainfall data, available for two of these areas (Fig. 4) shows the typical pattern of rainy summers and dry winters of the collecting areas in China and the more even pattern of rainfall in the Southern Appalachian area of the United States. Thus, the major climatic difference in the collecting area and the potential release area is the seasonal pattern of rainfall and cool summers.

Host Associations in Endemic Area

In addition to hemlock, other conifers and some angiosperms were sampled by beating limbs over an umbrella to determine the extent that alternate hosts may be used by the three *Scymnus* (*Neopullus*) species. Of the three species, only *Ss* was found on any host plant other than hemlock with a regularity that was not considered incidental (Table 1). During the first four years of collecting, the 5-needle white pine, *Pinus armandii* Franch, had heavy infestations of the adelgid *Pineus armandicola* Zhang, Zhong & Zhang, and the adelgid's egg-filled ovisacs were on the pine's needles in both spring and fall. In the spring and late fall, *Ss* was about one-fifth as abundant on the white pine as on hemlock, but *Sc* and *Sn* were not recovered from white pine. In Yunnan during September, when HWA was in diapause, *Ss* was more numerous on *P. armandi* than on hemlock. Numerous *Scymnus*

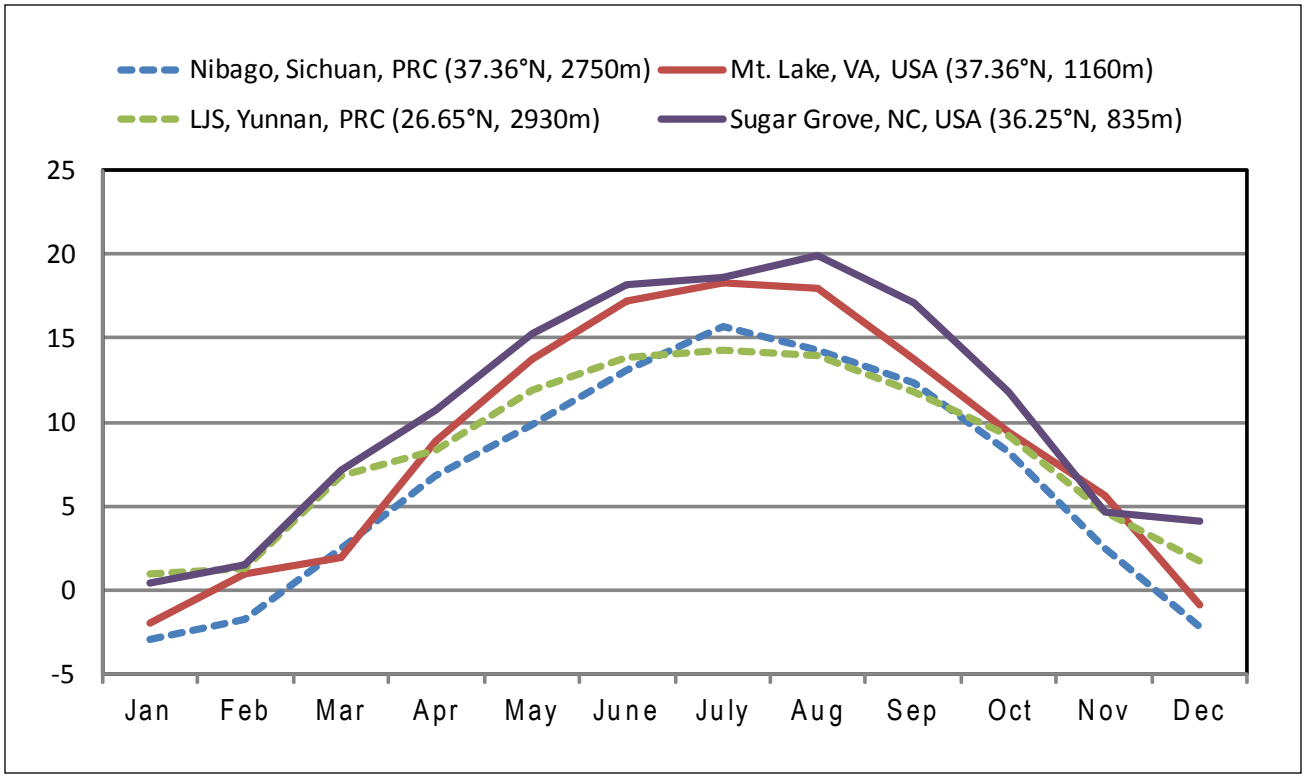

Figure 3. Average monthly temperatures (°C) of selected collecting locations in China and potential release sites in the United States. Latitude and meters altitude shown in brackets. Data collected by temperature recorders at the field sites for two or three year periods between 1999-2008.

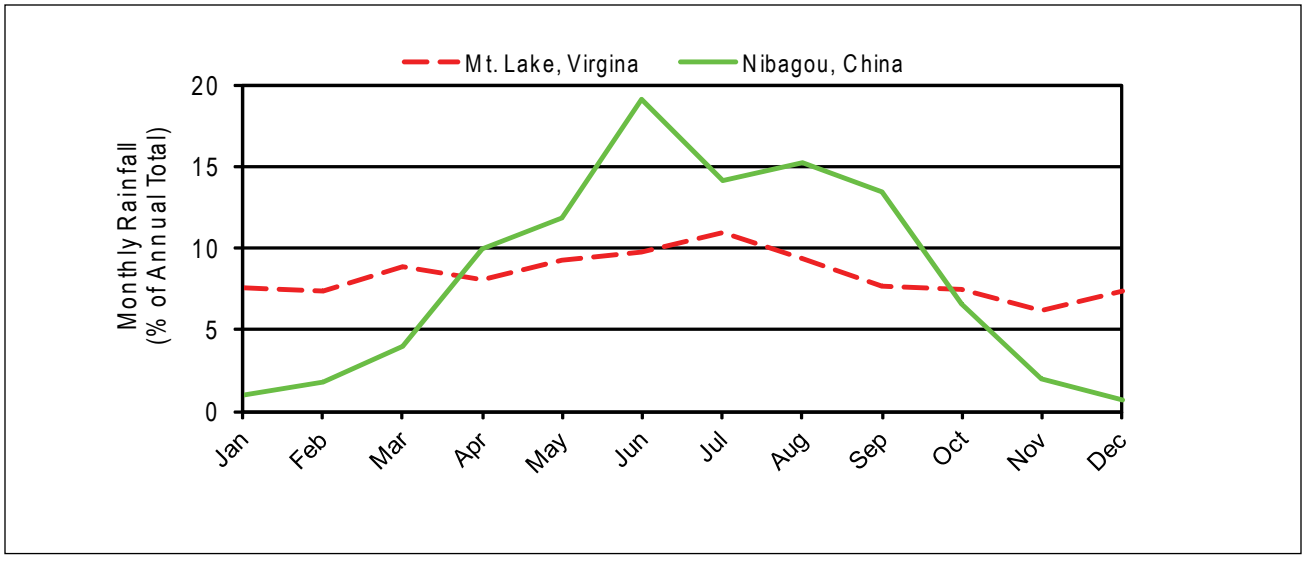

Figure 4. Average monthly precipitation as a percentage of total annual precipitation.

larvae collected from adelgid infested white pine at this time were identified as *Scymnus* (*Pullus*) *yunshanpingensis* Yu. Another *Pullus* species, *S.* (*P.*) *geminus* Yu and Montgomery, was abundant on white pine in the spring in Sichuan. Both of these *Scymnus* (*Pullus*) species will feed and oviposit on HWA, but their primary host seems to be pine adelgids. The other two species were not collected from white pine. It seems that the species in the subgenus *Pullus* may have pine adelgids as primary hosts whereas the three *Neopullus* species imported have HWA as the primary host, but may feed on pine adelgids to some extent.

HWA Phenology

Monthly sampling of HWA life stages and predators was done at three locations in Sichuan and Yunnan Provinces for one year. The number of ovisacs in each monthly sample was the most useful indicator of predator/HWA dynamics since the egg stage is targeted by most of its predators. As in the eastern United States, the onset of oviposition by HWA occurs earlier at more southerly latitudes. In Yunnan Province, the abundance of egg containing ovisacs peaks first in December and January and then in March whereas in colder Sichuan, the peak in abundance of ovisacs occurs in March and April (Fig. 5). In Sichuan, there is considerable overlap in eggs laid by the overwintering generation (sistens) and the spring generation (progrediens). In Sichuan, HWA ovisacs (and all nymphal stages) were found in low numbers throughout the summer and into early fall, especially in Nibagou, the coldest site. The cool summers of these regions may result in a longer period of survival and egg production by the progredientes. Other explanation for the presence of active stages throughout the summer include two progrediens generations or the progeny produced by migration of gallicolae from spruce. The difference in biology may also be related to the adelgids on hemlock in China being distinct genetically from HWA on other continents (Havill et al. 2006).

Predator Abundance and Phenology

Predators were collected by beating foliage over umbrellas. This technique is best suited for collecting adults. Usually 30 hemlock trees were beaten at each site every month. The closure of roads caused a switch to alternative sites in Sichuan; hence, the same three sites were not followed for

Table 1. Occurrence of adult *Scymnus sinuanodulus* by beating foliage in Lijiang Prefecture, Yunnan, People's Republic of China.

Tree Species	Occurrence	Hemiptera present
Tsuga dumosa Eichler	Frequent	*Adelges tsugae*, Diaspididae
T. forrestii Downie	Frequent	*A. tsugae*
Pinus armandii Franch.	Moderate	*Pineus* spp., Aphididae
P. yunnanensis Franch.	Rare	Diaspididae
Picea likiangensis Pritz.	Rare	Adelgid galls
Abies delavayi Franch.	Rare	*Adelges* sp., Aphididae
Larix potaninii Batalin	None	*Adelges* sp.
Taxus yunnanensis Chang et L.K. Fu	None	Pseudococcidae
Keteleeria evelyniana Mast.	None	Aphididae, Diaspididae
Quercus pannosa Hand.-Mazz.	None	Coccidae
Betula alnoidis Hamilt.	None	Aphididae
Populus yunnanensis Dode	None	None
Alnus ferdinandi-coburgii Schneider	None	None
Rhododendron spp.	None	None

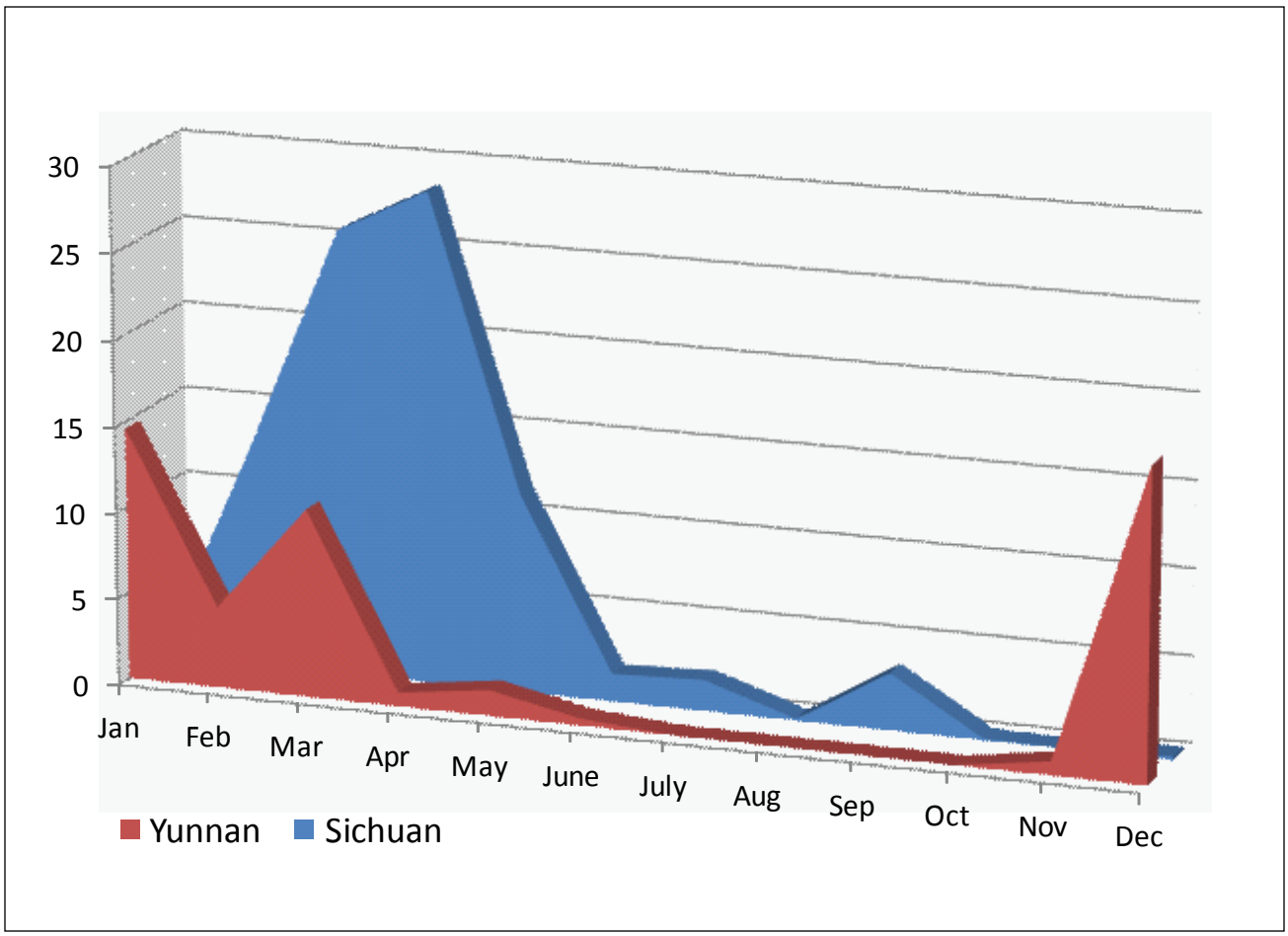

Figure 5. Seasonal presence of HWA ovisacs in Sichuan and Yunnan Provinces; values are the average number on a twig for 60 twigs from each of three sites in each province. Data collected by Jianhua Zhou in Sichuan and Li Li in Yunnan.

12 consecutive months. Overall 2,695 and 3,153 specimens were collected in Sichuan and Yunnan, respectively. This is an average of 2-3 predators per umbrella sample with a range of zero for all samples taken in January and February in Sichuan to 8-9 per sample for May in Yunnan and October in Sichuan. The overall abundance of categories of natural enemies in the two Provinces was dissimilar (Table 2). Only seven specimens of *Laricobius* sp. were collected, all in Sichuan Province; thus, based on abundance, *Laricobius* spp. do not appear to be important regulators of HWA in China. In both provinces, the majority of predators collected are in the family Coccinellidae. In Yunnan, 62% of the Coccinellidae were the species *Sc* and *Ss*. In Sichuan, 48% of the Coccinellidae were the species *Sc* and *Sn*. *Ss* was not found in Sichuan and only three *Sn* individuals were found in Yunnan. The two most abundant predators were the large, colorful coccinellid *Oenopia signatella* (Mulsant) in Sichuan and the anthrocorid, *Tetraphleps galchanoides* Ghauri, in Yunnan. Both the literature and laboratory host range studies indicate that these last two predators are generalists feeding on aphids, adelgids, and other Heteroptera; thus, they appear to be non-specific, opportunistic species.

Table 2. Total number of predators recovered from umbrella samples taken monthly in two provinces.

Species	Sichuan	Yunnan
S. camptodromus	240	232
S. ningshanensis	226	3
S. sinuanodulus	0	269
Oneopia spp.	549	23
Other Coccinellidae	746	436
Laricobius sp.	7	0
Anthocoridae	22	305
Other Predators	141	171

The seasonal abundance of the three predators in each province that seem to have the greatest role in HWA population dynamics is presented in Figure 6. Very few predators were collected during the coldest months of January and February in either province. There were two peaks in abundance of adult predators, one in the spring and another in the fall. In May and June, one site in Yunnan, which had the highest density of HWA, also had a high density of *T. galchanoides* nymphs and adults. Because this true bug pierces and sucks fluid from its prey and the HWA carcass remains attached to the stem, it was possible to assess, with the aid of a hand lens, that mortality of HWA exceeded 90 percent. The fall peak in predator abundance does not correspond well to HWA biology—HWA was still in diapause in Yunnan, but active stages of HWA were present in Sichuan—so its significance to HWA regulation is unclear.

The abundance of the adults of the *Scymnus* (*Neopullus*) species peak in early spring, then increase in late spring and early summer (Fig. 6).

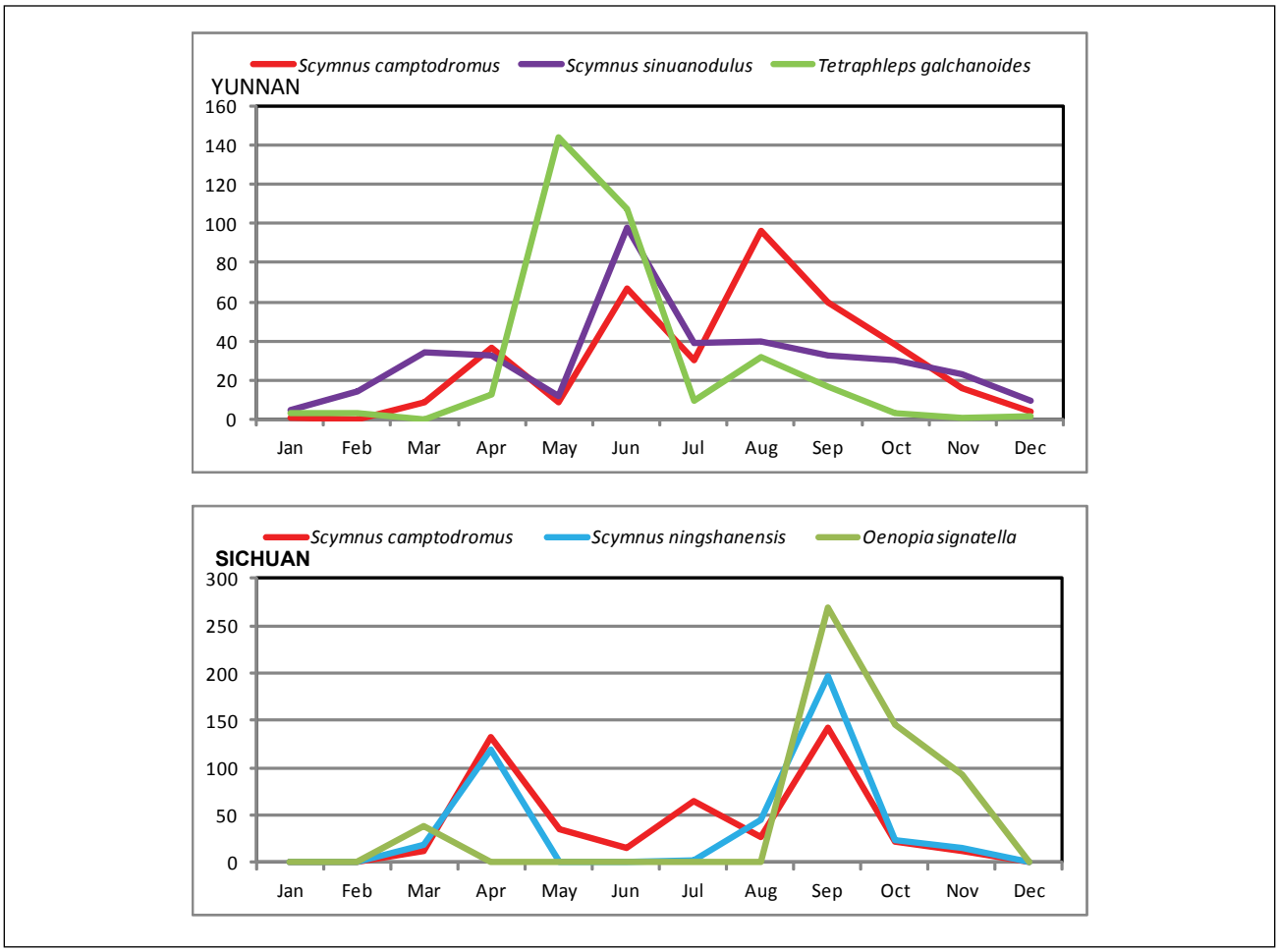

Figure 6. Seasonal presence of selected predator species recovered from monthly foliage beating samples.

This decrease in mid-spring likely corresponds to mortality of the overwintered lady beetles after egg laying and is followed by the appearance of the newly eclosed adults about two months later. The adults are abundant on hemlock in the fall and this is the best time to collect them for export. Adults of *Sc* collected in the fall or spring will immediately lay eggs in quarantine, but *Sn* and *Ss* collected in the fall will not begin to lay eggs until after a few months in cold storage. Because *Sn* and *Ss* lay eggs in the spring, adults collected then for export may have laid most of their eggs and thus may produce few eggs when brought into the quarantine.

Observations of immatures in the field (Yao and Hongbin 1999; Montgomery, pers. obs.) indicate that larvae were present during April and May in both provinces. At the end of May, larvae with waxy plumes were seen walking on the trunk and branches, and pupae were found under the bark on the bole of the tree (Fig. 7). Mating of *Ss* adults was observed in early April and they oviposited from then until late May. In Sichuan, oviposition by *Sc* was observed only during April, but eggs were found in the field in late summer. Based on the temperature data of the study locations (Fig. 3), development of the immature stages occurs when daily average temperatures are between 5 °C and 15 °C.

Figure 7. Scymnus (Neopullus) larva feeding on HWA (left) and pupae in bark crevices on the bole of a hemlock tree in Yunnan, China (photos by Guoyue Yu).

BIOLOGY STUDIES IN QUARANTINE

The *Scymnus* (*Neopullus*) lady beetles imported from China exhibit two different life history schemes that synchronize their development with that of HWA, including the approximately three month aestivation period of HWA. Two species, *Ss* and *Sn*, both have an extended pre-oviposition period; new adults mate soon after emergence but need some exposure to cool temperatures before females will initiate oviposition. They lay eggs only in the spring that quickly hatch. The other species, *Sc*, can begin to oviposit one month after emergence if mated, but these eggs enter diapause and do not hatch until the next spring. The only other record of an egg diapause in Coccinellidae is for another *Scymnus* species, *S.* (*Pullus*) *impexus* (Mulsant), which feeds on *Adelges piceae* (Ratzeburg) in Europe (Delucchi 1954). Details on these two strategies and the data that supports them are provided in the following sections.

Egg Deposition and Hatch

The females of all three species select concealed, protected places, near HWA to lay their eggs, but in the laboratory will lay eggs in exposed areas and white gauze (*Ss* and *Sn* only) if suitable oviposition sites are not available. Eggs are usually laid singly, but may be in groups if the adults are crowded. The eggs of all three species are first yellow-orange, but become darker in 2-3 days. As they near hatch, the outer shell (chorion) becomes transparent and reddish eye spots are visible. Just before hatching, the egg surface becomes iridescent as the chorion separates from the embryo. Eggs of *Sc* are generally deposited in more concealed locations and have a more leather-like surface than the other two species (Fig. 8). This may provide added protection because the eggs of *Sc* will not hatch until 4-8 months after being laid, whereas the eggs of *Sn* and *Sc* hatch in about two weeks after being laid.

The eggs of *Ss* and *Sn* will hatch in an average of 10 and 8 days, respectively when held at 18-20 °C. Egg hatch was about 90% for both these species. Storing eggs of these two species at 5 °C for two weeks does not affect hatch, but longer storage reduces percent egg hatch. Thus, the eggs of these two species are not able to overwinter.

Figure 8. Scymnus camptodromus egg inserted between bud scales of hemlock.

At temperatures ≥ 15 °C, the eggs of *Sc* remain yellow-orange and show no signs of embryo development until exposed to temperatures < 15 °C for 1-3 months (Keena and Montgomery 2010). There is some variation among individual eggs in amount of chill required to break diapause, and diapause seems to be broken most quickly when eggs are held at temperatures near 5 °C. Once diapause has been broken, the *Sc* embryo will begin to develop, even at temperatures near 0 °C, and the speed of development slowly increases with increasing temperatures up to 15 °C. At a constant 10 °C, the eggs will hatch after an average of 227 ± 32 days. Eggs will hatch after exposure to 5 °C and the percentage hatch increases with increasing time at 5 °C. The temperature regime for highest percentage hatch (90%) in the shortest time is 56 days at 5 °C followed by about 2 months at 10 °C. The optimal temperature for *Sc* egg hatch is near 10 °C. Thus, *Sc* eggs will spend the summer in diapause, develop only after exposure to the cool temperatures of fall, and hatch after HWA has begun laying eggs.

Larva and Pupa Development

The larvae have four instars, are elongate, yellowish to reddish brown, densely setaceous on the head and plates, and the body has tubercles, but lacks prominent spines (Fig. 9) (Lu et al. 2002). The newly-hatched larvae are transparent with the color of the hemolymph and recently consumed adelgid visible (Fig 10). The larvae grasp HWA crawlers with their mandibles and suck the prey contents and then expel it back and forth into the dead prey's exoskeleton to aid in digestion (Lu et al. 2002). By the last instar, the larvae produce a conspicuous waxy covering on the cuticle (Fig. 11). The pupae are naked, with the larval exuvium attached only to the last abdominal segment, but are covered with coarse setae with viscous droplets on the tips, which we believe are defensive (Fig. 12).

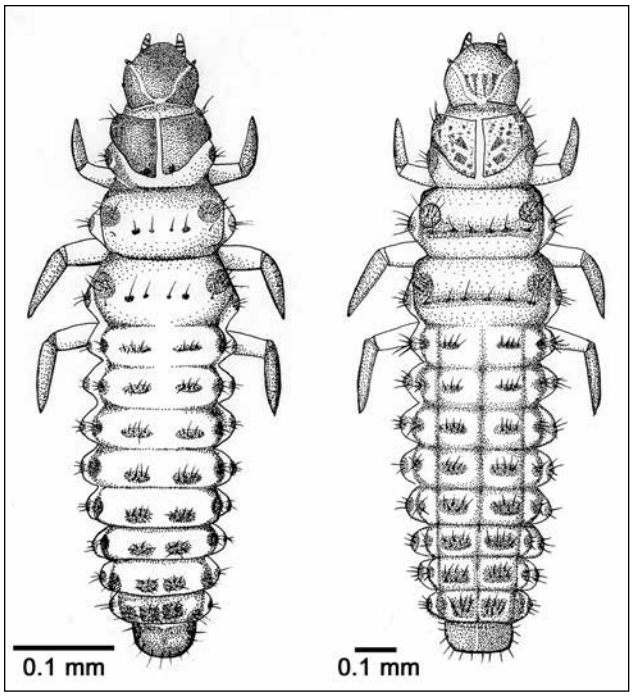

Figure 9. Drawings of Scymnus sinuanodulus larvae, first instar (left) and fourth instar (from Lu et al. 2002).

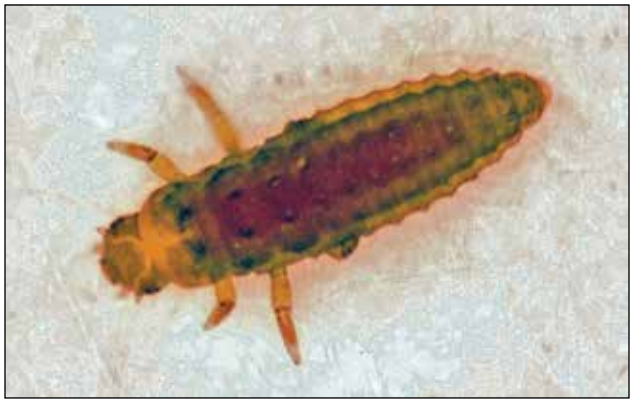

Figure 10. Scymnus camptodromus first instar larva.

Figure 11. Fourth instar Scymnus larva and HWA eggs.

Figure 12. Droplets at the tips of the setae covering Scymnus pupa.

The total time for development at 20 °C for the larval and pupal stages is about 20 days and 10 days, respectively, for *Ss* and *Sc*. *Sn* has a shorter period in the larval stages (Table 3). The fourth larval instar of *Sn* is shorter than the other two species, which spend about half of the last larval stage wandering or inactive (Lu et al. 2002). This behavior has also been observed in another lady beetle that feeds on adelgids, *S.* (*P.*) *impexus* (Delucchi 1954).

The larvae of *Sc* can develop at constant temperatures between 10 and 25 °C (Keena and Montgomery 2010). Larvae of *Ss* also complete development at temperatures between 15-25 °C, but rearing at lower temperatures was not attempted (Lu and Montgomery 2001). However, 10 °C and 25 °C may be sub-optimal, because survival to pupation for *Sc* larvae was only 50% at 10 °C, > 70% for 15-20 °C, and 25% for 25 °C (Keena, unpublished data). For *Ss*, survival from egg hatch to pupation was > 60% at 15-20 °C and only 5% at 25 °C (Lu and Montgomery 2001). The *Sc* larvae took an average of 90 ± 3 days and 20 ± 1 days to pupate, at 10 °C and 25 °C, respectively. In their indigenous environment, average temperatures range between 5 °C and 15 °C during the months the larvae and pupae are developing. The lab rearing indicates that these beetles should thrive at the warmer temperatures they would encounter in the Southern Appalachian Mountains (cf. Fig. 3).

Table 3. Development time (days) of larval and pupal stages of *Scymnus sinuanodulus* (*Ss*) and *S. camptodromus* (*Sc*) at 20 °C and *S. ningshanensis* (*Sn*) at 18-20 °C.

Stage	*Ss*[1]	*Sn*[1]	*Sc*[2]	*Sc*[3]
L1	3.1	2.8		
L2	2.5	2.5	20	20
L3	2.9	3.7		
L4	11.4	5.9		
Pupa	10.6	11.1	9.0	10
Total	30.5	26.0	29.3	30

Data sources: [1]Montgomery et al. 2002; [2]Keena and Montgomery 2010; [3]Lu and Montgomery 2000.

Mating and Oviposition

The adults of all three species are uniformly dull orange after emergence, with the elytral maculation that is distinctive for each species taking a few days to appear. After about three weeks, the adults of all three species become more active and are observed to mate and fly frequently (Fig. 13). Adult weights of the three species vary with larval food quality and rearing temperature, but *Sc* may be larger than *Sn* and *Ss* (Table 4). The greater weight of *Sc* may have been due in part to it being reared individually whereas *Ss* and *Sn* were reared in groups. Another factor may be that the weights of *Ss* and *Sn* were taken after they had been in lab culture for 12 and 11 generations, respectively, whereas the *Sc* colony was a mixture of populations established 5 to 4 generations ago. Measurements of the body size made on field collected specimens indicate that *Sc* and *Ss* are the same size, but *Sn* is a little smaller (Yu et al. 1997, Yu et al. 2000).

Female *Sc* first mate when about 3 weeks old and begin oviposition about a week later if active stages of HWA are available. Only a few eggs are laid if HWA nymphs are the only food and total egg production is better if females are held at < 10 °C until HWA eggs are available. When HWA eggs are available, oviposition will begin about 2 weeks after placement at > 10 °C. The females normally deposit eggs individually in concealed locations such as bud scales (especially ones that form a curl), but prefers the pollen cones of hemlock. When opened pollen cones are available, along with ample HWA eggs for food, they will average 10-14 eggs/female/week at 20 °C and often lay multiple eggs in a cone. If the food supply is not optimal, only 1-2 eggs are laid in a week.

Figure 13. Scymnus sinuanodulus with wings spread open as in flight (photo by Nathan Havill).

Table 4. Average adult weights (mg) of *Scymnus sinuanodulus* (*Ss*), *S. camptodromus* (*Sc*), and *S.ningshanensis* (*Sn*).

Sex	*Ss*	*Sn*	*Sc*
Male	0.90	0.96	1.30
Female	0.98	1.15	1.30

Females of the other two species (*Ss* and *Sn*) also mate at about 3 weeks post-eclosion, but have a prolonged pre-oviposition period that includes exposure to temperatures of 5-10 °C for at least two months (Lu and Montgomery 2001). After four months exposure to cool temperatures, they will lay eggs within 48 hours after being warmed to 20 °C and provided HWA eggs for food. Peak egg laying for *Sn* is 18 egg/female/week when held at 19 °C. The average number of *Sn* eggs produced per ovipositing female in the laboratory was 28 in China and 85 in the United States. This difference likely reflects the artificially extended laying period in quarantine in the United States and the availability of foliage with a higher density of ovisacs that had about 30% more eggs/sac. A reasonable expectation for oviposition in the laboratory is one to two eggs per day for a period of 5 to 10 weeks, if good quality and abundant food and oviposition sites are available. Both *Ss* and *Sn* oviposit single eggs in concealed locations such as bud scales and at the edge of ovisacs. If the ratio of oviposition sites to beetles is low or they are confined on the same foliage for several days, multiple eggs may be found in the same place and on the twigs, under dead needles on the bottom of the rearing container, and on substrates added to the cup such as gauze.

Two experiments showed that hemlock woolly adelgid eggs must be present for *Sn* to lay eggs (see Montgomery et al. 2002). Adults removed from cold storage were given foliage either with adelgid eggs or with only adelgid nymphs (third instar). Beetles provided adelgid eggs laid an average of

2.3 ± 0.8 eggs/week (N = 15) over a three-week period whereas the 15 lady beetles given only nymphs laid a total of three eggs for the entire three weeks. These three eggs were laid in the first week. When provided a diet simulating late winter conditions, with mostly adelgid nymphs and few hemlock woolly adelgid eggs, the lady beetle laid an average of only 6.42 ± 3.54 eggs during the 4 weeks following removal from cold storage, whereas 30.42 ± 8.98 eggs were laid by the beetles when they were provided adelgids at peak oviposition. Similar studies on *Ss* have shown that they will lay fewer eggs when provided only HWA nymphs than when provided HWA eggs (Lu and Montgomery 2001).

During the summer months when HWA is in diapause, the adults of all three beetle species feed little and are inactive during the summer. Survival is best during the summer when the adults are held at 10-15 °C, which is equivalent to the summer temperatures where they are indiginous. During the winter months, adults survive well at 5 °C and will feed occasionally on artificial diet. Both *Ss* and *Sn* adults have been found to have super cooling points generally between -12 and -20 °C so are well adapted to survive in cold climates (Costa et al. 2008). Since *Sc* eggs overwinter, it would be beneficial to know if *Sc* eggs can withstand sub-freezing temperatures as well as the adults.

Feeding Behavior on HWA

Scymnus adults prey on adelgid eggs by chewing them, often leaving smeared egg contents or partially consumed eggs. The beetles feed first on eggs exposed outside the ovisac, then gradually crawl into the ovisac until little of their body is visible. Often the adult adelgid is dislodged and subsequently dies. When adult beetles attack adelgid adults or older nymphs, they take a single bite, which causes dark brown hemolymph to ooze from the adelgid that the beetles then drink; they sometimes chew on the adelgid's body without entirely consuming it. The adult beetles feed little on the active crawlers, which easily escape when approached (Lu et al. 2002).

In China, adult *Ss* and *Sc* ate, respectively, an average of 22 and 31 HWA eggs daily, taking 60-80 seconds to consume an egg (Yao and Wang 1999). In quarantine at 20 °C, adult *Sn*, removed from cold storage, consumed 1.0 ± 0.29 nymphs, 0.8 ± 0.31 adults, and 5.5 ± 0.23 eggs/day when given a mix of stages (Montgomery et al. 2002). Both *Ss* and *Sn* adults fed at 0 °C and consumed 7-10 and 9-17 eggs in 20 hours when held at temperatures between 2.5 °C and 10 °C, respectively (Costa et al. 2008).

Scymnus larvae feed on all stages of adelgids, but mostly on eggs. When feeding on adelgid eggs, the larvae enter the adelgid ovisac and usually consume all of the eggs before leaving. Larvae suck the eggs, leaving the chorions, whereas adult beetles chew the eggs and do not leave the chorions. Although adelgid crawlers usually escape encounters with adult beetles, *Ss* and *Sc* larvae have been observed capturing and eating this active stage. The larvae appear to feed through a form of extraoral digestion on the crawlers. They bite and suck out the contents of the crawler, then regurgitate the contents back repeatedly into the crawler several times, before abandoning the empty corpse (Lu et al. 2002). When starved, 1st instar *Ss* larvae fed on crawlers of the hemlock scale *Fiorinia externa* Ferris, regurgitating in the same way (Lu et al. 2002). The 1st instar *Ss* beetle larva have also been observed attacking the aestivating 1st instar adelgid nymph by turning the prey on its side, piercing the underside of the thorax near the stylet, and sucking out the hemolymph. Large beetle larvae, starved for 1-2 days, were cannibalistic.

Larvae are voracious feeders. For example, *Sn* larvae in the third instar consumed 99.2 ± 11.7 adelgid eggs/day. Chinese colleagues reported that consumption of HWA ovisacs by each larval instar (I-IV) was 1.8 ± 0.6, 3.9 ± 1.5, 5.3 ± 2.0, and 11.2 ± 3.1, respectively (Montgomery et al. 2002). The adelgid ovisacs had an average of 31 ± 11 eggs/sac; thus, total consumption by a larva was 23 ovisacs, or 713 eggs. First instar larvae of all three *Scymnus* species do not survive if they do not have adelgid (or their own) eggs on which to feed.

HOST EVALUATION IN QUARANTINE

Methods for Host Preference Testing

The methods and results are a compilation and summary of previously reported experiments (Montgomery and Lyon 1996, Montgomery et al. 1997, Butin et al. 2002, Butin et al. 2004, Hoover et al. 2010), unpublished data of the authors, and personal communications from K. Hoover. Included in some of the tests for comparison purposes were two non-native coccinelid species established in the United States, *Harmonia axyridis* Pallas, a predator of arboreal aphids, and *Scymnus* (*Pullus*) *suturalis* Thunberg, a predator of pine adelgids. Feeding by adults was measured as it is the adults stage that make the choice of prey on which the larvae will feed. Tests were not conducted during the summer when the adults are normally inactive and feed little.

A series of sequential tests were used. First, no-choice tests are made where adult beetles are confined with a single type of potential prey—this indicates what they will not eat even when in a starved condition. Where warranted, this is followed by tests where the beetles are offered a choice between two to four prey items—this indicates relative preference. Unless indicated otherwise, prey was presented on a small section of host material in a Petri dish with a filter paper on the bottom. Prey were carefully counted before placement in the dish. Beetles usually were tested individually, and starved, but given water, prior to testing. The number of prey remaining was counted after a period of 20 hours or more, and the presence of fecal droplets on the filter paper was noted in no-choice tests.

The prey evaluated included insects that could be encountered on hemlock foliage (e.g. scales, psocids, and predatory dipteran larvae), tree feeding aphids, and other adelgids present in eastern North America. There are only five *Adelges* and seven *Pineus* species of adelgids reported from eastern North America; all of the *Adelges* are introduced species, while five of the *Pineus* species are native. One aphid tested, *Paraprociphilius tesselatus* (Fitch), is the primary prey of the only predaceous lepidopteran in the continental United States, *Feniseca tarquinius* F.; therefore, there was concern that the *Scymnus* beetles might attack this aphid.

Prey Acceptance (No-choice Test Results)

Four species of adelgids, five species of aphids, and representatives of non-aphidoid taxa were presented to the lady beetles (Table 5). *Harmonia axyridis*, a generalist predator, had a high acceptance rate for all the prey it was presented, except for *Fiorinia* elongate scale. It was the only predator that fed on the large aphid, *Cinara pinea*. This coccinellid is very active and capable of catching large, active prey. It also consumed the predaceous Diptera larvae that prey on aphids and adelgids. The *Scymnus* beetles were more restrictive in their prey acceptance. The aphids they were presented were all first instar nymphs, most of which were larger than HWA adults; only *Eucallipterus tiliae* and *Aphis gossypii* are comparable in size to first instar HWA nymphs. Aphids were not appreciably consumed by these *Scymnus* beetles, except for the consumption of *Aphis gossypii* by *Sc*. The first instar aphids were presented in a small container off its host and without filter paper, and may have been unable to escape the beetles. The other *Scymnus* species tested will also consume small aphids off host. The only species that preyed on all the adult adelgids was *Sc*. The pine adelgid, *Pineus strobi*, which is smaller than HWA, was consumed at rate equal to HWA.

Prey Preference (Choice Tests)

Because prey preferences among the adelgids were not clear based on single prey tests, HWA and another adelgid on their respective host plant were offered together in a Petri dish. Extensive choice tests by Butin et al. (2004) indicated that *Sn* prefers HWA over *Adelges laricis* and *A. cooleyi* and any aphid tested, but HWA and *P. strobi* were eaten equally.

When *Sc* females were presented with a choice of HWA and 2-3 other adelgids, they showed a strong preference for HWA. In the four-way choice tests, the relative preference was HWA > *Pineus strobi* >> *Adelges cooleyi* or *Adelges laricis*. The combination of other prey present, when only three were offered, resulted in significant differences in the relative feeding preference. *Sc* females were 6.6-fold more likely to eat HWA eggs over larch adelgid, 4-fold more likely to eat eggs of the combined group of *Adelges cooleyi*

Table 5. No-choice feeding[1] by adult lady beetles during 24-72 hour confinement with prey.

Prey Item	*Harmonia axyridis*	*Scymnus suturalis*	*Scymnus ningshanensis*	*Scymnus sinuanodulus*	*Scymnus camptodromus*
Adelgidae					
Adelges tsugae Annand					
egg	+++	+++	+++	++	+++
crawler	nt	nt	++	+	+
aestivating nymph	++	+	+	+	+
nymph III & adult	++	+++	+	++	++
Pineus strobi (Hartig)	+++	+++	+++*	++	+++
Adelges laricis	nt	nt	0*	nt	+++
Adelges cooleyi (Gillete)	nt	nt	++*	nt	+++
Aphididae					
Cinara pinea (Mordwilko)	+++	0	0	0	0
Eucallipterus tiliae (L.) nymph	+++	+	+	++	nt
Prociphilus tessellatus (Fitch)	+++*	nt	+*	nt	0
Eriosoma lanigerum (Hausmann)	nt	nt	nt	nt	0
Aphis gossypii Glover					+++
Diaspididae					
Fiorinia externa Ferris	0	0	0	0	0
Pseudococcidae					
Pseudococcus sp.	+++	0	0	0	nt
Psocoptera					
Pseudocaecilidae	+++	0	0	0	nt
Diptera					
Syrphidae (larva)	++	0	0	0	nt
Chamaemyiidae (larva)	++	0	0	0	nt

[1] Codes refer to the percentage of predators tested that fed on the prey: 0 = none; + = <33%; ++ = 33 to 67%; +++ = >67%; and "nt" = not tested.

* Indicates score based on the proportion of alternative prey eaten in 2-way choice test between indicated species and HWA.

and *Pineus strobi* over *Adelges laricis*, and 1.6-fold more likely to eat HWA over the combined group of *Adelges cooleyi* and *Pineus strobi* (Hoover et al. 2011). Beetles only laid eggs on hemlock infested with HWA during these choice tests.

Choice tests using only the host plant found that significantly more time was spent by *S. suturalis* on white pine than on hemlock, whereas hemlock foliage was preferred by *Ss* and *Sn* (Table 6). Thus, it appears that tree foliage itself may vary in attractiveness to *Scymnus* species.

In summary, all three species of *Scymnus* imported from China are adelgid specialists, but when adelgids are not present, adults will minimally feed on slower moving aphids that are similar in size to HWA. Larvae seem to require HWA eggs to complete development, but will feed on other adelgids. They prefer HWA over other adelgids in choice tests and the adelgid's host plays a role in host choice. They appear to more readily accept adelgids on hemlock than on pine, and on pine than on other conifers. Adelgid eggs are required for larvae to complete development and for optimal oviposition. Both adults and larvae will locate an HWA ovisac and generally feed on it until it is exhausted before moving to the next. They will feed on crawlers, nymphs, and adults, often only injuring or partially consuming the later.

Table 6. Foliage preferences by adult lady beetles given a choice between pine and hemlock for 240 seconds.

Predator species	No. tests	Time on foliage (seconds)		Probability[1]
		Tsuga canadensis	*Pinus strobus*	
S. suturalis	7	24.1	55.2	<0.001
S. sinuanodulus	13	93.9	41.6	<0.001
S. ningshanensis	8	80.7	29.3	<0.001

[1]Probability that means are equal, paired t-test

Since these beetles are specialized on adelgids, of which seven of the 12 species in eastern North America are non-native, and they have a strong preference for HWA, they are not likely to have any appreciable impact on non-target prey. In addition, when *Sc* were presented with eggs of various adelgids without host or waxy coverings, they ate three times more eggs than when the same adelgid eggs were presented with the female parent on host material in a 48 hour time period. This indicates that prey acceptance is at least partially based on host characteristics. *Sc* adults also showed a significant preference for the eggs of the overwintering generation of the adelgids (both HWA and larch adelgids) when compared to the second generation, which may indicate some host quality differences between generations.

ESTABLISHMENT

Field Evaluation in Sleeve Cages

Information on the efficacy of biological control agents can be difficult to obtain when they are released freely into the environment because they disperse and both the agent and its target are affected by external factors that cannot be controlled. Field cage studies are an effective method to evaluate efficacy of natural enemies in a controlled setting and are more realistic than the laboratory, although the cages may alter microclimate and interfere with some enemy/prey interactions (Luck et al. 1999). Between 1999 and 2008, a series of experiments were conducted using sleeve-cages (Fig. 14) to confine *Sn*, *Ss*, and *Sasajiscymnus tsugae* on HWA infested branches of eastern hemlock.

Figure 14. Sleeve cages used to confine lady beetles on HWA infested hemlock branches.

The recommended procedure, which was refined over several years of use, is as follows: sites should be selected several months before the cages and beetles will be placed on the trees. They should have healthy hemlocks that have branches reachable from the ground with a density of HWA ranging from 100-300 per 0.5 meter of branch. The sleeve cages are made of light weight "No-see-um" knitted polyester fabric (nylon is more susceptible to UV rot) and are 1 m long × 0.65 m wide. Each treatment should

have at least 30 replications, because variation within treatments is high. Just before the start of the test, branches are selected that appear to have between 150-350 ovisacs on the terminal 0.5 m. The number of ovisacs is estimated visually on each branch (it takes about 3 full days for two people to count 160 branches) and these are tagged where the open end of the bag will be closed over the branch. The branches are ranked by HWA density and the treatments assigned randomly within the sequential groups based on the number of treatments. (Initially, trees were considered blocks with complete sets of treatments assigned to each tree, but it was found that variation between trees was not a significant component of random variation.) Ideally, mature beetles held over winter in cold storage are used in the experiments. They may be removed from the cold and held as a group overnight for mating; however, mating just prior to placement in the field does not seem to increase fecundity. The beetles are sexed and placed individually in small containers that can easily be opened with one hand in the cage. Ideally, the sleeve cages are placed on the branches in April before HWA eggs have started to hatch and average daily temperatures are 5-7 °C, and removed in June, when the progredientes are just starting to lay eggs. The branch with the bag attached is cut from the tree and taken to the lab and placed in cold storage until the bags can be opened. The beetles and HWA are counted with the aid of a microscope. The statistic most useful in assessing impact on HWA is the per capita change in the population ($r=ln\ [Nt/No]$) where *No* was the initial population (sistens ovisacs) and *Nt* was the final population (progrediens ovisacs) in each sleeve cage.

In the first experiment, the sleeve cages were installed at two sites in Connecticut on 20 April and most were removed one month later. During this month, the eggs in the sistens ovisacs hatched and progrediens nymphs were present when the cages were removed. The HWA population increased in all the cages during the month but those with a single female *Ss* had, on average, lower HWA populations than the control bags without a beetle (Fig. 15). Note that there was a cage effect. HWA numbers were higher on the branch in the cage without a beetle than on the branch without a cage—the effect of the beetle was compared to the caged branch without a beetle. The branches where beetles produced progeny reduced HWA populations more than uncaged branches. Some of the cages in this experiment were allowed to remain on the branches until July 7. At this time, the HWA population in the cages with reproductive beetles was not significantly different, on average, than in the cages without beetles (Fig. 16). By July, the cages contained aestivating neosistentes and most of the beetles were dead, perhaps from the lack of suitable food. In cages without the beetle, the HWA populations increased in the first generation

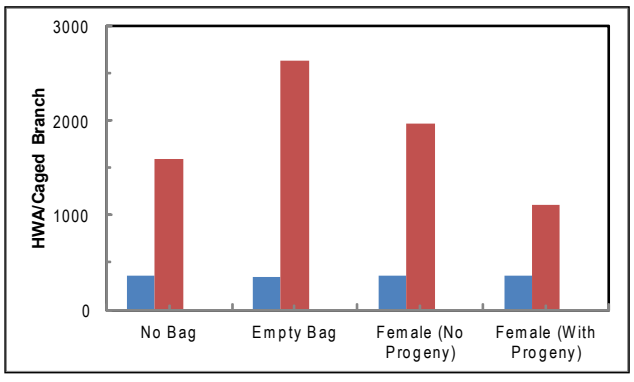

Figure 15. Population change of HWA in cages for one month (sistens ovisacs to progrediens nymphs).

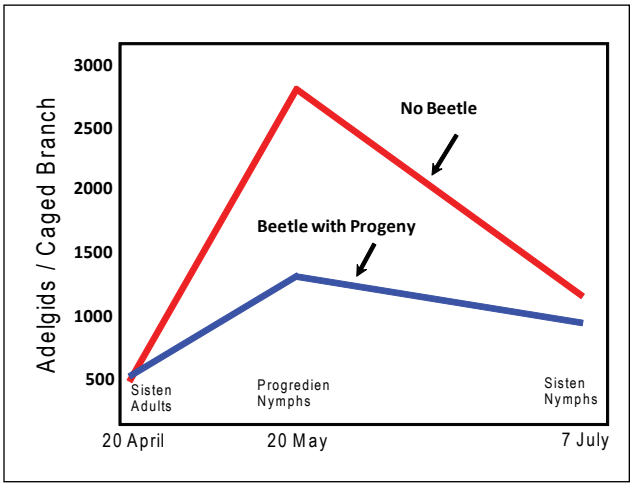

Figure 16. Population change of HWA in cages with or without beetles for two generations of HWA.

and then crashed in the next generation whereas bags with the beetle stabilized the population, but at a level still high enough to damage trees. Stabilization of pest populations below damaging thresholds is the goal of biological control.

In a similar caged study, the presence of *Sn* resulted in negative growth of HWA populations, whereas the population growth in the cages without the beetle was positive (Butin et al. 2003). This study also included *S. tsugae* as a treatment and this lady beetle also reduced HWA populations compared to the control, but not significantly. The *S. tsugae* were new beetles that had not been overwintered in the laboratory. The *S. tsugae* did not reproduce in the bags, whereas one-third of the *Sn* did. Butin also reported that, in the laboratory, the fecundity of *Sn* increased with increasing densities of HWA ovisacs. With both *Ss* and *Sn*, the beetle cages with the most beetle progeny were those with the highest number of HWA at the end of the experiment and most of the progeny were produced within the first week after the beetles were placed in the bags. These results indicated that plentiful, fresh food is needed for good beetle reproduction.

Other caged field trials, conducted from 2002 to 2006, were less successful. In some, the HWA population crashed in the control cages and neither the presence of *Ss* nor *Sn* was able to drive the HWA populations significantly lower (although many cages with beetles contained no HWA at the end of the feeding period). The beetles did not produce progeny on these declining populations. These trials showed the importance of "pre-conditioning" of the adult females placed in the cages. It was also found that degree day growth models could be used to predict when each stage of the lady beetle would be present in the cage and that the free released and caged beetles had the same development rates.

In 2007, three species of lady beetles (*Sn*, *Ss*, and *S. tsugae*) were evaluated in sleeve cages in northwestern North Carolina. All three species significantly reduced HWA compared to the cages without beetles (Table 7). However, there was not a significant difference in reduction in HWA populations among beetle species. For this test, all three species had been held over winter at 7 °C and short daylength at the Forest Service laboratory in Hamden. This test demonstrated that placement of overwintered, reproductively active lady beetles in the field, when day-night temperatures ranged from -2 °C to 12 °C, results in good oviposition by all three species and full development of their progeny, including maturation feeding of the new adults, before HWA enters aestivation.

In 2008, a sleeve cage evaluation focused on *S. tsugae*. For this trial, *S. tsugae* was obtained from the North Carolina Department of Agriculture rearing facility. These beetles are normally reared at 25 °C

Table 7. Populations of HWA (average/caged branch) when the cages were placed on the branches April 15 and removed June 19-22. The change in HWA population (r) is (*ln* Final – *ln* Initial). The number of progeny the female beetle produced in each cage is based on number of all beetle stages recovered at the end of the test, minus the initial female.

Treatments	Empty cage (no beetle)	*Scymnus ningshanensis*	*Scymnus sinuanodulus*	*Sasajiscymnus tsugae*
Sistentes (initial)	254.5	297.0	258.0	252.5
Progredientes (final)	132.7	49.5	1.5	17.6
HWA pop'n change (r)	-0.77	-3.79	-6.02	-5.85
HWA pop'n change (%)	-41.0	-84.1	-99.0	-95.0
Beetle progeny (avg)	na	4.8	3.2	7.9
Beetle progeny (max)	na	24	29	31
Replications (n)[1]	30	34	31	24

[1]Initally 30 controls and 34 cages for each treatment, but some cages were damaged.

and stored at 18 °C prior to release. The adults are usually less than one-month-old when field released. Thirty replicates of four different ages of *S. tsugae* were placed in cages on April 1. Complete sets of the four treatments were removed on a given day between July 5 and 16, and the progeny counted (Table 8). The two-week-old beetles did not produce any progeny and only one of the one-month-old beetles produced progeny. The beetles held for one month at 18 °C and then placed in cold storage for one month also did not produce progeny. The only beetles that reproduced well had been reared the previous year and had been producing progeny for field release prior to being placed in the bags. These beetles were noticeably smaller and much less active than the much younger beetles in the other treatments. The progeny of the oldest beetles were either adults or pupae, whereas the progeny from the one-month-old beetles were only in the larval stage, which indicates that they may not have been ready to oviposit when released. This experiment suggests that releasing mature, ovipositing *S. tsugae* beetles in the spring would be more successful than releasing beetles that became adults that same spring. With both *S. tsugae* and the *Scymnus* beetles, better reproduction occurred with adults that had been overwintered in the laboratory.

While sleeve cages are useful in defining release parameters and impact of lady beetles, the cages seem to inhibit their reproduction. In all the trials, the cages with the most progeny were the cages that had the most HWA at the end of the trial (control cages excepted). It seems that all of these lady beetles produce more progeny when more food is available.

Table 8. Production of progeny in sleeve cages by *Sasajiscymnus tsugae* varying in age.

Adult Age (Months)	Produced Progeny	Progeny (Avg. No.)	Progeny (max.)
0.5	0%	–	–
1	3%	16	16
1+1*	0%	–	–
>8	30%	32	64

*Held one month at 18 °C and then refrigerated one month

Environmental Releases

Free releases of *Ss* (Table 9) and to a limited extent of *Sn* (Table 10) have been made, but there is no record of recovery of these beetles in the years following their release. Following the early spring release of adult beetles that had been overwintered, both *Ss* and *Sn* larvae have been recovered and adults found until November, but not in the year following release. Thus, there is no evidence that either *Ss* or *Sn* are established in the eastern United States.

IMPLICATIONS FOR BIOLOGICAL CONTROL

The *Scymnus* (*Neopullus*) lady beetles that prey on HWA have either delayed reproductive maturation in the adult stage or an egg diapause. The periods of arrested development in all three *Neopullus* species coincide with the aestival diapause of the HWA neosistentes. The adults of these lady beetles prey on HWA during all periods when it is active, and their larvae are present in the spring when HWA eggs are present.

A key question is whether a species with a life history where adults exist for several months before they lay eggs that quickly hatch, or a species with a life history where eggs exist for several months before they hatch, is more suitable biological control agent for HWA. The adults of *Sn* and *Ss* spend eight months exposed to predators and the elements before they lay eggs that soon hatch in spring. On the other hand, *Sc* begins laying eggs soon after eclosion and has an extended oviposition period in which to stockpile eggs, but these eggs then remain in diapause for many months until they hatch in early spring. It is not possible to predict which survival strategy would result in better HWA control. However, in their native range in China, the abundance of *Sc* was more consistent among the sites and during the survey period than the other species.

Table 9. Environmental releases of adult *Scymnus sinuanodulus*.

Date	No. released	Location	Rearing Lab.	Condition
April, 2004	150	Rauben Co., GA	USFS, Hamden	10-wk-old adults
October, 2004	320	Rauben Co., GA	"	4-month-old adults
April, 2005	528	Fairfield Co., CT	"	reproductive adults
Spring, 2005	1,530	NJ	NJ Dept. Agric.	new adults
"	1,210	PA	"	"
"	750	NC	"	"
"	460	MD	"	"
"	460	WV	"	"
Spring, 2006	1,500	NJ	"	"
"	1,200	PA	"	"
"	1,000	NC	"	"
"	500	MD	"	"
"	1,000	WV	"	"
July, 2006	228	Watauga Co., NC	Sleeve cages	new adults
Spring, 2007	6,305	NJ	NJ Dept. Agric.	new and old adults
April, 2007	496	Avery Co., NC	USFS, Hamden	reproductive adults
May, 2007	45	Gt. Smoky Mtn. NP	UTK, Knoxville	post-reproductive
Late May, 2008	208	Chattahoochee NF, GA; 1 site	UGA, Athens	reproductive adults
Spring, 2008	7,480	Chattahoochee NF, GA; 9 sites	UGA, Athens	eggs & larvae
Spring, 2009	8,400	Chattahoochee NF, GA; 15 sites	UGA, Athens	eggs & larvae
Late May, 2009	165	Chattahoochee NF, GA; 1 site	UGA, Athens	reproductive adults
Spring, 2010	15,741	Chattahoochee NF, GA; 53 sites	UGA, Athens	eggs & larvae
Late May, 2010	100	Chattahoochee NF, GA; 1 site	UGA, Athens	reproductive adults
Spring, 2011	11,075	Chattahoochee NF, GA; 23 sites	UGA, Athens	eggs & larvae
Early Spring, 2011	800	Chattooga River Corridor; 7 sites	UGA, Athens	reproductive adults

The first predator imported from China for biological control of HWA was *Sc* in 1995. Unfortunately, this species has been very difficult to rear because of its egg diapause. The requirements for breaking its egg diapause have now been deciphered and colonies can now be maintained in the laboratory. This species has several characteristics that indicate it would make it a good biological control agent: (1) it has a true aestival diapause as does HWA; (2) it occurs over a broad geographic area and in diverse habitats in its native range; (3) its larvae are present at a key point in the life cycle of HWA; and (4) its adults feed on HWA throughout most of the year.

Table 10. Environmental releases of reproductive adult *Scymnus ningshanensis* reared at the USDA Forest Service lab in Hamden, CT.

Date	No. released	Location
April, 2007	300	Hampshire Co., MA
April, 2007	300	Hampden Co., MA
April, 2007	300	Hartford Co., CT
April, 2009	500	Avery Co., NC

The other species of *Scymnus* (*Neopullus*) imported from China have been environmentally released, but appear not to have established. *Ss* has been released in considerable numbers in several areas and does not seem to merit further effort at establishment. The other species, *Sn*, has been released only in very low numbers and may merit further effort. A significant problem with this species is that the colony was founded on a single collection of a low number of specimens, which declined to a colony of less than ten individuals before being multiplied to sufficient numbers for release.

In addition to the *Scymnus* (*Neopullus*) lady beetles imported, there were other predators in China that appear when HWA is at its highest densities. These predators are mostly opportunists that feed on a broad range of prey. This non-specificity makes them unsuitable as biological control agents, but the significance of opportunistic predators in driving down high HWA populations where it is indigenous needs to be recognized. It is not surprising that native, HWA specific predators are lacking in the eastern United States considering than HWA is a relatively recent introduction; what is surprising is the lack of native opportunistic predators preying on HWA.

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Chapter 6: Laricobius nigrinus Fender (Coleoptera: Derodontidae)

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INTRODUCTION

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is a native herbivore in western North America (Havill et al. 2006). It feeds on western hemlock, *Tsuga heterophylla* (Raf.) Sargent, but it is not considered a forest pest (Furniss and Carolin 1977), in complete contrast to its pest status in eastern North America. Observations in Washington State suggest that hemlock resistance and predators limit HWA densities to innocuous levels (Mausel 2005, McClure and Cheah 1999). The importance of predators is underscored by the long-term survival of eastern hemlock trees in western arboreta (Fig. 1). Clearly, detailed demographic studies would be useful to identify the key factors that regulate HWA populations in this region.

To determine what types of biological control agents may be needed in the eastern U.S., if any, HWA natural enemies were surveyed in Virginia and Connecticut, but few were found and they had no impact on HWA (Montgomery and Lyon 1996; Wallace and Hain 2000). In western North America, exploration for potential classical biological control agents was carried out in western Washington and Oregon to comprehensively document and characterize HWA predators (Kohler et al. 2008). Of the 55 predator species identified, *Laricobius nigrinus* Fender and *Leucopis* spp. (Diptera: Chamaemyiidae) (see Chapter 8) were the most abundant natural enemies at low and high HWA densities.

Figure 1. A healthy eastern hemlock tree in the Washington Park Arboretum, Seattle WA (No. # 119-49-B, planted in 1949) has innocuous HWA densities suggesting that factors other than host-resistance, such as natural enemies, explain its non-pest status in western North America (photo by D.L. Mausel in 2002).

Laricobius nigrinus was prioritized early on for evaluation because it was frequently collected from HWA-infested trees in Victoria, British Columbia, Canada (Humble 1994) and all *Laricobius* species with known biology feed exclusively on adelgids (Leschen [in press]). Since 1998, studies on *L. nigrinus* determined major aspects of its life history, environmental risk, and potential efficacy. After approval for release in 2000, field releases began in 2003 and populations have established from the southern Appalachians to New England spanning the invaded range of HWA, but remain isolated. Beetle population growth has been considerable and thousands of adults have been mass-collected at some sites for release in nearby forests to enhance dispersal. Post-release monitoring has detected an *L. nigrinus* impact on HWA populations, but more time is needed to definitively state in what situations, if any, *L. nigrinus* saves hemlock trees from decline and probable death.

SYSTEMATICS AND TAXONOMY

Within the Polyphaga supergroup of beetles, the Derodontidae family has archaic morphological features and is one of the earliest lineages (Hunt et al. 2007). The Derodontidae is a small family known as the tooth-necked fungus beetles due to the dentate or flattened lateral margins of the pronotum (der = neck; odon = tooth). There are four genera in the family, with three being fungus feeders (*Derodontus* Leconte, *Peltastica* Mannerheim, *Nothoderodontus* Crowson) and one, *Laricobius* Rosenhauer, that is predaceous on adelgids (Lawrence 1989, Leschen 2000). To date, 21 *Laricobius* spp. have been described worldwide in the temperate latitudes (Leschen [in press]). *Laricobius nigrinus*, *L. rubidus* Leconte, and *L. laticollis* Fall are native to North America and *L. erichsonii* Rosenhauer was introduced from Europe (Downie and Arnett 1996, Hatch 1962). *Laricobius* species feed exclusively on the Adelgidae, and all adelgid species feed on Pinaceae hosts (Havill and Foottit 2007). The etymology of the genus *Laricobius* is rooted in Laric-, referring to *Larix* spp. or Larch, which was historically used as a generalized term for trees in the Pinaceae family.

Laricobius nigrinus was first collected and described from Bear Springs, Oregon (Fender 1945). Adults are small (2-3 mm), shining black, covered with fine ashy hairs, have striate elytra (10 rows, oval), 11-segmented antennae (scape, pedicel, and nine annuli), and 5-5-5 tarsal segmentation (Fig. 2). The life stages have been described by Zilahi-Balogh et al. (2006). Other beetles that look similar to *L. nigrinus* and commonly collected from hemlock include *L. rubidus*, silken fungus beetles (Coleoptera: Cryptophagidae), and minute brown scavenger beetles (Coleoptera: Lathridiidae).

RANGE AND COLLECTION AREAS

Laricobius nigrinus is native to the western United States and Canada with records from British Columbia, Alberta, Idaho, Washington, Oregon, and California (Bright 1991, Fender 1945; Hatch 1962, Lawrence 1989). Specimens of

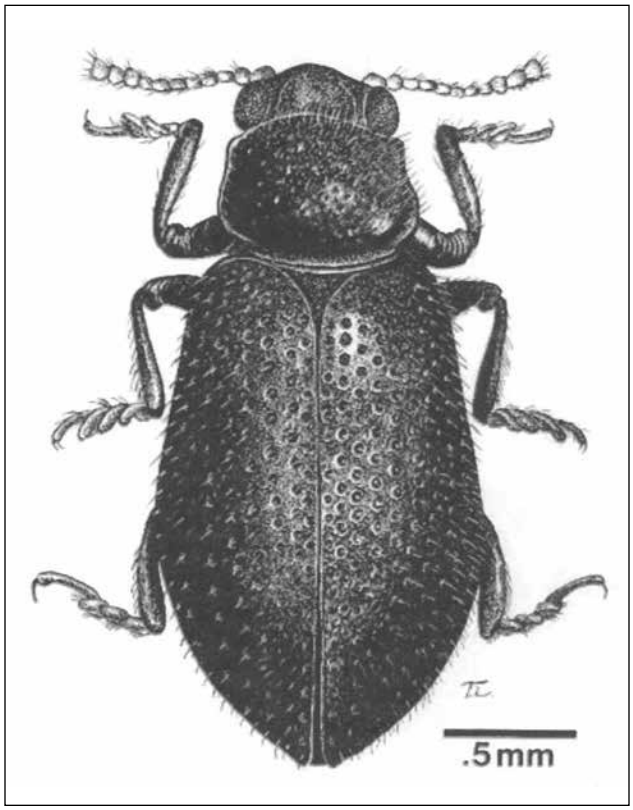

Figure 2. Laricobius nigrinus is a predator native to western North America, where it feeds and reproduces primarily on HWA (illustration by Terry Lawrence).

L. nigrinus in the U.S. National Museum of Natural History, Washington D.C. have been collected from Wyoming and the southeastern Yukon (M. Montgomery, pers. comm.). The distribution of *L. nigrinus* likely overlaps the range of western hemlock and other western conifers that host adelgids.

Initial collections of *L. nigrinus* from a western hemlock seed orchard in Victoria, British Columbia in 1997 were identified by Donald E. Bright (Agri-Food Canada, Systematic Entomology Section). From 1997 to 2004, 2,710 beetles were collected from this site for quarantine evaluation, mass rearing and releases in the eastern United States. From 2005 to 2011, 54,878 beetles were collected from western hemlock in Washington State (principally Seattle, WA) for mass rearing and releases (D. McDonald, pers. comm.). In addition, from 2007-2010, 4,601 beetles were collected in northern Idaho and northwest Montana. Equal numbers of beetles were collected from western hemlock and western white pine (Callahan et al. 2008), and the majority of beetles were collected in Idaho (97%). The identification of *L. nigrinus* from these locations was morphologically confirmed by G. Zilahi-Balogh (Canadian Food Inspection Agency) and N. Vanderberg (U.S. Department of Agriculture, Agriculture Research Service), and by mtDNA by N. Havill (U.S. Department of Agriculture, Forest Service).

BIOLOGY AND ECOLOGY

L. nigrinus has one generation per year (Fig. 3), which is synchronized with the phenology of HWA in its native and introduced range (Mausel et al. 2008, Zilahi-Balogh et al. 2003a). Both the beetles and HWA are active in the fall, winter, and spring, and dormant in the summer. Lamb et al. (2007) investigated the factors influencing summer dormancy and determined that temperature was the most important cue for its termination, while photoperiod was a modifying factor. Adult beetles emerge from the soil in the fall, disperse to hemlock branches, and feed on sistens (summer-

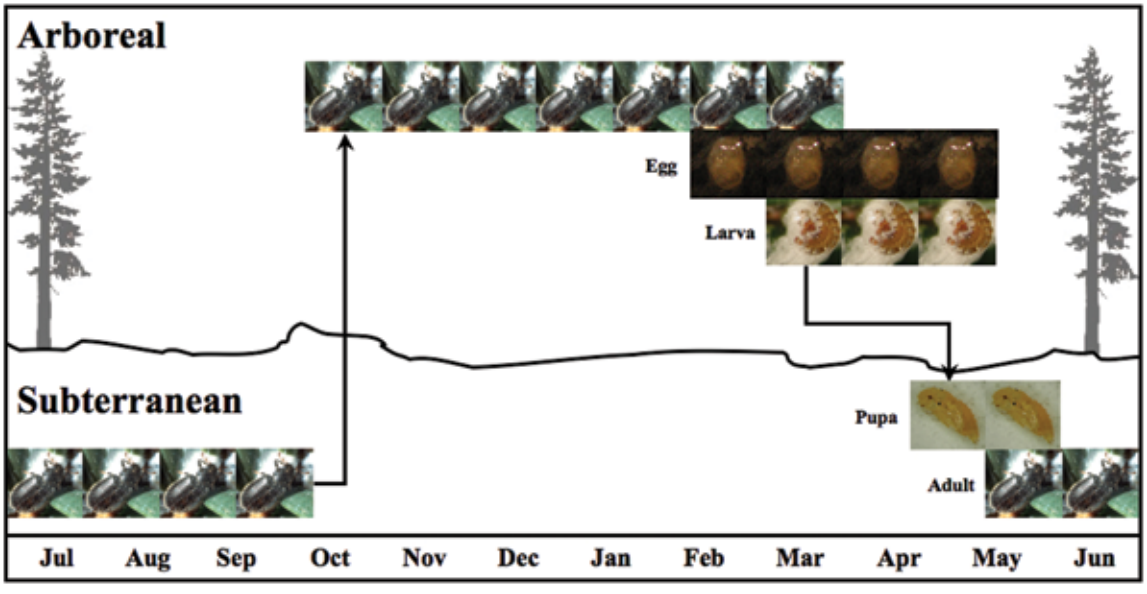

Figure 3. Generalized life-cycle of Laricobius nigrinus. Development rate varies based on temperature as influenced by latitude, physiography, and elevation. Arrows indicate major dispersal events and arboreal adults search tree canopies for HWA from fall-early spring to feed and lay eggs. Adults are dormant in earthen cells during the summer months.

early spring HWA generation) nymphs, reproduce in early spring, and then die (Zilahi-Balogh et al. 2003b, c). The adults pierce HWA nymphs with pointed mandibles and consume their hemolymph and organs. In the lab, females oviposit up to 396 eggs (mean = 101), typically one per HWA sistens ovisac, but more than one is common if prey density is high. The larvae have four instars and feed on hundreds of progrediens (Spring HWA generation) eggs to complete larval development, which may require more than one ovisac. The most apparent sign of *L. nigrinus* feeding is the disturbed ovisacs due to larval feeding (Fig. 4). In spring, mature larvae drop to the soil, enter a pre-pupal and pupal stage in an earthen cell. Development from egg to adult takes 65 days at 15 °C.

Laricobius nigrinus is cold hardy and active during fall-early spring as long as temperatures are above freezing (Zilahi-Balogh et al. 2003a). Immature stages have minimum developmental thresholds at or below 5.0 °C (Zilahi-Balogh et al. 2003b). Adults from Victoria, BC are physiologically able to avoid dying down to -16 to -19 °C (Humble and Mavin 2005). Eggs can survive -27.5 to -26.9 °C. Larval survival diminished from -22.1, -17.0, -15.0, and -13.0 °C for each consecutive instar. Beetles from northern Idaho appear even more cold tolerant (Mausel et al. [in press]-b).

Studies on *L. nigrinus* host searching behavior is in its infancy. The basic sequence of adult behaviors and its visual ability have been described in the

Figure 4. HWA ovisacs that were fed on by Laricobius nigrinus larvae from a release site in western Massachusetts (left), compared with intact HWA ovisacs from a non-release site (right).

laboratory (Flowers et al. 2007, Mausel et al. [in press]-a). Antennal morphology and preliminary behavioral studies suggest that *L. nigrinus* use host volatiles in long-range host searching behavior and/or mate location (Broeckling and Salom 2003).

Few natural enemies are known to affect *L. nigrinus* in the eastern United States. Generalist predators (i.e., spiders and ground beetles) likely prey on beetles in the canopy and in the soil, respectively. Other previously released HWA predators probably do not drastically compete or prey on *L. nigrinus* due to phenological differences (Flowers et al. 2006). In Europe, *L. erichsonii* was reported as a host of *Earobia* sp. and *Phrudus* spp. (Hymenoptera: Ichneumonidae) parasitoids (Franz 1958). A pathogen that causes fatal disease in larvae was observed by Franz (1958). No parasitoids have been observed in North America to date, but a microsporidian parasite caused mortality in a mass-rearing laboratory (see Chapter 12).

NATIVE RANGE STUDIES

Field studies were conducted in western North America to determine if *L. nigrinus* has biological characteristics that could be damaging to HWA populations. The phenology of *L. nigrinus* was monitored for 2 yr and it was highly synchronized with HWA (Zilahi-Balogh et al. 2003a). This suggests that *L. nigrinus* is host-specific and showed that the beetle has a unique niche, as it fed on both HWA generations during fall-spring when most other predators are inactive. Adult and egg/larvae density appears to increase with increasing HWA density (Kohler et al. 2008, Mausel 2007) and this relationship is an important trait observed in successful biological control agents (Huffaker 1974). The actual impact of *L. nigrinus* on HWA populations in western North America has not been experimentally tested (i.e., predator exclusion study). However, an exclusion study may not accurately predict *L. nigrinus* impact where it is introduced, as that will depend on the abundance that it reaches.

HOST RANGE EVALUATION

During field collections in the native range of *L. nigrinus*, adults are primarily collected from HWA-infested western hemlock in the Puget Trough Region (Seattle, WA and Victoria, BC) and western hemlock and western white pine in northern Idaho. During informal surveys of other hosts in the Okanogan region of British Columbia (G.M.G. Zilahi-Balogh, unpub. data), western Washington (D. McDonald, unpub. data), and northern Idaho (D.L. Mausel, unpub. data), adults have been collected from *Pineus similis* (Gillette)-infested western white pine, *Adelges lariciatus* (Patch)-infested western larch, and *Adelges cooleyi* (Gillette)-infested Douglas-fir and Engelmann spruce. Larvae have been collected from *P. similis*-infested western white pine and *Adelges lariciatus*-infested western larch.

The *L. nigrinus* adults used in quarantine host range tests were field collected from HWA-infested western hemlock from Victoria, BC and imported to Virginia in 2000 for evaluation (Zilahi-Balogh et al. 2002). The test prey, six hemipteran species in three families (Adelgidae, Aphididae, Diaspididae), were used in choice and no choice host acceptance and suitability tests (Table 1). Host acceptance tests determine whether a candidate biological control agent will feed and/or oviposit on a host. Host suitability tests determine whether the biological control agent is able to complete development to the adult stage and produce viable offspring on a particular host.

In both the no-choice and paired-choice oviposition tests, *L. nigrinus* females laid significantly more eggs in HWA ovisacs over other test prey. In the paired-choice tests, no eggs were laid on host plant twigs housing the non-adelgid test prey, *Chionaspis pinifoliae* (Diaspididae), *Cinara pilicornis* (Aphididae) and *Myzus persicae* (Aphididae). Oviposition was more than five times greater on HWA than on the adelgid test prey, *Adelges piceae*, *Adelges abietis* or *Pineus strobi* in the paired-choice tests. These differences indicate an ovipositional

Table 1. Summary of *Laricobius nigrinus* host-range testing results with adelgid, aphid, and scale prey (Hemiptera)

Test prey	Acceptance[a]		Suitability	Final host status[b]
	Oviposition	Adult feeding	Larval development	
Adelgidae				
Adelges tsugae	+	+	+	Yes
Adelges piceae	+	+	–	No
Adelges abietis	+	+	–	No
Pineus strobi	+	+	–	No
Aphididae				
Cinara pilicornis	+	x	–	No
Myzus persicae	–	x	x	No
Diaspididae				
Chionaspis pinifoliae	+	x	–	No

[a] + = positive response on test prey; – = negative response on test prey; x = test not conducted.
[b] Whether the species could serve as a host to *L. nigrinus*.

preference for HWA over these other adelgids. In no-choice adult feeding tests, eggs of all the test adelgids were fed on by adult *L. nigrinus*. Significantly more HWA eggs were consumed than *A. piceae* and *P. strobi* eggs, but not *A. abietis* eggs. Though not statistically significant, *L. nigrinus* adults consumed on average of two times more eggs of HWA (48) than *A. abietis* eggs (25).

Laricobius nigrinus only completed development to the adult stage on a diet of HWA (Table 1). *Adelges piceae* and *P. strobi* supported larval development to the fourth instar, providing evidence of larval feeding, but did not support further development. *Laricobius nigrinus* larvae that fed on *A. abietis*, *C. pilicornis* or *C. pinifoliae* did not survive beyond the first instar. Host suitability tests with HWA and *P. strobi* were repeated using *L. nigrinus* collected from western hemlock and western white pine in northern Idaho (D.L. Mausel, unpub. data). As in Zilahi-Balogh et al. (2002), beetles fed and developed to the fourth instar on either host, but only completed development on HWA. Survival from the egg to 4th instar was significantly greater on HWA than on *P. strobi*.

Previous reports describing members of the genus *Laricobius* as adelgid specialists (Leschen [in press]) support the quarantine test results. Based on these data, *L. nigrinus* is host specific on the family Adelgidae and prefers HWA over other adelgids tested in this study. These results contributed to approvals for release of *L. nigrinus* from quarantine by APHIS, and for field releases by the U.S. Forest Service, U.S. Fish and Wildlife Service, National Park Service, and several eastern States.

FIELD EVALUATION

Field studies at high elevation sites in southwestern Virginia showed that *L. nigrinus* survived the winter, oviposited, fed and reduced HWA densities on eastern hemlock within mesh cages (Lamb et al. 2005, Lamb et al. 2006). Furthermore, interactions among *L. nigrinus*, *Sasajiscymnus tsugae* (see Chapter 4), and *Harmonia axyridis* did not decrease survival, feeding, or reproduction indicating that these species are complementary (Flowers et al. 2006). An open release of *L. nigrinus* adults on planted hemlock trees artificially infested with HWA established successfully, increased in abundance over three years, had a significant impact on HWA populations, and revealed a density-dependent relationship between the predator and prey (Mausel et al. 2008).

RELEASES MADE

The first *L. nigrinus* release utilized a strategy that liberated an estimated 10,344 eggs in early spring 2003 (Lamb et al. 2006). In fall 2003, an open release of 258 adults at a "field insectary" was conducted to produce field-acclimated beetles to supplement mass rearing operations (Mausel et al. 2008). From 2003-2005, a large-scale exploratory release of 9,225 adults at 22 sites was conducted to investigate the effect of climate, release size, and release season on establishment (Mausel et al. 2010). With increased mass-rearing productivity (see Chapter 11) and collections of adults in Seattle, WA, 102,069 adults were released at hundreds of sites in 14 States from 2003-2010 (see Chapter 18). From 2007 to 2010, 2,686 adults from Idaho and Montana were released in the northeast and mid-Atlantic States. Over 258,747 eggs have been released in the extreme southern range of eastern hemlock to further evaluate this release approach (M. Dalusky and L. Burgess, pers. comm.).

EVALUATION OF PROJECT OUTCOMES

Establishment and Spread

The first *L. nigrinus* egg release resulted in recovery of adults two years post-release (Lamb et al. 2006). The field insectary release successfully established as well and after three years, hundreds of adults were relocated to HWA-infested forests in Pennsylvania and Maryland (Mausel et al. 2008). The large-scale exploratory release study established 13 *L. nigrinus* populations from Tennessee to Pennsylvania and release size and minimum winter temperature were positively related with establishment (Mausel et al. 2010). A site in western North Carolina and western Maryland were sampled intensely from the 3rd to 7th year post-release and thousands of adult beetles have been collected and redistributed to nearby HWA-infested sites to enhance dispersal (D. McDonald and B. Thompson, pers. comm.). Program releases are considerably adding to the number of established sites and results can be accessed via an online database (see Chapter 18). According to the database at this time, *L. nigrinus* has been recovered in at least 11 states, from northern GA to coastal ME. A cold-tolerant biotype of *L. nigrinus* from Idaho and Montana was evaluated for release in cold-regions where beetles sourced from Victoria, BC had a low probability of establishment (Mausel et al. [in press]-b). Idaho and Montana beetle recoveries one or two years post-release have been made at 4 of 14 sites. One of these sites is in Vermont, 10 miles south of the most northerly known HWA infestation in the region (D.L. Mausel, unpub. data). As such, the entire invaded range of HWA has established but isolated *L. nigrinus* populations.

To determine the rate at which *L. nigrinus* disperse to the upper hemlock crown, vertical movement of adults was evaluated during the spring, for two years following beetle release, in Virginia and Pennsylvania (G. Davis, unpub. data). Beetles were capable of dispersing into the upper crown (>15 m) for oviposition and approximately 88% of the offspring recovered from the branch samples were located above 7 m. The proportion of second-generation larvae collected above 7 m was 98%. If monitoring for *L. nigrinus* is limited to the lower hemlock crown, its presence may be overlooked or underestimated. Horizontal movement of *L. nigrinus* was monitored for six generations at eight locations, one each in Tennessee and North Carolina, two in Virginia, and four in Pennsylvania. Each spring 16 hemlock trees were sampled at various distances from the release areas, with limits of 100 m for the first two generations and 300 m for third through sixth generations. Its rate of spread increased from 50 m/yr for the first generation to 75 m/yr by the fifth. Other observations suggest that *L. nigrinus* can disperse greater distances. For example, D. McDonald (pers. comm.) did not limit sampling distance and recovered *L. nigrinus* from at least 1.6 km from the release area, five years post-release. This equates to an approximate spread rate of 320 m/yr. By the fifth generation, *L. nigrinus* was recovered more frequently where densities of HWA were highest, regardless of distance from the release.

Non-target and Unanticipated Effects

Because *L. nigrinus* has recently established permanent populations in the eastern U.S., its impact on non-target organisms has not yet been comprehensively evaluated in the field. Informal monitoring has recovered *L. nigrinus* from *P. strobi*-infested eastern white pine (D. McDonald, pers. comm.), but it is unclear if they are reproducing on this host, as laboratory tests suggest they will not (Zilahi-Balogh et al. 2002). An unexpected risk from the release of *L. nigrinus*, is the recent field, lab, and DNA evidence that *L. nigrinus* and the native *L. rubidus* are very closely related, hybridize, and have the ability to produce viable offspring (Klein et al. [in press]). The effects of this interaction on *L. rubidus*, native adelgids, and the potential for biological control of HWA are unknown, but under careful study.

Suppression of HWA

In Virginia, a predator exclusion experiment detected a significant *L. nigrinus* impact on HWA in the field after a small release two years before (Mausel et al. 2008). During beetle dispersal studies, HWA density was reduced nearly seven-fold in the upper crown stratum, while *L. nigrinus* abundance increased 15-fold (G. Davis, unpub. data). The direct impact of *L. nigrinus* (F4-F7 larvae) on HWA was also evaluated at two paired release and control sites, one each in North Carolina and Pennsylvania. Release sites had a greater abundance of *Laricobius* spp. and reduced HWA survival compared with the control sites. *Laricobius* spp. densities at the release sites have reached levels observed in its native range. However, the ratio of *Laricobius* spp. to HWA remains much lower at the release sites than observed in their native range at this time.

Recovery of Hemlock

Hemlock canopy health (i.e., crown transparency) was assessed from trees that *L. nigrinus* was released upon at 12 paired release and control sites at the time of release and either five or seven years post-release (G. Davis, unpub. data). In addition, before and after photographs were taken of release and control trees. This assessment of *L. nigrinus* impact on tree health has detected the typical hemlock decline and mortality (Figs. 5 and 6). Crown transparency was similar between the release and control sites at the onset of the study and increased at a similar rate five to seven years later. Substantial evidence showing that the progression of hemlock decline has materially changed at sites where *L. nigrinus* has established has not been observed, to date. At beetle release and control stands in NY, MA, VT, NH, and ME, permanent plots were installed for long-term monitoring of *L. nigrinus* and HWA populations, hemlock health, and forest vegetation structure (D.L. Mausel, unpub. data).

RECOMMENDATIONS FOR FUTURE WORK

1. Collect *L. nigrinus* adults in the western US and screen for entomopathogens and parasitoids before field releases and redistribution from established eastern sites.
2. Continue to evaluate and test existing and new release strategies to improve program efficiency and effectiveness.
3. Initiate long-term monitoring of *L. nigrinus* and HWA populations, hemlock health, forest structure and vegetation, and non-target impacts on permanent plots in the mid-Atlantic and southern Appalachian States where results may differ from plots that have been established in the northeast.
4. Evaluate *L. nigrinus* impact on HWA and non-target organisms via rigorous predator exclusion and life table experiments.

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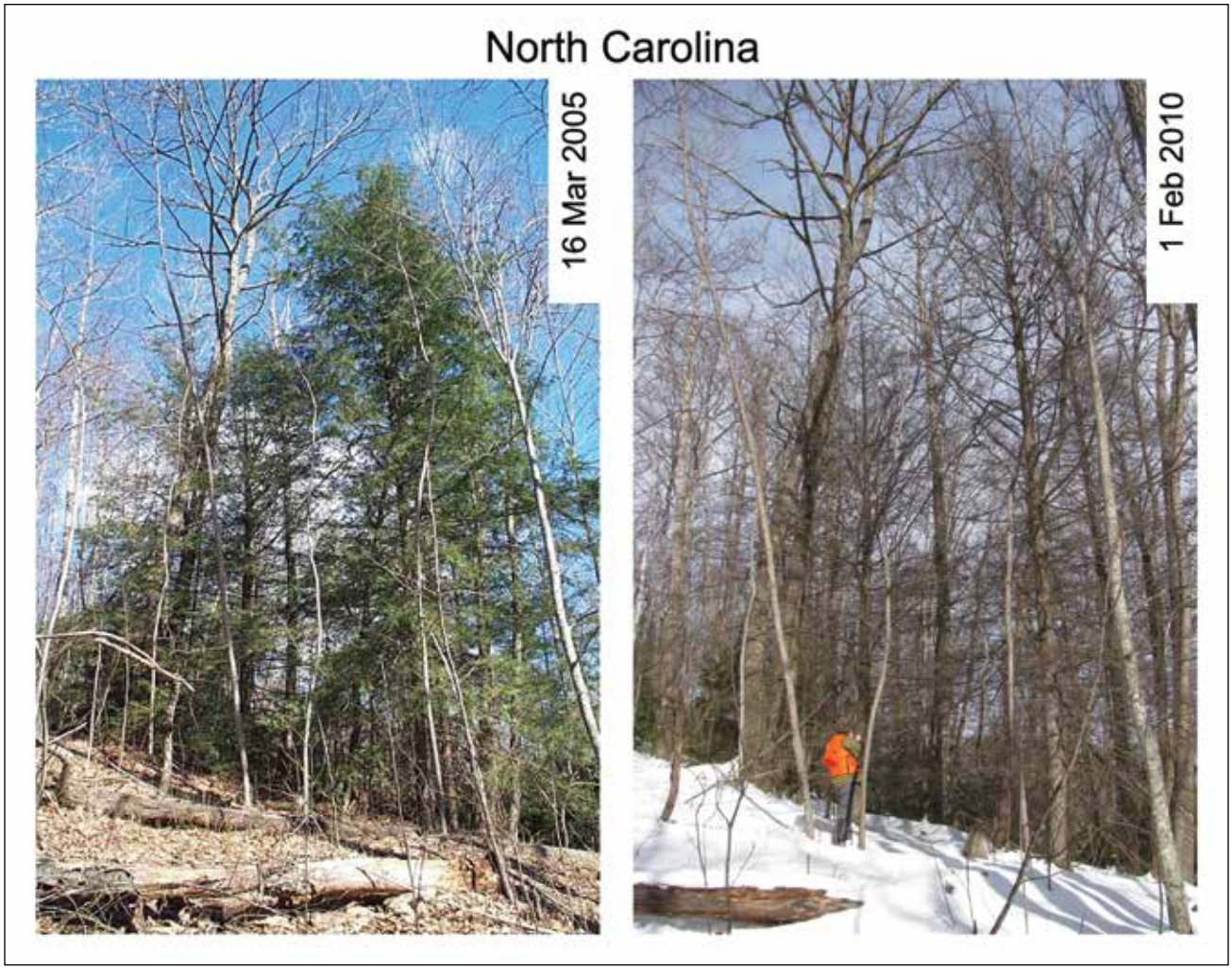

Figure 5. Relatively rapid eastern hemlock decline in the southern Appalachians presents a challenging scenario for classical biological control, as seen here in the Pisgah National Forest where 300 L. nigrinus were released on 12 Jan and 13 Mar 2005. The beetle established but was not able to prevent these trees from dying. The effect of the beetle on surviving trees at the site and future tree cohorts remains to be determined (photos by D.L. Mausel and G.A. Davis).

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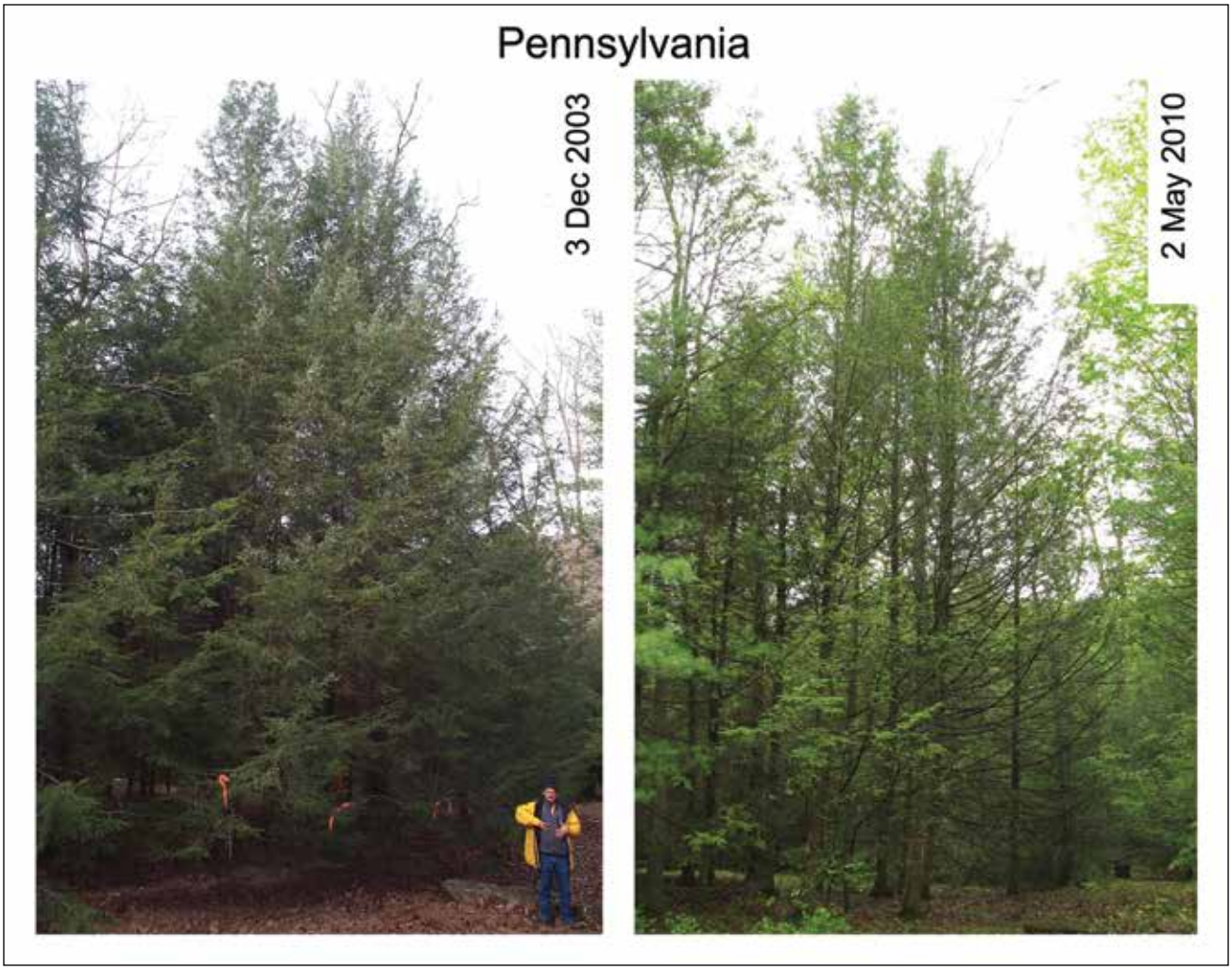

Figure 6. Typical eastern hemlock canopy decline and mortality is slower in the mid-Atlantic and northeast States and presents a less challenging scenario for classical biological control, as seen here in the Rothrock State Forest where 300 L. nigrinus were released on 4 Dec 2003 and 20 Apr 2004. The beetle established, yet tree decline does not appear to have reversed course, to date (photos by D.L. Mausel and G.A. Davis).

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Chapter 7: Laricobius osakensis, a Hemlock Woolly Adelgid Predator from Japan

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INTRODUCTION

The approach for the biological control of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, has been to release multiple species of host-specific predators in order to reduce HWA populations below damaging thresholds. Beetles in the genus *Laricobius* prey excusively on adelgids and have life histories matched closely to that of their prey (Lawrence and Hlavac 1979). Thus, this group has high potential for biological control of adelgids.

The genus has 21 recognized species—1 native to Europe, 3 native to North America, and 17 native to Asia (Leschen 2011). The European species, *L. erichsonii* Rosenhauer was released widely in North America for biological control of the balsam woolly adelgid, *A. piceae* (Ratzenburg), and although it initially established, it did not thrive and disappeared (Schooley et al. 1984). The only *Laricobius* species native to eastern United States, the target area for biological control of HWA, is *L. rubidus* Leconte, a predator of pine bark adelgid, *Pineus strobi* (Hartig). A western North American species and known predator of HWA, *L. nigrinus* Fender, has recently been introduced in the eastern United States (Mausel et al. 2010). Because of the presence of two congeners in the eastern United States, it is important that distinctions between the species and how they would interact is known prior to the introduction of another *Laricobius* species.

Recently, *Laricobius osakensis* Shiyake and Montgomery was discovered in Japan (Shiyake et al. 2008, Montgomery et al. 2011). It is very promising for biological control because it comes from the same location as the source of the population of HWA in the eastern United States (Havill et al. 2006). Therefore, this species has evolved with the HWA strain affecting *T. canadensis*. This chapter will review information on its (1) identification, (2) the phenology and distribution of the predator in its native habitat and (3) experiments conducted in a quarantine laboratory to assess its host-specificity and compatibility with a native and introduced congeneric species.

IDENTIFICATION

Laricobius osakensis, like other members of the family Derodontidae, is a small beetle between 2-3 mm in length, elongate, dorsally convex and ventrally flattened, with a vestiture of fine hair, a head with large pores or canals and 11-segmented antennae with a 3 segment club, a narrow prothorax with esplanate sides, elytra seriate or striate, and an abdomen with five visible sternites. Like other members of the genus, it has strongly lobed tarsi, open procoxal cavities, and elyta with a scutellary striole and 10 rows of punctures. Distinguishing *L. osakensis* from the other two

Laricobius species now in the eastern United States can be difficult in the field with live specimens, but fairly easy with dead, mounted specimens in the laboratory. A detailed description of *L. osakensis* can be found in Montgomery et al. (2011).

Live adult *L. osakensis* have two color morphs—one has reddish elytrae with darker maculation on the lateral edge, similar to *L. rubidus* (Fig. 1A), whereas the other color form has nearly black (not piceous) elytrae, similar to *L. nigrinus* (Fig. 1B). When a group of beetles are sorted according to color and then dissected to confirm the sex by examination of their genitalia, about 90% of the reddish specimens were female, while about 80% of the dark-brown specimens were male. Following death, the reddish form becomes various shades of brown. The eggs are light yellow and about 50% larger than an HWA egg (Fig. 1C). The larvae are covered by white wax and usually feed inside the HWA woolly ovisac (Fig.1D).

There are several characteristics, visible under a good stereomicroscope at high power, that can be used to distinguish *L. osakensis* from the other *Laricobius* species already present in the eastern United States. Most Derodontidae have two small ocelli on the head, each mesal of the compound eyes. *Laricobius osakensis*, *L. taiwanensis* Yu and Montgomery, and *L. kangdingensis* (Zilahi-Balogh and Jelenik 2003) are exceptions to this rule and have no ocelli (Montgomery et al. 2011). *Laricobius rubidus* and *L. nigrinus* have small rudimentary ocelli. Careful

Figure 1. Laricobius osakensis life stages. (A) Adult female, (B) adult male, (C) two eggs beside dead adult HWA, and (D) fourth instar lava feeding on HWA eggs.

examination of the frons area of the head is the most reliable way to distinguish *L. osakensis* from *L. rubidus* and *L. nigrinus* (Fig. 2). The patterns of the deep pits and punctures on the head are variable and are not a sure way to separate these three species. There are small differences in the dimensions and shape of the pronotum of the three species, but these are difficult to discern (see Montgomery et al. 2011). If the genitalia are dissected and the specimen is a male, *L. osakensis* can readily be distinguished from the North American species by its narrow, acutely pointed parameres and slender median lobe (Fig. 3).

Based on PCR-based molecular diagnostics, *L. nigrinus* and *L. rubidus* are closely related sister species but *L. osakensis* is in a separate clad with other Asian species (Montgomery et al. 2011). Molecular information is not only a way to identify adults, but can be also used to identify larvae.

DISTRIBUTION

Laricobius osakensis has been collected in 14 prefectures on the Japanese islands of Honshu, Kyushu and Shikoku (Fig. 4) at elevations from 80-1850 m on both hemlock species native to Japan. The southern Japanese hemlock, *Tsuga sieboldii* (Carrière), grows naturally at elevations between 200-1500 m on the southern half of Honshu and the more southern islands. The northern Japanese hemlock *T. diversifolia* (Maxim.)

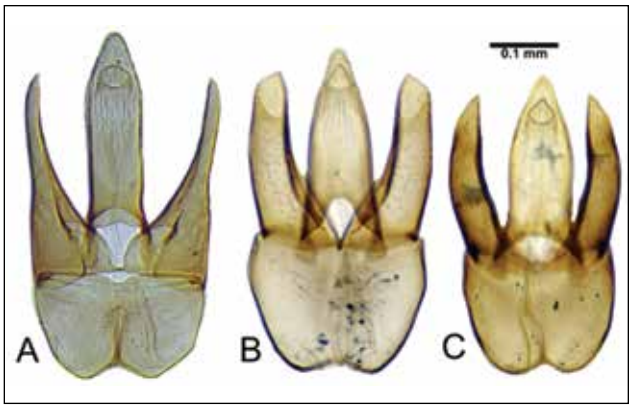

Figure 3. Sexual organs of Laricobius males: (A) Laricobius osakensis, (B) L. rubidus, and (C) L. nigrinus.

Masters grows at elevations from 700-2100 m, mostly in the more northern areas of Honshu Island. Both hemlocks are grown as ornamental or landscape trees, and HWA and the beetle are more common in landscape plantings or semi-natural settings compared to forest sites.

PHENOLOGY

The seasonal history of *L. osakensis* and HWA was monitored in the Kansai area of Japan to predict its ability to thrive in the eastern United States. HWA-infested *T. sieboldii* trees in 3 prefectures were sampled weekly for 2 years. At each sample period, beat sheet samples were conducted at each

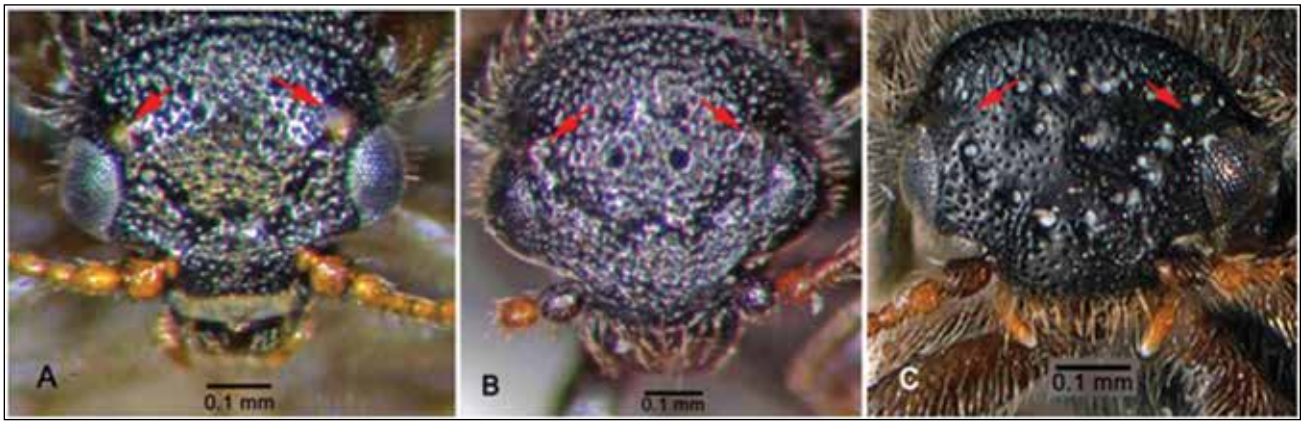

Figure 2. (A) Laricobius rubidus and (B) L. nigrinus with arrows pointing to ocelli; (C) L. osakensis with arrows showing where ocelli are absent.

Figure 4. Map of Japan indicating the prefectures where Adelges tsugae has been collected (yellow shading) and locations where Laricobius osakensis has been collected (red dots).

tree to determine the presence of adult predators. In addition, branch samples were removed from the trees and examined microscopically for immature stages of the predator and to determine the stages of HWA. Both species were present and active on the hemlock foliage during all months except for June through October (Fig. 5). *L. osakensis* adults first appeared on the trees in the Kansai region in mid-November, approximately one week after HWA had resumed feeding. These adults remained on the trees throughout the winter and early spring. In late December, adult *L. osakensis* began laying eggs in the woolly masses, in synchrony with HWA oviposition. *L. osakensis* continued to lay eggs throughout the winter and early spring. *L. osakensis* eggs hatched from January to April and larvae fed on HWA eggs. After developing through four instars, the larvae dropped from the hemlock branches in the early spring and pupated in the soil. *L. osakensis* aestivate as adults from May through October in the Kansai region. Phenology of *L. osakensis* is synchronized with the winter generation of HWA on *T. sieboldii* in Japan. Both species are present and active from

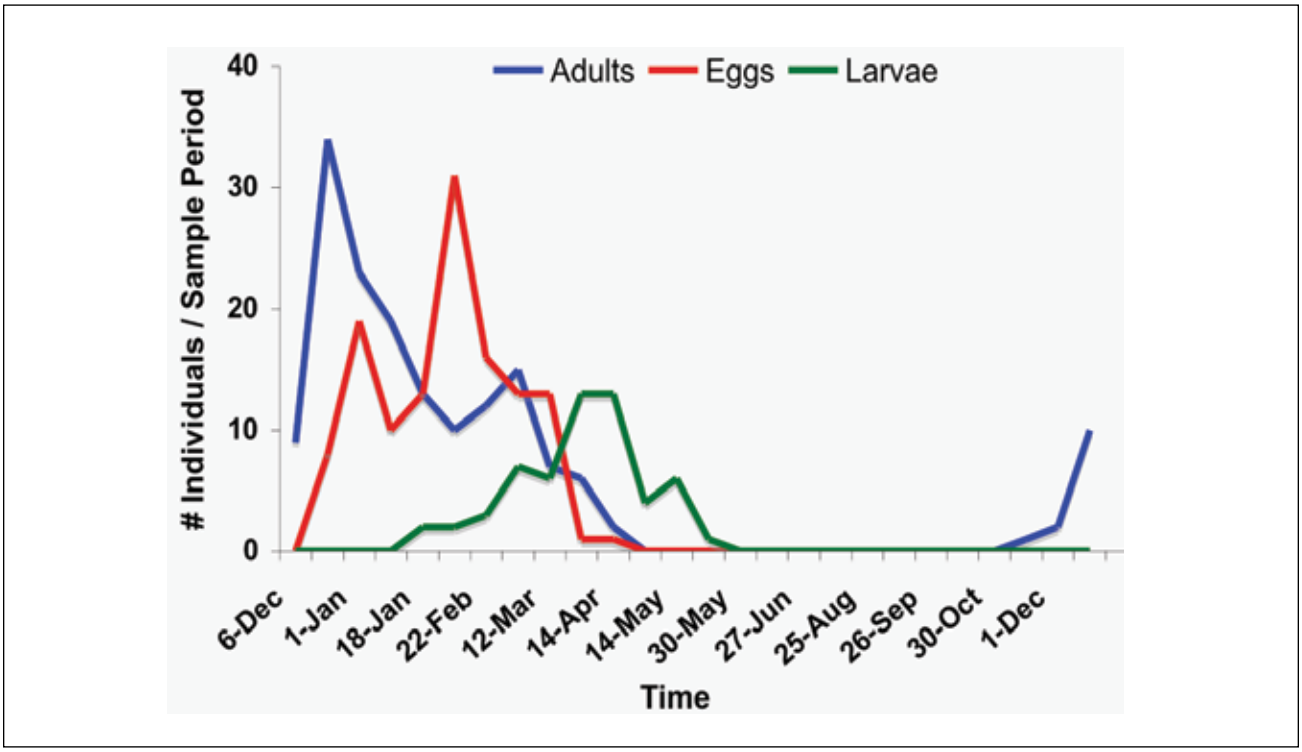

Figure 5. The number of Laricobius osakensis adults (blue), eggs (red), and larvae (green) found at each sample period on Tsuga sieboldii in the Kansai region of Japan.

November through May, feeding and ovipositing at the same time. In addition, both species undergo a dormant period throughout the summer months.

The genotype of HWA in this area is identical to the genotype of HWA in the eastern United States (Havill et al. 2006). The seasonal temperatures of the study area in Japan are comparable to the temperatures of potential release areas in North America (Fig. 6). In the Kansai region of Japan, *L. osakensis* adults became active in mid-November, a little later than expected based on emergence in British Columbia (Zilahi-Balogh et al. 2003) and emergence of *L. nigrinus* and *L. rubidus* in Virginia (Mausel et al. 2008). The delay in emergence of *L. osakensis* in Japan is likely because HWA on *T. sieboldii* in Japan breaks dormancy slightly later, probably due to the extended summer at low latitudes in Japan.

Phenology of *L. osakensis* on *T. diversifolia* at higher altitudes has not been studied in detail. Adults have been found on *T. diversifolia* in late October and early May. Trees were extensively sampled in early April, but adults were not collected. Since temperatures are very low and a large amount of snow falls in these high mountain areas, *L. osakensis* may not be active throughout the winter. Although more sampling is needed to determine the phenology of *L. osakensis* in colder regions, it's clear that it can survive in areas with average winter minimum temperatures below -20° C.

IMPACT ON HWA IN JAPAN

Since *Tsuga* species in Japan do not appear to be injured by HWA, the impact of predatory species on HWA populations were of interest. A predator exclusion study was conducted at 2 sites in the Kansai area of Japan. At one site, 48 HWA-infested branches were chosen and adult predators were removed by beat-sheeting. Half of the branches were caged from January to April. In April, 2 branch samples (10 cm in length) were removed from each branch and the density of

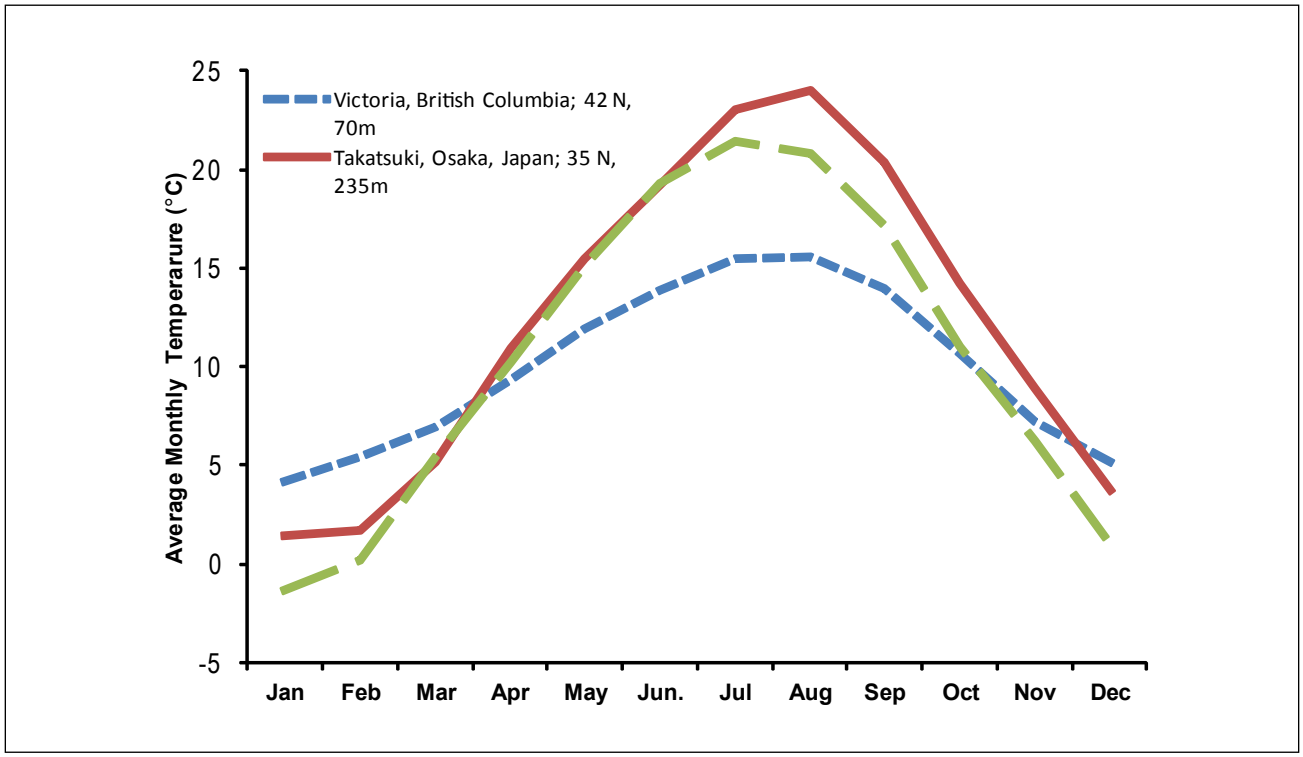

Figure 6. Average monthly temperatures (°C) of seasonal phenology study areas of Laricobius nigrinus in British Columbia and L. osakensis in Japan compared to a representative, prospective release area for both species in the eastern United States.

HWA was determined. On the caged branches (not exposed to predators), approximately half the HWA were alive and produced ovisacs, whereas only 5% of the adelgids were alive on the open branches. Thirty branches were selected at the second site. Half of these branches were caged after predators were removed by beat-sheeting. The cages were left on the branches from May until the following June. The branches that were caged for the 14-month period were heavily infested with adelgids (>3/cm), whereas branches left exposed had almost no adelgids (<0.1/cm).

Additionally, uncaged branches were sampled for predators throughout the year. *Laricobius osakensis* was the primary predator during the winter months at both sites. During the spring and summer, many generalist predators were present on the trees (Coccinellidae, Cantharidae, Elateridae, Syrphidae, Reduviidae, and others) (Shiyake et al. 2008). One Coccinellid species of particular interest found in these samples was *Sasajiscymnus tsugae* (Sasaji and McClure), a predator that has been released extensively in eastern North America since 1997 (Cheah et al. 2004). *S. tsugae* was collected fairly consistently on several, but not all, of the study trees during May and June. This beetle was not consistently present throughout the other months of the year.

The exclosure experiment suggests that the predators in Japan contribute to population control of HWA in the Kansai region. The sampling for predators indicates that *L. osakensis* is the primary predator during the winter and early spring. In late spring and early summer, generalist predators can be abundant, especially on hemlock with dense HWA infestations.

EVALUATION OF HOST RANGE AND DEVELOPMENT IN THE LABORATORY

Development tests were conducted by placing *L. osakensis* eggs on hemlock with HWA or on an alternate host. Larvae were observed for survival and maximum developmental stage reached. Choice and no-choice tests were set up in petri dishes and the amount of host consumed and number of eggs laid on each host were determined. The alternate hosts used were: balsam woolly adelid (BWA), pine bark adelgid (PBA), eastern spruce gall adelgid (ESGA), *Adelges abietis* (Linneaus), woolly alder aphid (WAA), *Paraprociphilus tessellates* (Fitch), elongate hemlock scale (EHS), *Fiorina externa* Farris, and pine needle scale (PNS), *Chionaspis pinifoliae* (Fitch). In choice tests, single adults were put in petri dishes containing a known number of HWA and an alternate host. The amount of prey consumed and number of eggs laid on each were counted after 7 days. In the no-choice tests, single adults were put in petri dishes with a known number of HWA or alternate host and the number of host consumed and eggs laid were determined after 5 days.

Laricobius osakensis consumed more HWA and laid more eggs on HWA than on any other host in both the choice and no-choice tests. In addition, these predators were only able to complete development to adults on HWA. They were only able develop up to 4th instar larvae on the adelgid alternate hosts and no development occurred on the non-adelgid alternate hosts. The host range tests indicate that this predator is host-specific on HWA and does not develop on other species tested (Vieira et al. 2011).

CURRENT STATUS IN UNITED STATES

These data suggest this predator poses no risk to native fauna within the eastern United States. This information was provided in the petition to release *L. osakensis* from quarantine. In May 2010, this predator was issued a FONSI (Finding Of No Significant Impact) and is no longer required to be in quarantine. In October 2010, a large field collection of *L. osakensis* was brought to Virginia Tech for mass rearing. The University of Tennessee is also rearing a small colony. Further research will be conducted at Virginia Tech and based on the results, *L. osakensis* will be tested in the field. If field tests are favorable and large numbers can be reared, field releases of this beetle will begin. Given what we know about its life history, *L. osakensis* has the potential to contribute to biological control of HWA in the future.

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Chapter 8: Chamaemyiid Predators of the Hemlock Woolly Adelgid from the Pacific Northwest

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The family Chamaemyiidae (Diptera), known colloquially as "silver flies" due to the silvery-gray adult body color of most species, includes two subfamilies, Cremifaniinae and Chamaemyiinae, with 28 described genera and subgenera, and over 330 described species worldwide, over 1/3 of which are in the single subgenus *Leucopis* (*Leucopis*) Meigen. The family has a cosmopolitan distribution, but more than half the described species are Palearctic, at least in part. Both subfamilies occur in the Nearctic region, with only 10 of the genera present, and fewer than 100 described species, although three new genera are currently being described by the second author, and many new species are known. The taxonomy and systematics of the family, particularly the genus *Leucopis*, has been challenging due to their small size (1-4 mm) and similar appearances, with a lack of clear, external morphological characters to distinguish species in most genera (Bennett 1961; Brown and Clark 1956a; Gaimari and Turner 1996a; Greathead 1995; McAlpine 1960, 1971, 1977, 1987; McAlpine and Tanasijtshuk 1972; Sluss and Foote 1971, 1973). Consequently, most species can only be determined by examination of the male genitalia, and females are usually not possible to identify without close association with males. Identification resources are most complete for the Palearctic region, where the fauna has been extensively studied (Tanasijtshuk 1986), and Australia, where Tanasijtshuk (1996) has made a first pass at describing the fauna. Also, several new species of Nearctic *Leucopis* were described by Tanasijtshuk (2003, 2005, 2006). At the genus level, several regional keys are available, including for the Nearctic region (McAlpine 1987), the whole New World (Gaimari 2010), and the Palearctic region (McLean 1998), although for the latter Cremifaniinae was treated as a separate family (Papp 1998).

Larvae are known to attack members of all superfamilies of sternorrhynchous Hemiptera except Aleyrodoidea, but most commonly aphids, adelgids, scales, and mealybugs. As a group, they display a spectrum of feeding strategies from active predation within free-living prey colonies to completely sessile predation on eggs within a single coccoid egg sac. Gaimari (2010) provided a review of the known feeding habits and general biology for the family, which includes the following generalities about the feeding habits for certain genera. These include: mealybugs in leaf sheaths of grasses by species of *Chamaemyia* Meigen (Tanasijtshuk 1970, Sluss and Foote 1973, Raspi 1983), *Parochthiphila* Czerny (Tanasijtshuk 1963, 1968; Raspi 1983), and *Pseudodinia* Coquillett (Barber 1985); adelgids on gymnosperms by most species of *Neoleucopis* Malloch (McAlpine 1971, Sluss and Foote 1973, Gaimari et al. 2007), *Anchioleucopis* Tanasijthuk (Tanasijtshuk 1997, 2001), and *Cremifania* Czerny (Delucchi and Pschorn-Walcher 1954, Clark and Brown 1962); scales and mealybugs on dicots

by species of *Melaleucopis* Sabrosky (Beingolea 1957) and *Leucopis* (*Leucopella*) Malloch (Malloch 1927, James 1934, Gaimari and Raspi 2002); on eggs within ovisacs of coccoids by species of *Echinoleucopis* Gaimari and Tanasijtshuk (Griot 1954, Gaimari and Tanasijtshuk 2001) and *Leucopomyia* Malloch (Malloch 1922, Tanasijtshuk 1959, Babaev and Tanasijtshuk 1971, Kaydan et al. 2006). Other genera have less specialized feeding habits, including *Leucopina* Malloch which feed on the whole variety of coccoid hosts, and *Leucopis* (*Leucopis*) which feed on the entire range of available prey. Tanasijtshuk (1986) also provides considerable biological data, with host records for many of the Palearctic species.

As a result of their specialization as predators of Sternorrhyncha, they have been studied as natural controls on herbivore populations and as biological control agents of pest insects in those groups. Biological control efforts against adelgid pests are detailed in this paper, but chamaemyiids have also been utilized against Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae) in the Pacific Northwest (Gaimari and Turner 1996b, 1996c, 1997; Noma et al. 2005), and one species, *Leucopis verticalis* Malloch, was recognized as an important predator in the galls of grape phylloxera *Daktulosphaeira* (as *Phylloxera*) *vitifoliae* (Fitch) (Stevenson 1967).

Although the biology and ecology of most adelgid-feeding chamaemyiid species are poorly known, some generalizations can be drawn from those that have been studied (Amman and Spears 1971; Brown and Clark 1956b, 1957; Clark and Brown 1957, 1962; Delucchi and Pschorn-Walcher 1954; Eichhorn 1968; Greathead 1995; Grubin 2011; Kohler 2007; Kohler et al. 2008a; McAlpine 1971, 1978; McAlpine and Tanasijtshuk 1972; Mitchell 1962; Sluss and Foote 1973; Smith 1958; Smith and Coppel 1957; Tanasijtshuk 1986, 2001, 2002; Wilson 1938). Most species appear to have 1-3 generations per year that are synchronized with the seasonal life history of their prey species. Larvae feed on eggs, nymphs, and adults of adelgid hosts with many species capable of feeding and developing on several species of adelgids. Often, pupariation and oviposition occurs near larval feeding sites, and the puparia are firmly attached to host trees. Overwintering is in the larval and, more commonly, puparial stages, and diapause may occur during the summer or winter.

The potential value of chamaemyiids as biological control agents for adelgids has been recognized for a long time. Following the seminal work of Trägårdh (1931), detailing the life history and effectiveness of *Neoleucopis obscura* (Haliday) against *Adelges picea* (Ratzeburg) in Europe, this species became the first chamaemyiid introduced to control the exotic adelgid in North America in the 1930's. In another case, following the accidental introduction of *Pineus pini* (Macquart) into Australia, Wilson studied the natural enemies of *P. pini* and *Pineus strobi* Hartig in England (Wilson 1938). He identified *Neoleucopis obscura* as the most efficient predator of these species and suggested that it "…should be introduced [into Australia] at the earliest possible moment." Subsequently, a number of chamaemyiid species have been considered for biological control of *Pineus* Shimer and *Adelges* Vallot species throughout the world with varying degrees of success (Greathead 1995, Mills 1990, Zilahi-Balogh et al. 2002a).

At least seven species of chamaemyiids were studied as biological control agents for balsam woolly adelgid, *Adelges piceae*, in North America, although the exact number released is unknown because of poor knowledge of the taxonomy of the group (McAlpine 1971, 1978). Chamaemyiid species were introduced into eastern Canada (Balch et al. 1956; Brown and Clark 1956a, 1956b, 1957; McAlpine 1978; Smith 1958; Smith and Coppel 1957), North Carolina (Amman and Spears 1971), Oregon and Washington (Mitchell and Wright 1967), and British Columbia (Harris and Dawson 1979, Humble 1994, McAlpine 1978). With the exception of North Carolina, one or two of the following four species became established in each geographic area: *Cremifania nigrocellulata* Czerny, *Neoleucopis obscura*, *Neoleucopis atratula* (Ratzeburg), and *Leucopis hennigrata* McAlpine (Balch et al. 1956; Brown and Clark 1956b, 1957; McAlpine 1971, 1978; Mitchell

and Wright 1967; Humble 1994; Schooley et al. 1984). Although none of these species significantly reduced *A. piceae* populations or associated damage to host trees, several developed large populations and dispersed rapidly. *Neoleucopis obscura* reportedly dispersed an average of 6 miles per generation in eastern Canada (Brown and Clark 1957), and within 14 years had spread throughout the infested areas of the Maritime Provinces (Brown 1947, Balch et al. 1956) and into Maine (Thomas 1968), and was recovered about one mile from a release point in the Pacific Northwest (Mitchell and Wright 1967). *Cremifania nigrocellulata* spread 2-3 miles from release points in 3-5 years, and was found to begin feeding slightly earlier than *Neoleucopis obscura* (Balch et al. 1956). Collectively, the inability of these chamaemyiid species to significantly impact *A. piceae* populations was attributed to feeding too late in the host developmental period, limited searching ability of larvae, slow rate of population increase relative to the prey population, higher rate of overwintering mortality than the prey, inability to persist in light infestations, and/or lack of seasonal synchrony of predator and prey populations.

Although less well documented, some other attempts to use chamaemyiids for biological control of adelgids have apparently been more successful than those with *A. piceae*. In Hawaii, there was a strong correlation between densities of *Neoleucopis tapiae* (Blanchard) (originally reported as *Leucopis obscura*) and an introduced adelgid, *Pineus pini* over a two-year period 3-5 years after introduction of the predator (Culliney et al. 1988, Greathead 1995). In New Zealand, *Neoleucopis tapiae* (originally reported to be *Leucopis obscura*) was responsible for the control of *Pineus boerneri* Annand on *Pinus radiata* D. Don (Rawlings 1958, Zondag and Nuttall 1989). And, in Chile, *Neoleucopis obscura* (which may also be *Neoleucopis tapiae*; see Greathead 1995) provided effective control of *P. boerneri* (Francke-Grosmann 1963; Zúñiga 1985). In all three of these cases, the target *Pineus* species feed primarily on twigs near the base of needles, unlike *A. piceae* which forms dense colonies on the main bole of the host trees. It may be that chamaemyiids are more important in the population dynamics of twig feeding adelgids than those that feed on the bole. However, attempts in the 1970's to introduce *Neoleucopis tapiae*, *N. nigraluna* McAlpine and *Leucopis* spp. into Kenya for control of the twig feeding *Pineus boerneri* on *P. radiata* and *Pinus patula* Schiede ex Schltdl. & Cham. were unsuccessful (Greathead 1995, Day et al. 2003). In this case, Greathead (1995) speculated that the numbers of insects released may have been too small to ensure establishment. Day et al. (2003) also reported that two other species, *Neoleucopis manii* Tanasijtshuk and *Leucopis argenticollis* Zetterstedt, were imported into Kenya but never released, although they may have been released in Tanzania along with the species released in Kenya.

The predator community associated with hemlock woolly adelgid, *Adelges tsugae* Annand, infested western hemlock, *Tsuga heterophylla* (Raf.) Sarg., in Oregon and Washington was studied by Kohler et al. (2008a). In surveys of 116 infested trees at 16 sites from January 2005 to November 2006, chamaemyiids were the second most abundant group of predators to the Derodontidae (Coleoptera), predominantly *Laricobius nigrinus* Fender. In total, 1,039 adult and immature *Leucopis* spp. were collected compared to 2,723 derodontids and 531 Hemerobiidae (Neuroptera), the third most abundant group. Since we do not know the efficiency of beat sampling used to collect these specimens, we do not know whether this represents the true differences in abundance among these predator species. However, the ratio of immatures to adults was over three times higher for the chamaemyiids (9.2) compared to the derodontids (2.6) or hemerobiids (3.1) suggesting that beat sampling was less efficient at collecting adult chamaemyiids, and that chamaemyiids are more abundant relative to the other predators than indicated by the counts from beat samples.

Two species of *Leucopis* were collected in samples from *A. tsugae*-infested western hemlock, *L. argenticollis* and *L. piniperda* Malloch (as *L. atrifacies* Aldrich) (Kohler et al. 2008a). Unfortunately, the misidentification as *L. atrifacies* by S.D. Gaimari was not recognized until he began to study the more extensive materials collected by

Grubin (2011). This was the first record of either *L. argenticollis* or *L. piniperda* associated with *A. tsugae*. Eighty-seven percent of the chamaemyiids that were collected as adults or reared to the adult stage were *L. argenticollis*, which is a Holarctic species previously found in colonies of several *Pineus* species in Russia, India, Japan, Canada, and the United States (McAlpine and Tanasijtshuk 1972). *Leucopis piniperda* is widespread in North America, and has been collected from colonies of several species of *Adelges* and *Pineus* (Tanasijtshuk 2002). Although never collected associated with *A. tsugae*, *Leucopis atrifacies* is restricted to western North America where it has been found associated with several species of *Pineus* and *Adelges* (Greathead 1995, Tanasijtshuk 2002). Following the introduction of *A. piceae* into North America, *L. argenticiollis* was found associated with *A. piceae* in eastern Canada (McAlpine and Tanasjitshuk 1972), and both *L. atrifacies* and *L. piniperda* were found associated with *A. piceae* in western North America (Tanasjitshuk 2002). This indicates that all three species are capable of searching for, locating, and potentially utilizing novel adelgid prey species within their native habitats.

Since Kohler et al. (2008a) were unable to distinguish *L. argenticollis* and *L. piniperda* larvae, it was not possible to record separate observations of the biology for each species except in the cases where they were reared to the adult stage. There were two peaks in larval abundance, one in the spring and one in the early summer coinciding with the two periods of *A. tsugae* oviposition, suggesting that these *Leucopis* spp. have two generations that are synchronized with the *A. tsugae* life cycle. *Leucopis* spp. larvae that were brought to the lab fed on eggs and nymphs of the progrediens and sistens generations of *A. tsugae*. Puparia were always firmly attached to the twigs near the larval feeding sites. Immature *L. argenticollis* that were collected in November 2005 and held on *A. tsugae* infested twigs in an environmental chamber simulating outdoor conditions did not emerge as adults for four months. Larvae of both *Leucopis* spp. that pupariated in the spring emerged as adults within 4 weeks.

Nonparametric analyses of insect community structure demonstrated a strong positive correlation between both *Leucopis* spp. larvae and *L. argenticollis* adult abundance and *A. tsugae* population density (Kohler et al. 2008a, 2008b). Among the nine predator taxa that were positively correlated to *A. tsugae* population density, the strength of the correlation with *Leucopis* spp. larvae and *L. argenticollis* adults was similar to or greater than that of all the others including *Laricobius nigrinus* adults and *Laricobius* spp. larvae. However, these data do not distinguish whether the *Leucopis* spp. are regulating *A. tsugae* populations or simply reaching high densities where their prey are abundant. Further studies will be needed to elucidate the nature of the ecological relationships among *Leucopis* spp. and *A. tsugae* populations.

In 2009 and 2010, several no-choice feeding trials were conducted with *Leucopis* spp. larvae collected in Oregon and Washington from *A. tsugae*-infested western hemlock to determine the suitability of alternative adelgid prey (Grubin 2011). Survival was always higher on *A. tsugae* than other species of *Pineus* and *Adelges*, although the differences were not always statistically significant. Also, some *Leucopis* spp. survived to the adult stage on each of the four alternative adelgid prey species. Thirty-eight of the *Leucopis* spp. larvae survived to the adult stage and were identified to species. Seventy-one percent were *L. argenticollis* and the remaining 29% were *Leucopis piniperda*. It is worth noting that only 15% of the larvae used in the feeding trials survived to the adult stage.

Although species of native and introduced chamaemyiids are found in eastern North America, none have been shown to significantly impact *A. tsugae* populations (Montgomery and Lyon 1996, Wallace and Hain 2000). The two species of *Leucopis* found associated with *A. tsugae* in the Pacific Northwest, *L. argenticollis* and *L. piniperda*, also occur in eastern North America (McAlpine and Tanasijtshuk 1972, Tanasijtshuk 2002). However, populations of these predators in such widely separated geographic regions with different native hosts (e.g., *A. tsugae* is native to the West,

but introduced in the East; Havill et al. 2006) are likely distinct. Consequently, the introduction of individuals from Pacific Northwest populations of these *Leucopis* spp. to the eastern United States may be beneficial to the ongoing biological control efforts for *A. tsugae*. Furthermore, the wide geographic range of these species suggests that individuals from the West would be able to adapt quickly to environments in the East (Mills 1990).

Prior to the introduction of non-native chamaemyiids in the Pacific Northwest for biological control, Mitchell (1962) found three unidentified, native "*Leucopis*" spp. (one of which is *Neoleucopis ancilla* McAlpine (McAlpine 1971)) associated with *A. piceae*. All three of these species were generally found at low densities, but, at one site, one species (not *Neoleucopis ancilla*) was found in the "…heaviest predator population ever seen associated with the balsam woolly adelgid" (Mitchell 1962), although this species only seemed to have one generation per year. The presence of these native species in *A. piceae* colonies, in some cases at high densities, within 30 years of the discovery of this introduced species suggests that they have vagile host searching behaviors and wide diet breadth within the Adelgidae. Other records of the host ranges of adelgid-feeding chamaemyiids would suggest that these are common attributes among the group (McAlpine 1971, McAlpine and Tanasijtshuk 1972, Tanasijtshuk 2002). More recently, *Neoleucopis tapiae* were collected from both *A. piceae* on grand fir, *Abies grandis* (Douglas ex D. Don) Lindl., and *Pineus coloradensis* (Gillette) on western white pine, *Pinus monticola* Douglas ex D. Don, in western Oregon in 2007 (Kohler 2007, G.R. Kohler, unpublished data). The ability to utilize several adelgid species as prey would allow potential biological control agents to survive at times and in places where their primary host was unavailable.

There are many known parasitoids of Chamaemyiidae, particularly in the Chalcidoidea, where 26 species of 13 genera in five families (Aphelinidae, Encyrtidae, Eulophidae, Pteromalidae, Signiphoridae) have been reported (Noyes 2010), including at least nine species of the pteromalid genus *Pachyneuron* Walker. Several parasitioids have been reared from the adelgid-feeders, which could potentially interfere with their effectiveness as biological control agents. Wilson (1938) reared *Dendrocerus carpenteri* (Curtis) (Hymenoptera: Megaspilidae) (as *Lygocerus testaceimanus* Kieffer), *Amblynotus longitarsus* Reinhard (Hymenoptera: Cynipidae), and *Syrphophagus aphidivorus* (Mayr) (Hymenoptera: Encyrtidae) (as *Aphidencyrtus aphidivorus*) from puparia of *Neoleucopis obscura* collected in England. *Pachyneuron altiscutum* Howard (Hymenoptera: Pteromalidae) was reared from puparia of native and introduced chamaemyiid species in eastern Canada (Brown and Clark 1956b, 1957), with parasitism of *Neoleucopis obscura* by *P. altiscutum* at 21.3% across two generations. Puparia of the native species *Neoleucopis pinicola* (Malloch) were parasitized by *Pachyneuron virginicum* Girault in northeastern Ohio (Sluss and Foote 1973), and in eastern Canada by *P. altiscutum* (Brown and Clark 1956b) and *Melanips iowensis* Ashmead (Hymenoptera: Figitidae) (Clark and Brown 1957). In Oregon and Washington, Mitchell (1962) reared unidentified species of *Pachyneuron* and *Dendrocerus* Ratzeburg (as *Lygocerus* Förster) from *Neoleucopis ancilla* (as *Leucopis* sp. I) and one of the additional unidentified *Leucopis* species collected from *A. piceae* colonies, and Kohler et al. (2008b) reared two *Pachyneuron* spp. and a species of *Melanips* Giraud from *Leucopis* spp. puparia collected from *A. tsugae*-infested western hemlock. In the latter study, the parasitism rate was 21-23% over two years. If *Leucopis* spp. from the Pacific Northwest are ever introduced in the eastern United States, care should be taken to avoid introducing associated parasitoids by releasing only adults or immatures reared in parasitoid-free colonies. Furthermore, any Pacific Northwest *Leucopis* spp. populations that become established in the eastern United States should be monitored to assess parasitism rates by indigenous parasitoids.

Successful biological control of *A. tsugae* in the eastern United States will likely require a suite of predators (Montgomery and Lyon 1996, Cheah et al. 2004). In addition to the predators that have already been released, including *Laricobius nigrinus*

and two Coccinellidae (Coleoptera), *Sasajiscymnus tsugae* (Sasaji and McClure) and *Scymnus sinuanodulus* Yu and Yao, it is likely that other candidate biological control agents will need to be identified, tested, and released to achieve ecologically and economically significant reductions of *A. tsugae* populations. Since *Leucopis* spp. found in the Pacific Northwest feed on both progrediens and sistens eggs and nymphs, they will likely compliment *Laricobius nigrinus* imported from the same region which feeds only on progrediens eggs and nymphs (Kohler et al. 2008a, Zilahi-Balogh et al. 2002b). However, species of *Scymnus* Kugelann may prey on *Leucopis* spp. larvae and reduce their effectiveness (Mills 1990). Given the widespread occurrence and abundance of *Leucopis* spp. associated with *A. tsugae* in the Pacific Northwest, they should be studied in more detail to understand their roles in the population dynamics of *A. tsugae* in the West as well as their potential for biological control in the East. In particular, it would be valuable to develop rearing techniques to facilitate studies of the biology and ecology of each of the species that have been found in association with *A. tsugae*.

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Chapter 9: Insect-Killing Fungi for HWA Management: Current Status

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ECOLOGICAL CONSIDERATIONS

Fungi play an active and diverse role in forest and other ecosystems. Some are decomposers, breaking down and recycling formerly living material so that the nutrients are again available for plant growth. Some can cause serious diseases of living plants, while others are even cultivated by ants in subterranean gardens. A number of fungal species impact insect populations, sometimes causing major disease outbreaks (epizootics) that drastically reduce insect numbers. Insect-killing fungi, called entomopathogens, are found naturally in most terrestrial habitats.

The ultimate goal of a biological control program based on the use of insect-killing fungi is to have the fungus circulating within a target insect population and naturally achieving sufficient levels of pest suppression. Fungi and other pathogens, such as viruses and bacteria, are registered through EPA as biopesticides, but when living propagules are present, they may better reflect biological control approaches instead of traditional insecticides. Like introduction or augmentation strategies using predators or parasitoids, ideally, after several releases the biological agent should integrate and work to bring about a check to insect outbreaks that damage or kill their hosts. Additionally, like predators and parasitoids, insect-killing fungi often work best as members of a complex of agents effective upon different stages and densities of an insect lifecycle. What makes insect-killing fungi unique is that most are readily mass produced, and, when developed and registered as biopesticides, can be released in large amounts to obtain some degree of immediate pest suppression. This strategy in turn can benefit adoption of other biological measures that have longer lag times to build up to effective levels. This is particularly critical in the case of eastern and Carolina hemlocks that are rapidly overcome by explosive outbreaks of HWA populations soon after initial invasion.

The successful implementation of insect-killing fungi for HWA suppression requires close attention to biological properties and limitations of fungal agents, their interaction with the insect target and potential non-target hosts, and important influence of abiotic factors, particularly moisture, temperature and sunlight. Effectiveness of a management program could be seriously impaired by simple factors, such as targeting of an insect life stage less vulnerable to infection, the susceptibility of fungi applied midday to intense UV light (Fargues et al. 1996), or the lack of sufficient moisture for spore germination after application. Practical considerations include the need for sufficient amounts of fungus to come into direct contact with the target insect to initiate infection. In the case of HWA, this means having spray droplets penetrate through the canopy to deposit at the base of the needle underside where HWA are located when non-motile, or timing the application to coincide with emergence of HWA crawlers so that fungal/insect contact is achieved as the motile crawlers seek a suitable place to settle and continue development. As with most biological products, e.g., *Bacillus thuringiensis* and the nucleopolyhedrosis virus (Gypchek) for gypsy moth suppression, adherence to concrete application guidelines will be critical to fruitful implementation of fungi for HWA management.

FUNGAL DISEASE DYNAMICS

When viable fungal spores (conidia) come in contact with a susceptible insect host they adhere to its skin/cuticle and are stimulated to germinate (Fig. 1). The germinating spore sends out a growing tube that penetrates into the insect. It does this through a tremendous amount of force focused on the growing point, often using enzymes that digest a hole though the surface. Once the fungus enters the body cavity it rapidly proliferates as it overcomes insect immune responses. Within several days, depending on temperature, the insect dies, after which the fungus emerges from the cadaver to release large amounts of new spores that can go on to infect other insects. If enough susceptible insects come into contact with the spores under favorable environmental conditions, the prevalence of disease can spread rapidly, initiating an epizootic and subsequent collapse of the insect population.

Insect-killing fungi are less abundant in the environment between epizootic events, causing

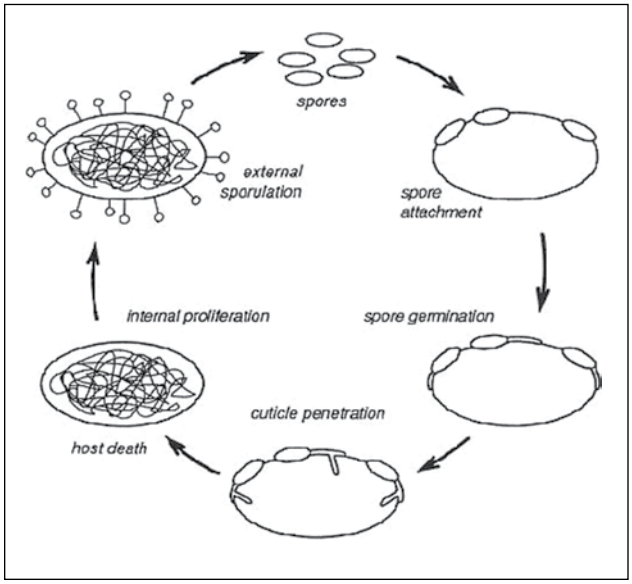

Figure 1. Generalized infection cycle of an insect-killing fungus. Spores (smaller circles) adhere to the surface of insect cuticle (larger circle) and then germinate. Germ tubes penetrate the insect and fill the body cavity, causing insect death. The fungus grows out of the dead insect and releases new spores to continue the infection cycle in new insects.

relatively low levels (enzootic) of insect infections. Reid (2003) found several insect-killing fungi, including *Beauveria bassiana*, *Paecilomyces farinosus*, and *Verticillium lecanii*, associated with field collected hemlock woolly adelgid populations. However, in examining over 6,100 cadavers, these 3 species of fungi were recovered from barely 3% of the specimens. An adequate level of fungal inoculum, i.e., the number of spores available for causing infection, is a requisite factor leading to the development of a fungal epizootic in insect populations. As the invasion front of HWA enters new hemlock stands, there appears to be insufficient time for the level of fungi to increase and suppress HWA before serious tree damage occurs. However, in some northern regions where lower winter temperatures periodically impact HWA population growth (Costa et al. 2005), fungi appear to be causing significant reductions in survival of HWA sistens during summer aestivation (Gouli et al. 2011; Costa, personal observations). Interestingly, after several years of conducting fungal trials at Mount Tom State Reservation, Holyoke, MA, high mortality in untreated controls made further use of this site unreliable for experimental applications of fungi (Costa, personal observation). Although insect-killing fungi are ubiquitous in most terrestrial environments, augmenting their prevalence in hemlock forests through inundative applications is a first step toward facilitating timely development of disease outbreaks. This can only be legally accomplished through the use of fungi registered as biopesticides by the U.S. Environmental Protection Agency that are labeled for the intended use.

In addition to a sufficient level and adequate distribution of fungi in the environment, there must be adequate moisture and temperature to initiate spore germination and fungal growth, with an insect that is susceptible to infection. A relative humidity between 85 and 100% is generally required for most species of fungi to germinate. In hemlock forests, these levels, if not apparent during daytime, can be achieved as temperature declines overnight (Fig. 2), which may also lead to extended periods of needle wetness that favor fungal germination. Optimal temperatures for insect-killing fungi

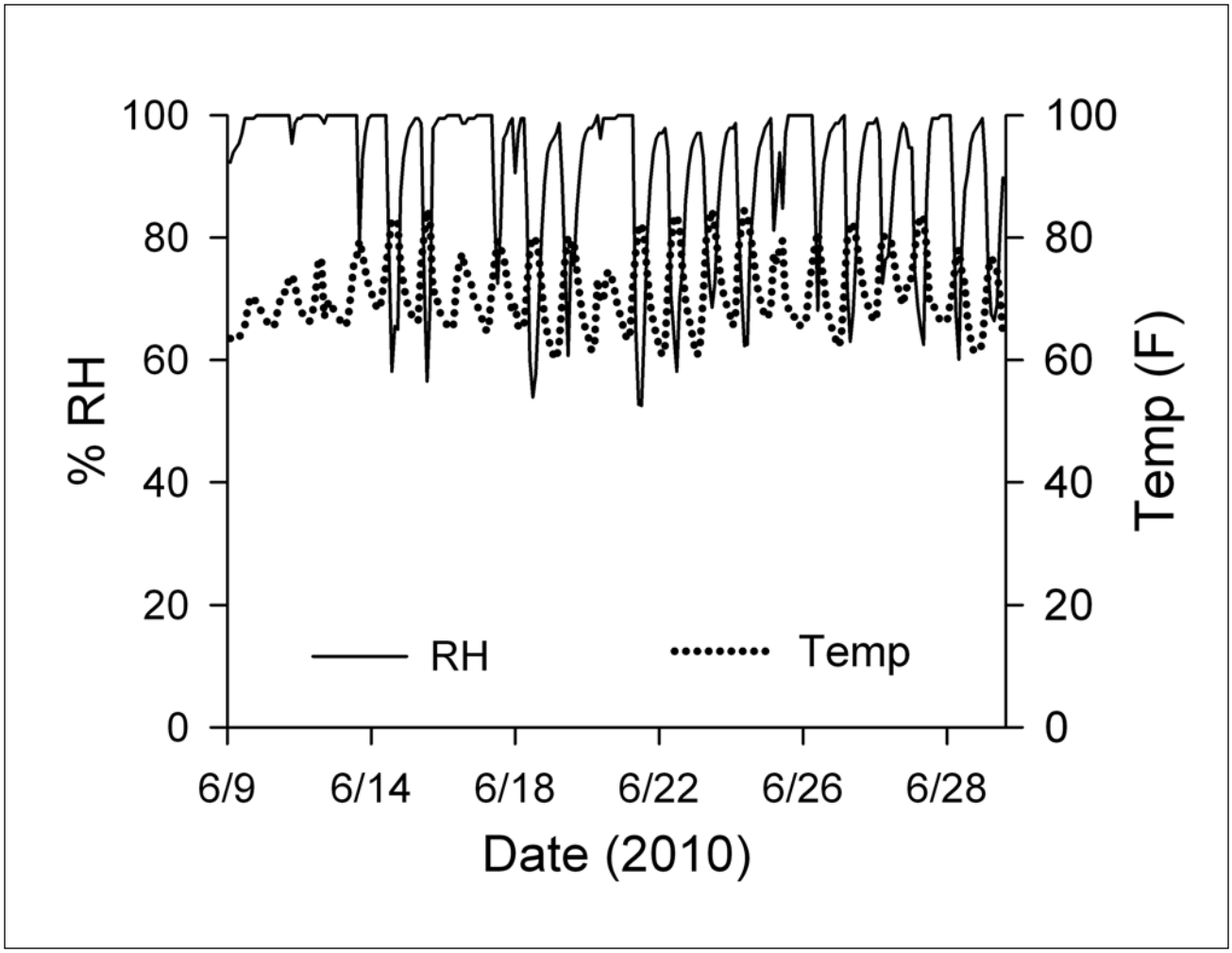

Figure 2. Temperature (dotted line) and relative humidity (RH – solid line) recorded in a hemlock forest at Tennessee Wildlife and Recreation Area, Titus Creek, during a critical period post fungal treatment (2010 Pilot Study). Note extended periods of high humidity and repeated increases in humidity as temperatures cycle lower.

generally range from 15-30 °C depending upon the species and isolate of fungus examined. Most fungi are intolerant of higher temperatures, which is a factor contributing to their general safety for warm blooded animals and humans (Vestergaard et al. 2003). Although several fungal isolates have been found to be active at lower temperatures (De Croos and Bidochka 1999; Fargues et al. 1997), temperature considerations and experience suggest that fungal applications against HWA should be directed against progrediens adults and their sistens offspring as temperatures warm in mid to late spring, but well before temperatures decline in fall.

FUNGAL BIOPESTICIDE DEVELOPMENT

While all insect-killing fungi by definition are considered pathogenic to at least some species of insects, there is variability in the susceptibility of different insect species to a particular fungus. Certain insect-killing fungi, such as *Beauveria bassiana*, have a broad spectrum of activity against insects, in that they kill many different kinds of insects from a variety of insect orders. Others have relatively narrow or even very restricted host ranges, only infecting a small number of closely related insect species, as is the case with *Entomophaga*

miamaiga, a pathogen that periodically devastates gypsy moth populations. Even within one species of fungus, a single isolate may be more virulent in that it kills insects at lower concentrations than other related isolates. In the laboratory, the virulence of fungi as biopesticides can be characterized similarly to chemical pesticides by determining LC_{50} and LD_{50} values against a target pest population. While initially valuable in earlier stages of research, the artificial conditions generally used in laboratory assays favor the fungus and give only a limited indication of how well it will perform under field conditions.

Considerable effort has been made not only to identify fungi for suppression of HWA, but also to develop operational formulations and evaluate application technology and timing for delivery into hemlock forests. In earlier stages, domestic and foreign explorations were carried out to identify potential candidates to target HWA (Gouli et al. 1997; Ried 2003). Later small scale field tests on single branches or small trees (<3 meters) found fungi in the genus now classified as *Lecanicillium* (formerly *Verticillium lecanii*) to hold the greatest promise (Reardon et al. 2004); strains of *Beauveria bassiana* and *Metarhizium anisopliae*, some commercially available in the United States, were ineffective. Field testing for non-target effects on adults of the predatory beetle *Sasajiscymnus tsugae* also found the *Lecanicillium* sp. (ARSEF 6010) to be a promising candidate, whereas *M. anisopliae* caused significant predator mortality (Reardon et al. 2004). Regretfully, ARSEF 6010 was an experimental isolate and did not possess any background research for environmental safety that would allow a US biopesticide registration without considerable expense and delay.

An alternative was found in Mycotal, a commercial formulation of *L. muscarium* registered in several European and Asian countries by Koppert Biological Systems (Netherlands). The biopesticide registration package for Mycotal has been recently updated for the European Union. This extensive package of environmental assessment, a previous history of release for testing in the United States, and documented isolation of the same species in hemlock forests in the Eastern United States made Mycotal a favorable candidate for importation and release. After careful review, permits were secured through USDA Plant Protection and Quarantine for importation and experimental release into hemlock forests. The availability of a full registration package also dramatically reduces the costs associated with obtaining a biopesticide registration within the United States, which is a small fraction of the cost for synthetic chemical pesticides. Hemlock being a riparian species with close proximity to forest streams makes the health and environmental safety of Mycotal particularly attractive for suppression of hemlock woolly adelgid.

Historically, fungal biopesticides are generally applied using high volumes of water with the intention of enhancing spray coverage and providing adequate moisture to allow spore germination. The use of high volumes of carrier is operationally unacceptable for aerial application in forest systems. Adoption of rotary atomizers (Micronair AU6539, Micron Sprayers Ltd, UK) to generate fine mists allowed dramatic reductions in spray volumes (10 liters (2.6 gallons)/acre) (Costa et al. 2011) and facilitated aerial (helicopter) spray penetration into the hemlock canopy, even onto the underside of hemlock needles (Reardon and Costa, unpublished data). At the same time, frequent occurrence of high levels of relative humidity within the hemlock forests supplanted the need to apply additional moisture to support fungal germination (for example, Figure 2). These factors contribute to the likelihood that Mycotal could be suitable for operational use in HWA suppression.

Insect-killing fungi as a tool for pest management has faced a major hurdle in delivering an efficacious amount of fungi over large acreage at a reasonable cost. As part of the program to deploy fungi for HWA management, an operational formulation was developed based on the incorporation of sweet whey, an inexpensive byproduct from cheese production. The whey additive in this fungal microfactory formulation (patent pending: MycoMax™ fungal enhancer) serves as a nutrient

to encourage the growth and reproduction of *L. muscarium* in spray deposits. The result is a dramatic increase (10- to100-fold) in the number of spores and a corresponding reduction in the amount of fungal biopesticide that needs to be applied (Grassano 2008). In a sense, the use of MycoMax™ fungal enhancer transfers a significant portion of fungal mass production out of the factory into the forest, with the potential for cost savings through reduction in biopesticide application rates.

PILOT STUDY EFFICACY TRAILS—DESIGN OVERVIEW

In 2009 and 2010, pilot studies using aerial applications of Mycotal into hemlock forests were conducted at the Tennessee Wildlife and Recreation Area, Titus Creek. An additional ground based hydraulic application (where individual trees were treated) was conducted in 2010 at Townsend State Park, VT. A complete description of these field trials will be published elsewhere. Without issuance of an EPA Experimental Use Permit, Federal regulation restricts acreage treated with non-registered pesticides to no more than 10 acres/year. In both years, replicated plots (1-1.25 acres) of mixed hemlock/hardwood forest were treated aerially (10 liters/acre) by Helicopter Applicators, Inc. using a Bell Jet-Ranger helicopter with a mounted AU6539 Micronair nozzle spray system. Targeting of treatments was accomplished with an on-board navigation system and ground-truthed plot locations. Two applications were made each year to enhance spray coverage; sampling of deposits within the hemlock forest indicated penetration of sprays through the canopy.

In 2009, treatments were made in the evening and the following morning and consisted of a no spray control, Mycotal alone (*L. muscarium* at 1×10^8 spores/ml), and Mycotal formulated with MycoMax™ fungal enhancer (5% w/v). The oil adjuvant Addit (0.25% v/v : Koppert Biological Systems) and the sticker Hyperactive (0.05% v/v: Helena Chemical) were also added to both fungal treatments. In 2010, the rate was quadrupled: the treatment using Mycotal alone was dropped, and applications were only made in the evening. In Vermont, only a single hydraulic application in evening was made (38 liters/tree at 1×10^7 spores/ml). Treatments are made later in the day to reduce effects of UV radiation on survival of fungal spores. Assessment of treatment effects were made for preselected individual trees (10/plot) by evaluating HWA population levels in the lower and mid canopy (5 samples/elevation) both before and after treatment. The timing of the sampling provides evaluation of the overall change in the population of HWA sistens from one year to the next so that suppression by fungal treatments was assessed relative to populations in controls. A similar criterion was used for the ground based trial at Townsend State Park, VT.

PILOT STUDY EFFICACY TRAILS—EFFICACY SUMMARY

Aerial application of Mycotal reduced hemlock woolly adelgid population growth. The 2009 pilot study found that after nearly a year, plots treated with the MycoMax enhanced fungal formulation had less than half the growth in population than untreated plots (Fig. 3); those treated with the standard Mycotal formulation were intermediate (Costa, 2011). A subsequent application at a higher rate to a portion of these plots the following year, along with appropriate controls, did not provide any additional benefit (Fig. 4). However, the data did suggest that while HWA populations remained higher in the controls over the 2 year period, in the treated plots population growth had been arrested. A separate pilot study in 2010 found more than 75% reduction in HWA population growth (Fig. 5). The actual population in control plots increased from an average of 8 HWA/branchlette to nearly 12, whereas with almost 9 HWA/branchlette in treated plots the increase was only 10%. In both years, these differences were statistically significant ($P \leq 0.05$) when trees were evaluated as experimental units, which is justified given the similarity in variance between trees and plots. This approach was taken to compensate for variability associated with density-dependant effects on HWA population levels.

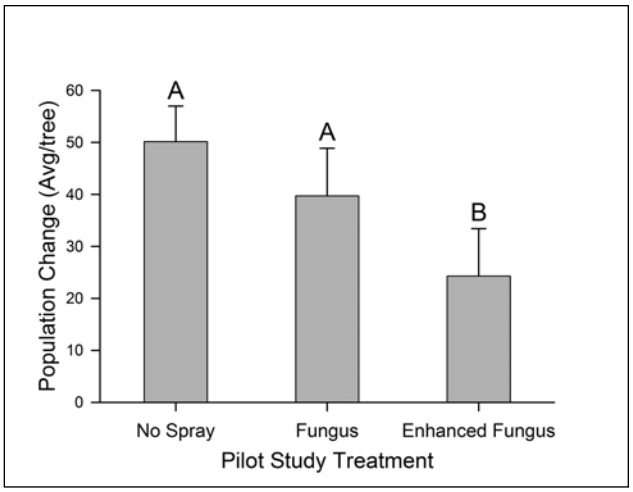

Figure 3. Response of HWA populations to aerial application of insect-killing fungi (Mycotal, L. muscarium), with and without a fungal enhancer (MycoMax), into hemlock forest plots for the 2009 Pilot Study. Bars represent the differences between pre- and post-treatment HWA counts (sistens generation); capped lines indicate the standard error and different letters denote significant (P≤0.05) differences between treatment means. HWA populations in the no spray and fungus alone plots grew at a faster rate than when enhanced fungus was applied.

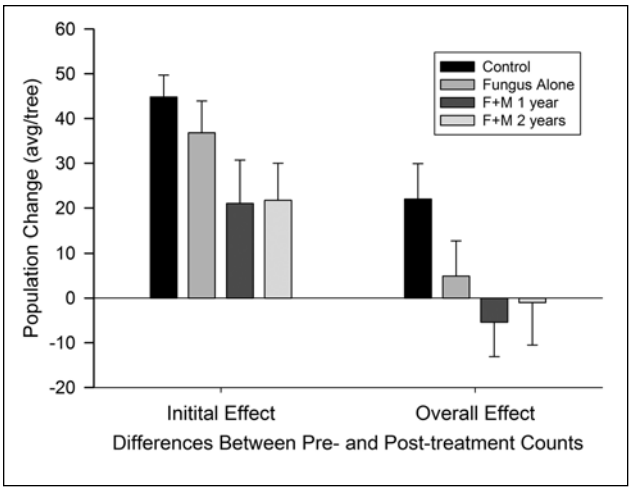

Figure 4. Response of HWA populations in a subset of the 2009 Pilot Study plots following a second year of aerial application of insect-killing fungi (F: Mycotal, L. muscarium), with and without a fungal enhancer (M: MycoMax), into hemlock forest plots. The "initial effect" represents changes in HWA population from pre-treatment levels after the first year and the "overall effect" is the change between the second year HWA counts and those taken before the year 1 treatment. Only the "F+M 2 years" treatment had a second aerial application, which was at 4 times the initial application rate; the data for those plots is broken out in the "initial effect" section for comparison. The treatment effect was significant (P≤0.05) for both initial and overall response data, but a significant interaction with HWA density precludes presenting mean separations. Application for a single year at a lower rate was sufficient to suppress HWA populations.

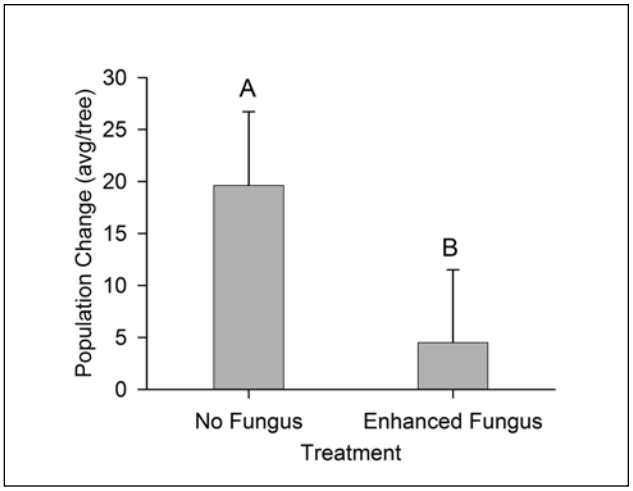

Figure 5. Response of HWA populations to aerial application of insect-killing fungi (Mycotal, L. muscarium), formulated with a fungal enhancer (MycoMax), into hemlock forest plots for the 2010 Pilot Study. Bars represent the differences between pre- and post-treatment HWA counts (sistens generation); capped lines indicate the standard error and different letters denote a significant (P≤0.05) difference between treatment means. HWA population growth was suppressed by treatment with enhanced fungi.

Ground-based treatment in VT using the enhanced fungal formulation arrested (P≤0.05) HWA population growth, while those on control trees continued to expand (Fig. 6). The actual number of HWA in control samples more than tripled from 15 to 50 per tree sample, whereas on fungal treated trees their number declined slightly after treatment; before treatment populations in the control trees and those to be treated did not differ significantly (P>0.05). Gouli et al. (2008) also did ground applications with whey and/or oil enhanced formulations of *B. bassiana* and *L. muscarium*, but employed an ULV sprayer. The total level of fungi delivered was similar ($1x10^{11}$ spores/tree) to the VT study, although the trees were approximately 1/10 the size, and HWA mortality ranged from 85-90 percent. The ability to suppress HWA using fungi applied from the ground helps corroborate the findings from aerial applications and holds promise for application to landscape plantings and those not suitable for aerial treatment.

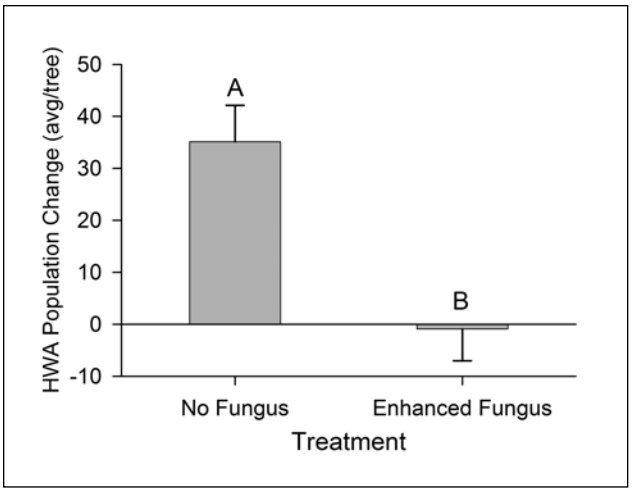

Figure 6. Response of HWA populations to ground application of insect-killing fungi (Mycotal, L. muscarium), formulated with a fungal enhancer (MycoMax), onto individual hemlock trees in Vermont (2010). Bars represent the differences between pre- and post-treatment HWA counts (sistens generation); capped lines indicate the standard error and different letters denote a significant (P≤0.05) difference between treatment means. HWA population growth was suppressed by treatment with enhanced fungi.

INSECT-KILLING FUNGI—MANAGEMENT POTENTIAL

Over the course of development, multiple strategies have been attempted to increase the opportunity for successful deployment of insect-killing fungi for HWA management. Applications that target the sistentes generation of crawlers seem to hold the most promise. Ambient temperatures are more suitable for fungal germination and growth when this generation emerges in late May through June. Treatments applied in late summer have proved inconsistent because declining temperatures are less suitable, particularly for benefiting from the microfactory approach with whey-based formulations. Although targeting the crawlers arising from overwintering sistens populations has not been attempted, temperature records suggest conditions are less favorable for fungal development. Another advantage in targeting sistens crawlers is their movement across the foliage as they seek to settle undoubtedly increases opportunity for insect fungal contact. The growth of *L. muscarium* from microfactory spray droplets should also facilitate HWA contact with a level of fungal inoculum that might not otherwise be sufficient to cause mortality.

The influence of the observed effects of *L. muscarium* (Mycotal) on longer-term HWA population dynamics is uncertain. Costa (2011) reported the results of an elementary modeling exercise incorporating the fungal impact obtained in the 2009 pilot study (~50% suppression in expansion) into a Hemlock Woolly Adelgid Population Simulator (Trotter 2011). Appreciable inhibition to HWA populations over a 4-year cycle was projected, although biannual spikes in crawler population still occurred. Interestingly, with an increase in fungal impact to the level observed in 2010, crawler spikes begin to subside and overall populations decreased over time! Modeling exercises cannot be substituted for real world evaluations, but they can provide insights into potential interaction of mortality factors on longer-term population dynamics.

Insufficient time has passed since the 2009 and 2010 pilot studies to determine if the observed impact on HWA populations will result in concurrent

improvement in tree health and survival; data collected during 2011 should begin to address this question. While dramatic knockdown is hoped for through making "inundative" applications of fungal spores, the benefits of broadly inoculating HWA populations to enhance prospects for future disease outbreaks should not be ignored. It may be that without other biological agents integrated into the system, the effect of fungus alone will be insufficient. One hypothesis is that enduring HWA populations will provide food for predatory beetles to maintain stability of their populations. Ecologically relevant non-target effects from *L. muscarium*, particularly to introduced predators, have not been identified and additional testing is underway.

There is a pressing need for implementation of effective tactics for protecting hemlock forests from the rapid decline experienced, particularly in southern range, as HWA becomes established. Currently, an experimental use permit is being sought from the EPA to allow expanded testing of Mycotal on larger acreage—approval seems probable. Biopesticide registration of Mycotal for forestry and other uses is likely only a minor hurdle due to its overseas regulatory history and a parent company committed to its development for HWA management. Recently, the economics of various management approaches was evaluated in a structured decision making exercise (Blomquist et al. 2010). A fungal biopesticide approach compared favorably with release of predators and silvicultural practices. However, the economic viability and efficacy of Mycotal seems dependant on adopting microfactory formulation technology to reduce application rates and to enhance insect/fungal contact.

The inaccessibility and rugged terrain often accompanying growth of hemlock makes aerial application a requisite tactic. Recent changes in EPA-NPDES regulations that specifically target forestry application of biopesticides highlight the need for forward planning if use of insect-killing fungi is to be broadly adopted, particularly because of riparian habitats hemlock occupy. A realistic appraisal suggests that with a concerted effort toward expanded testing, obtaining federal and state biopesticide label registrations, and early incorporation into strategic management plans, insect-killing fungi will become available for HWA management.

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Chapter 10: Other Species Considered

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INTRODUCTION

This chapter reports on predators which have only preliminary information or were found to have a host range that was too broad to be considered as safe biological control agents. The predators reviewed here include predaceous anthocorid bugs from China and western North America, a *Laricobius* beetle from China, and a lady beetle native to western North America. For each of the species, information on their taxonomy, biology, and host range is provided and their potential as a biological control agent for *Adelges tsugae* Annand, the hemlock woolly adelgid (HWA), is evaluated. Key questions for the reader to ponder are how host specific should HWA predators be and can successful biological control be achieved using only predators that are specific only to HWA.

SPECIES CONSIDERED

Tetraphleps galchanoides Ghauri (Hemiptera: Anthocoridae)

The Anthrocoridae is a family of 500-600 worldwide species that are mostly predaceous, a few of which have been deliberately introduced as biological control agents (Lattin 1999). Within the Anthrocoridae, the genus *Tetraphleps* has about 14 described species worldwide which feed primarily on aphids and adelgids (Lattin 2000). There are five species of *Tetraphleps* native to North America and four of these have been observed to prey on the balsam woolly adelgid (Kelton 1978). *Tetraphleps raoi* Ghauri and *T. abdulghani* Ghauri, both native to Pakistan and India, were introduced to North America for biological control of the balsam woolly adelgid, but apparently did not establish. Bu and Zheng (2001) attributed the failure to establish to a lack of climatic suitability. While *T. raoi* was not successfully introduced into North America, it was established in Kenya for control of *Pineus pini* (Macquart), where it is believed to be responsible for a decline in the density of the adelgid (Lattin 2000).

Tetraphleps galchanoides (Fig. 1) is known from India and China (Bu and Zheng 2001). The first report in China was a specimen collected from *Tsuga chinensis* in Baoxing County, Sichuan Province (Yao and Wang 1998). They reported that an adult could eat 2.7 nymphs and 5.6 eggs of HWA per day. In the spring of 2007, a very dense population of HWA was observed in Yunnan Province that was preyed on heavily by both adults and nymphs of *T. galchanoides* (McAvoy et al. 2008). Laboratory experiments showed that *T. galchanoides* preying on HWA was a Type II Holling functional response—predation rate increased as prey densities increased (Li et al. 2008).

Figure 1. Adult Tetraphleps galchanoides.

From 2002-2008, basic biology and host feeding studies were conducted in quarantine to determine the potential of *T. galchanoides* as a biological control agent of HWA (McAvoy et al. 2007, McAvoy et al. 2008). In 2002, eggs and nymphs were recovered from hemlock foliage collected in the fall in Sichuan, China that were shipped to the quarantine facility at Virgina Tech. When newly hatched nymphs were fed only the balsam woolly adelgid, *Adelges piceae* Ratz., none develped to the next nymphal stage; but when fed exclusively pine bark adelgid, *Pineus strobi* (Hartig), or woolly alder aphid, *Paraprociphilus tessellatus* (Fitch), they successfully developed to the adult stage. Research (unpublished) in China indicated that *T. galchinoides* preyed on the bean aphid placed in Petri dishes.

Tetraphleps galchanoides females normally oviposit their eggs into the lower side of the hemlock needle tissue. When offered a choice of foliage of white pine (*Pinus strobus*), red spruce (*Picea rubens*), and Fraser fir (*Abies fraseri*), *T. galchanoides* oviposited in white pine and fir but not spruce needles. However, in a no-choice test with spruce only, *T. galchanoides* did lay eggs in spruce needles. In a choice test with all four conifer species, *T. galchanoides* oviposited only in hemlock needles, indicating that hemlock is the preferred host, but will lay eggs in the other three non-target species, spruce being the least preferred species. Eggs were also oviposited in leaves of smooth alder (*Alnus serrulata*) in a no-choice test. Unlike the eggs inserted into the conifer needle tissue, eggs in the alder test were laid on the surface of the leaf and not inserted into the leaf tissue. Several of these eggs hatched.

Tetraphleps galchanoides fed and oviposited on non-target adelgids and aphids. Additional feeding tests found that *T. galchanoides* adults will feed on *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) larvae. Due to the suitability of several non-target homopteran species as hosts and its feeding on the larvae of a potential biological control agent, this species is not considered at this time to be a suitable candidate for release as a biological control agent.

Anthocoris nemoralis Fabricius and A. antevolens White

The genus *Anthocoris* occurs primarily in the Northern Hemisphere and consists of about 70 species. The Palearctic has 47 species, and 12 species occur in Canada and the United States (Lattin 2000). Only one species that is native to North America, *Anthocoris antevolens* White, is known to use adelgids as prey and this is considered incidental (Lattin and Stanton 1992). In North America, the hemipterans *Daecocororis pinicola*, *D. piceicola*, and *D. nubilus* in the family Miridae prey on adelgids on pine and spruce (Wheeler 2001).

Several species of Anthocoridae were collected by R. McDonald from October to February, 2008-2011, in Seattle, WA while he was collecting *L. nigrinus* for release in the eastern United States. The most abundant species was *Anthocoris nemoralis* (Fab.) with 55 adults collected or 89% of the total *Anthocoris* collected. This species was intentionally introduced from Europe as a biological control agent in orchards against aphids, psyllids, thrips, and moth eggs and larvae (Kelton 1978). It became established in the 1950s and is found in British Colombia, Ontario, Washington, Oregon, and California (Lewis et al. 2005). The second most common species was *A. antevolens* White, with seven adults found (11%). This species is native to North America and is found across Canada and western United States. This is the first report of finding *Anthocoris* species on *Tsuga*.

Adults shipped to the Virginia Tech quarantine facility were reared on *Tsuga canadensis* foliage infested with HWA at 15 °C. The mean length of time that the adults lived was 36 days. The number of females collected was low with only 1 female for every 6 males, and no oviposition was observed on hemlock. No nymphs were found during beat-sheet sampling of hemlock. It appears that the adults of these two species of *Anthocoris* may only use HWA as an occasional food source, but not for oviposition.

Laricobius kandingensis Zilahi-Balogh and Jelínek

The genus *Laricobius* (Coleoptera: Derodontidae) has 21 described species, all of which prey only on adelgids (Leschen 2011). Two of these species were discovered as a result of an expedition to China that specifically targeted this genus. In April 2002, an expedition to Sichuan Province, China found a new species in Baoxing County and another new species in Kangding County. These were described, and named according to where they were found: *L. baoxingensis* Zilahi-Balogh and Jelínek, and *L. kangdingensis* Zilahi-Balogh and Jelínek (Zilahi-Balogh et al. 2007). Both species were found on adelgid infested hemlock (*T. chinensis* (Franchet) Pritzel) in April 2002. Only female adults (n=5) of *L. baoxingensis* were collected and these died without reproducing after being shipped to the quarantine laboratory at Virginia Tech in Blacksburg, Virginia. Only larvae (n=23) of *L. kangdingensis* were collected and these completed development on *T. canadensis* infested with HWA in the quarantine.

Quarantine studies of *L. kangdingensis* at Virginia Tech (Gatton et al. 2009) found it to be univoltine, have 4 larval instars, a low temperature development threshold for eggs (0 °C), larvae (1.6 °C), and pre-pupae (5.8 °C), yet completed development only at temperatures between 12-15 °C. Host-range studies showed it preferred HWA over all other species tested.

This species has not been pursued as a biological control agent due to problems with rearing the beetles in quarantine. The largest challenge was the result of the small size of the founding colony and ultimately the colony could not be maintained. Efforts to start new colonies were not successful because only small numbers could be collected in China, and collected beetles did not survive shipment to the quarantine in the United States. In the meantime, another species, *Laricobius osakensis* Montgomery and Shiyake, was discovered in Japan and it was much more abundant and widespread in its native habitat; thus, work with the Chinese *Laricobius* was discontinued.

Scymnus (Pullus) coniferarum (Crotch)

Scymnus is the largest genus of lady beetles (Coccinellidae) with over 600 species worldwide. This large genus is divided into six subfamilies of which the subgenus *Pullus* is the largest with more than 300 described species. Although a large subgenus, *Scymnus (Pullus)* has only three species that are known to be specialists on adelgids (Whitehead 1967). Two of these species, *S. (P.) impexus* (Mulsant) and *S. (P.) suturalis* (Thunberg), are native to Eurasia and have been introduced to North America, and one species, discussed here, is native to the western North America.

Scymnus (Pullus) coniferarum Crotch (Coleoptera: Coccinellidae) was described in 1874 from specimens collected from pine in California. It is a small lady beetle, about 2 mm in length, that is clothed in fine, short pubescence with a black head and pronotum and reddish-yellow brown elytra that is piceous along the suture and at the base (Fig. 2a). Its larvae are covered in a white woolly wax (Fig. 2b). Whitehead (1967) and Gordon (1976) provide full descriptions with figures.

The known native geographical range of *S. (P.) coniferarum* is western North America (Gordon 1985). Specimens have been collected from various species of pines in British Columbia, Arizona, California, Colorado, Idaho, Nevada, New Mexico, Oregon, South Dakota, Utah, and Wyoming (Gordon 1976). Recently several hundred specimens have been collected from western hemlock in Washington State (Montgomery et al. 2009, McDonald 2010). It has recently been recovered from Monterey pine in Chile and Peru (Gonzalez 2006), probably an accidental introduction since pine and adelgids are not native to South America. There is a report of it in the eastern United States (Malkin 1945), but this may be a misidentification. Gordon (1976) recorded it in Pennsylvania, but later clarified that these were *S. (P.) suturalis* (Gordon 1985). Considering that the species has spread intercontinentally, has been found as far east as the Black Hills of South Dakota, and occurs in a variety of habitats

Figure 2. Adult (a) and larva (b) of Scymnus (Pullus) coniferarum.

in western North America, it is possible that it is already established in eastern North America.

The seasonal occurrence of *S. (P.) coniferarum* and another predator, *L. nigrinus*, as well as their prey, HWA, in the Seattle, WA area is shown in Table 1. Other information indicates that the production of progrediens eggs continues during July in western Washington (Kohler et al. 2008). The adults of both predators are present and presumably feed on HWA during the late fall and winter months. Larvae of *L. nigrinus* appear in March and complete development by mid-May. The larvae of *S. coniferarum* appear near the end of May and continue to feed on the progrediens and their sistens eggs into July. In severe winter weather, adults seek shelter in bark crevices on the bole of the tree.

The only hosts of *S. (P.) coniferarum* reported in the literature are adelgids that feed on pine (*Pineus* spp.) (Whitehead 1967, Gordon 1976). Recent observations in the Seattle area have found it abundant on hemlocks infested with HWA, and to a lesser extent on western white pine, but it was not found on several species of hard pines and spruce trees (Montgomery et al. 2009, Montgomery and McDonald 2010).

Table 1. Seasonal occurrence of HWA progediens stages (blue shading) and HWA sistens stages (yellow shading) and their predators *Laricobius nigrinus* (*Ln*) and *Scymnus coniferarum* (*Sc*) in the Seattle, WA area (light pink = relatively few, dark pink = peak abundance). Observations not made July-Sept.

Month	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
HWA adults									
HWA eggs									
HWA nymphs									
Ln adults									
Ln larvae									
Sc adults									
Sc larvae									

Laboratory studies indicate that *S. (P.) coniferarum* feeds specifically on adelgids and does not attack other Homoptera to a significant extent. In the laboratory, the beetle completed development from egg to adult on HWA and the pine bark adelgid, but mortality was greater on the latter host. In no-choice tests *S. (P.) coniferarum* adults did not feed on the native woolly alder aphid, *Prociphilus tessellatus* (Fitch) or the lime aphid, *Eucallipterus tiliae* (L.). In the laboratory, *S. (P.) coniferarum* has been reared for several successive generations on HWA growing on eastern hemlock, and studies on its potential to feed on non-target species are ongoing.

IMPLICATIONS FOR BIOLOGICAL CONTROL

Tetraphleps galchanoides is an example of an opportunistic predator. These bugs migrate to high prey densities where they can capture prey efficiently. Because of their feeding efficiency at high prey densities and relatively large size, these opportunistic predators are very effective in reducing outbreak populations in the adelgids' native environment. After the food supply is depleted, they disperse in search of another abundant source of suitable prey. In Japan, several opportunistic predators in the beetle families Elateridae, Cantharidae, Melyridae, and Coccinellidae also attack the progrediens/sexupara generations during May (Shiyake et al. 2008). Thus, we see in Asia that late spring predation by opportunistic generalists may play a critical role in reducing high outbreak densities of HWA to below damaging thresholds. Why similar predation on HWA by opportunistic generalist predators does not occur in the eastern United States is unclear. There are several species of native predators in the families Anthocoridae, Miridae, Elateridae, Cantharidae, and Melyridae that potentially could prey on HWA.

Scymnus (P.) coniferarum merits further study to better assess its potential as a biological control of HWA. Its larvae appear after *L. nigrinus* larvae have stopped feeding and have migrated to the soil. The late spring feeding of *S. (P.) coniferarum* indicates its impact on HWA population dynamics should complement that of *L. nigrinus*. Late spring predation of HWA may be critical in obtaining suppression of HWA below damaging levels (see Chapter 2). A question that remains to be addressed is the extent that *S. (P.) coniferarum* would feed on other adelgids, including native species present in the eastern United States. Except for *Pineus floccus* Patch, most of the adelgid species present in the eastern United States also occcur in the western United States. Its feeding on alternative prey, particularly species such as *Pineus coloradensis* Fitch that are active during the summer, may allow it to sustain higher populations and thus be a more effective predator of HWA.

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SECTION III LABORATORY REARING FOR FIELD RELEASE

Chapter 11: Rearing labs and distribution of predators for release

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REARING LABS

There are currently eight primary labs rearing five different predators of hemlock woolly adelgid. The labs include Clemson University, New Jersey Department of Agriculture (Philip Alampi Beneficial Insect Laboratory), North Carolina Department of Agriculture and Consumer Services, North Georgia College & State University, University of Georgia, University of Tennessee (Lindsay Young Beneficial Insect Laboratory), Virginia Tech, and Young Harris College. Species currently being reared include *Laricobius nigrinus* Fender, *Laricobius osakensis* Montgomery and Shiyake, *Scymnus coniferarum* Crotch, *Scymnus sinuanodulus* Yu et Yao and *Sasajiscymnus tsugae* Sasaji and McClure. Table 1 details the species currently being reared by each lab as of fall 2011. This has been a dynamic list and will likely continue to change.

DISTRIBUTION OF PREDATORS FOR RELEASE

Predators of hemlock woolly adelgid have been released across 17 states in the Eastern United States. Operational releases began as early as 1998 and have continued steadily since then. Releases take place primarily on public lands, including state and national forests, parks and Hemlock Conservation Areas. Figure 1 depicts release locations of three primary predators (*S. tsugae*, *S. sinuanodulus*, and *L. nigrinus*) which have been entered into the newly created HWA Predator Release and Monitoring Database. Entry of this data is currently ongoing and many releases have yet to be represented. Additional species will also be added.

Table 1. Rearing labs

Rearing Lab	L.n.*	L.o.	S.c.	S.s.	S.t.
Clemson University	x				x
New Jersey Department of Agriculture	x				
North Carolina Dept. of Agriculture and Consumer Services					x
Northern Georgia College & State University					x
University of Georgia	x			x	
University of Tennessee	x	x			x
Virginia Tech	x	x	x		
Young Harris College					x

*L.n. = *Laricobius nigrinus* Fender; L.o. = *Laricobius osakensis* Montgomery and Shiyake; S.c. = *Scymnus coniferarum* Crotch; S.s. = *Scymnus sinuanodulus* Yu et Yao; S.t. = *Sasajiscymnus tsugae* Sasaji and McClure

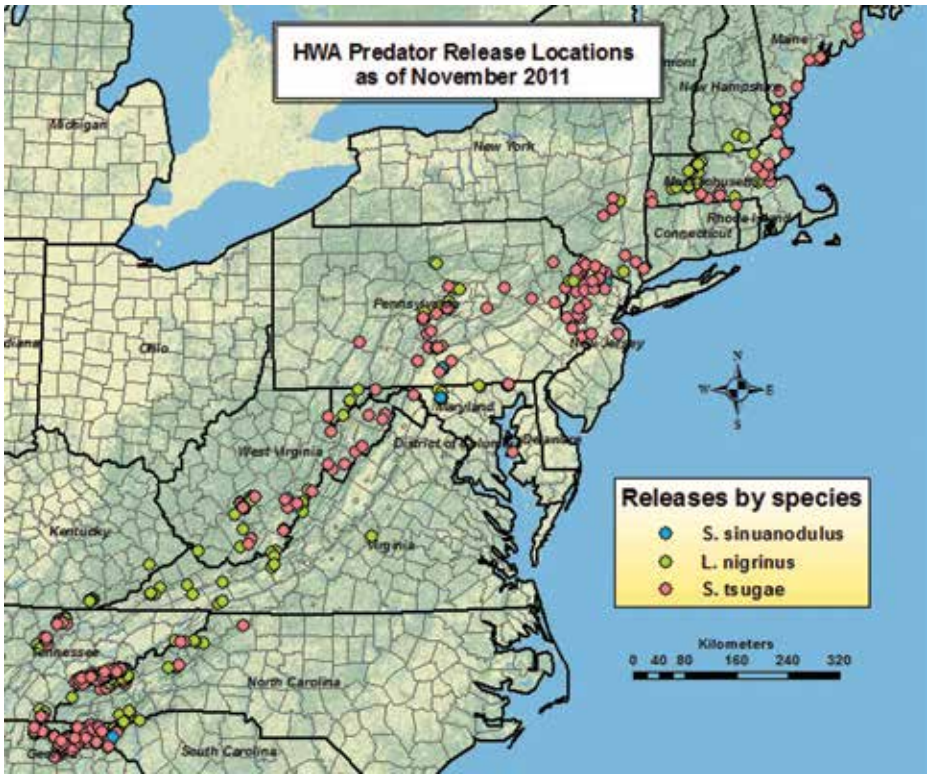

Figure 1. Map of the Eastern United States showing S. tsugae, S. sinuanodulus, and L. nigrinus releases which have been entered into the HWA Predator Release and Monitoring Database.

Laricobius nigrinus Release Locations

Clemson: Georgia, North Carolina, and South Carolina

New Jersey Department of Agriculture: Pennsylvania and New Jersey

University of Georgia: U.S. Forest Service Hemlock Conservation Areas throughout Georgia

University of Tennessee: Tennessee – Great Smoky Mountains National Park, Cherokee National Forest; Tennessee State Parks and Natural Areas, and Tennessee Wildlife Management Areas

Virginia Tech: Connecticut, Georgia, Maine, Maryland, Massachusetts, New Hampshire, New York, North Carolina, Pennsylvania, Rhode Island, Vermont, Virginia, West Virginia

Sasajiscymnus tsugae Release Locations

Clemson University:
- South Carolina - Sumter National Forest, Jocassee Gorges, Mountain Bridge Wilderness Area, Oconee State Park;
- North Carolina – Nantahala National Forest
- Georgia – Chattahoochee National Forest

New Jersey Department of Agriculture: Connecticut, Maryland, Massachusetts, Maine, New Hampshire, New Jersey, North Carolina, New York, Pennsylvania, Rhode Island, South Carolina, Tennessee, Vermont, Virginia, West Virginia

North Carolina Department of Agriculture and Consumer Services: North Carolina, Maine

North Georgia College & State University:
- Georgia – U.S. Forest Service Hemlock Conservation Areas throughout North Georgia on National Forest and State lands

University of Georgia: U.S. Forest Service Hemlock Conservation Areas throughout Georgia

University of Tennessee:
- Tennessee – Great Smoky Mountains National Park, Cherokee National Forest; Tennessee State Parks and Natural Areas, and Tennessee Wildlife Management Areas
- Georgia – U.S. Forest Service study

Young Harris College:
- Georgia – Chattahoochie National Forest and State properties in North Georgia

Scymnus sinuanodulus Release Locations

Clemson University: Chattooga River – Bull Sluice Site (Georgia/South Carolina border)
New Jersey Department of Agriculture: Maryland, New York, North Carolina, Pennsylvania, Tennessee, Vermont, West Virginia
Northern Georgia College & State University: Georgia – Hemlock Conservation Areas throughout North Georgia on National Forest and State lands
University of Georgia: U.S. Forest Service Hemlock Conservation Areas throughout Georgia
University of Tennessee: Tennessee – Great Smoky Mountains National Park

Scymnus coniferarum Release Locations

No releases yet.

A summary of release totals is shown in Table 2.

REARING PROCEDURES

Laricobius nigrinus

Dr. Ashley Lamb developed *Laricobius nigrinus* rearing procedures at Virginia Tech from 2000-2005 while working toward her Ph.D. and later as a postdoctoral associate. They were adapted for mass rearing, and have since been adjusted to maximize production. Each lab has manipulated their rearing protocols to fit their individual facility limitations such as space availability and equipment. In general, mass rearing begins in late January or early February when both HWA and *L. nigrinus* begin to lay eggs. New adults are collected each year in their native habitat, the Pacific Northwest area of the United States. These adults are brought back to rearing labs and serve as founders for that year's colony. Collecting fresh beetles each year helps add to the genetic diversity of released populations. Additionally, wild-caught beetles are often more fecund which results in higher laboratory production numbers. Once adult *L. nigrinus* beetles are brought back to the lab, they are held in clear, plastic feeding containers on hemlock bouquets (Fig. 2). These bouquets usually consist of roughly 20-25 branches of highly infested hemlock roughly 20-25 cm in length. These are nested in Parafilm wrapped floral foam for hydration.

Adults are randomly placed in containers, since sex cannot be determined through visual observation. They are held at densities of roughly 20 beetles per container to ensure minimal competition between individuals for feeding and egg laying sites. Adults will remain on these bouquets for a

Table 2. Release totals

Rearing Lab	*L. nigrinus*	*S. coniferarum*	*S. sinuanodulus*	*S. tsugae*
Clemson University	14,605 (adults) + 44,678 (eggs)		52 (adults)	940,721 (adults) + 82,256 (eggs)
New Jersey Department of Agriculture	75 (adults)		23,348 (adults)	536,290 (adults) + 22,744 (eggs) + 360 (larvae)
North Carolina Dept. of Agriculture and Consumer Services				484,800 (adults)
Northern Georgia College & State University			2,080 (adults)	204,206 (adults)
University of Georgia	141,580 (eggs)		32,089 (eggs)	Minimal egg releases made in 2006
University of Tennessee	17,656 (adults)		98 (adults)	736,733 (adults) + 89,084 (eggs)
Virginia Tech	72,489 (adults)			
Young Harris College				120,034 (adults) + 100,092 (eggs)

period of one week at specific temperatures and daylength depending on the time of year (Fig. 3).

Adults are removed after one week and the foliage is placed into floral foam blocks with fresh twigs. These blocks are then nested into galvanized steel funnels or other larval rearing systems where eggs are allowed to hatch and subsequent larvae develop (Fig. 4).

If labs are releasing eggs, they are placed in the field at this time. Typically, temperatures during this phase of development are between 12-15 °C with a 12:12 L:D daylength. It takes approximately 4-6 weeks for larvae to hatch and develop through 4 instars. Since their natural tendency is to drop to the soil when development is complete, larval rearing systems are designed to have a catch area such a drawer or jar under the foliage, where the larvae can congregate. These are checked daily until roughly July when production typically stops.

Collected larvae are immediately placed into summer aestivation containers. These containers are relatively small (approximately 15 cm × 15 cm) plastic, and ventilated on at least two sides for adequate air circulation (Fig. 5).

Approximately 6-7.5 cm of soil is placed into these containers with moisture levels at or close to 35% since the pre-pupae and pupae prefer a wet soil. A 2:1 ratio of peat moss and sand is used to create the most optimal soil mix. Mature larvae are simply placed on the soil surface and they eventually burrow down to create a pupation cell. Once in the soil, containers are held at roughly 12-15 °C for approximately 6 weeks while pupae develop into adults.

After pupae eclose, containers are then moved into 19 °C (16:8 L:D) temperatures for the summer months. These higher temperatures again mimic conditions in their native habitat and encourage the adults to stay in the ground. Soil moisture levels are kept very low at this point. Containers are watered very lightly on a weekly basis.

Figure 2: L. nigrinus feeding containers.

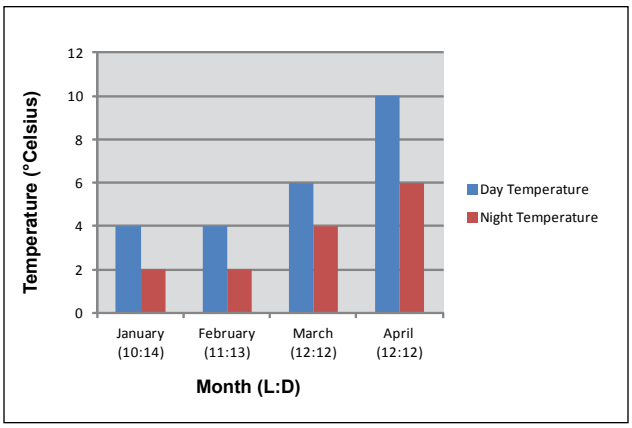

Figure 3. L. nigrinus adult feeding temperatures and daylength during oviposition.

Figure 4: L. nigrinus larval rearing system utilizing steel funnels.

When HWA has broken its summer diapause in the field in early October, *L. nigrinus* aestivation containers are then moved to lower temperatures to encourage emergence (12-15°C; 12:12 L:D). Adults will typically begin emerging in large numbers about 2 weeks after the temperature is dropped and will continue to emerge from these containers until mid to late December. If labs are doing adult releases, they are typically placed in the field during this time.

Figure 5. L. nigrinus soil aestivation containers.

Laricobius osakensis

Protocols for the mass rearing of *L. osakensis* are currently being developed at Virginia Tech. Dr. Ashley Lamb has completed initial groundwork, which is being followed by Ph.D. student Ligia Cota Viera and the Virginia Tech mass rearing staff. Many procedures will be identical to that of *L. nigrinus*; however, oviposition temperatures will likely be slightly lower.

Sasajiscymnus tsugae

Carole Cheah and the staff at the Connecticut Agricultural Experiment Station (CAES) developed initial small-scale rearing procedures for *S. tsugae*. These methods were then built upon and adjusted for mass rearing by Dan Palmer and Jennifer DeSio at the New Jersey Department of Agriculture (PABIL) and now stand as the foundation for procedures utilized at other rearing labs today. Each lab has tweaked protocols to suit their facilities' capabilities and limitations. Ovipositioning adults are typically held on hemlock bouquets placed in soaked floral foam in 1 gallon glass jars with modified, ventilated lids. Eight to ten twigs, 15-20 cm in length, comprise one bouquet (Fig. 6).

Gauze is placed between foliage to help estimate egg totals, as roughly 50% of the total number of eggs will be laid there. Beetles are typically held at densities of 15 per container with a 10:5 female: male ratio. Honey is added as a supplemental diet. Glass jars are held on their sides on racks at 24-25 °C, 55-65% RH, 16:8 L:D (Fig. 7).

Adults are removed after one week and the foliage and gauze are placed into a rearing cage. Adults are placed on fresh bouquets and the process is repeated. If egg releases are being made and if outdoor temperatures are consistently above 15 °C, they are done at this time.

Figure 6. S. tsugae ovioposition jars.

Figure 7. S. tsugae oviposition jars on racks.

Foliage and gauze containing eggs are placed in rearing tents (BugDorm – 152 cm ×152 cm × 152 cm) until an estimated 1500 eggs (750 from gauze) is reached (Fig. 8).

Twigs are placed into foam blocks with the gauze sandwiched between. Fresh, infested hemlock branches are added 2 times per week and after 20 days a honey strip is placed in each cage 1 time per week.

Any adults found during this time are collected and placed into storage cages. Rearing cages are broken down after roughly 30-50 days. All foliage is examined and any beetles found are collected. Up to 400 adults are placed into storage cages at 18-20 °C, 55-65% RH, 16:8 L:D at least 3 weeks prior to release to ensure they are sexually mature. Adult releases begin when outdoor temperatures stay above freezing.

Figure 8. BugDorm rearing tents.

Adults are stored in summer with protocols similar to those used for the rearing cages. Roughly 400 adults are held in each cage at temperatures of 14-20 °C, 55-65% RH, 12:12 L:D, and foliage blocks are replaced every 45-60 days.

Scymnus sinuanodulus

Since it is so similar to *S. tsugae*, protocol development for the mass rearing of *S. sinuanodulus* was again based on the foundations built by Carole Cheah at CAES and Dan Palmer and Jennifer DeSio at PABIL. Also, as with *S. tsugae*, individual labs have tweaked various procedures to meet their particular needs. More specifically, a multi-lab collaborative effort to fine-tune protocols for operational egg releases was headed up by the staff at the University of Georgia rearing facility. Rearing begins in February after HWA is confirmed to be developing eggs in the field. Adults are held at densities of 9 (6:3 female:male) on 5 twigs of infested hemlock. Temperatures run from roughly 12-14 °C, RH 65%, 12:12 L:D. Gauze is also used as egg indicator in oviposition jars but *S. sinuanodulus* will only lay roughly ¼ of its eggs there. Twigs are removed and refreshed weekly through June. Egg releases are done with the resulting twigs. If rearing to the adult stage, resulting twigs will be placed in BugDorms for larval development. The use of ample quantities of fresh, heavily infested hemlock is critical since larvae can become cannibalistic. Fresh foliage is added weekly. Larval rearing temperatures are set to 20-22 °C, 65% RH and 16:8 L:D. Adults will begin to appear in 7-8 weeks. They are collected and stored at 20-22 °C for 4 weeks at 16:8 L:D, 65% RH. These conditions allow maturation and mating to occur. They are then moved to 12-14 °C at higher densities (50-60 adults) until October when beetles are released.

Scymnus coniferarum

S. coniferarum is currently being studied in quarantine at Virginia Tech to confirm suitability as a biological control agent of HWA. Preliminary rearing techniques are being developed; however, rearing will likely be similar to other *Scymnus* species. Adults will typically lay more eggs at 26 °C but will die off sooner. Adults are therefore held at 18-20 °C as a compromise. They're held at densities of roughly 12 with a 2:1 female:male sex ratio, although it is difficult to distinguish between sexes. Gauze is also used in oviposition bouquets to estimate the number of eggs laid. Larvae are reared at 18 °C and adults emerge in roughly 50 days at this temperature.

GENERAL ISSUES AND PRODUCTION LIMITATIONS

Mortality before Release (S. sinuanodulus and S. tsugae)

Adults are typically held for several weeks before release to allow for mating and feeding; however, mortality is often high during this time.

Early Emergence (L. nigrinus)

A significant challenge when rearing *L. nigrinus* is that of early emergence of adults before HWA has broken diapause in the field. *L. nigrinus* adults will not feed on aestivating HWA nymphs so a great deal of mortality can occur if beetles are emerging from the soil early in the lab. A small level of early emergence typically occurs at all labs each year; however, some years have been particularly high. This is thought to happen when *L. nigrinus* larvae do not receive ample nutrients during their maturation process. Other unknown factors likely play into this as well. Mortality levels can be devastating. Holding early-emerged adults at low temperatures and offering supplemental artificial diets helps to stave off high mortality levels before HWA breaks. Holding temperatures of at least 4 °C greatly increased survival.

Staffing

High turnover rates have been shown to negatively affect success rates at the various labs. The rearing of each species is very labor intensive and challenging. *L. nigrinus*, for example, only has one generation per year. It takes several seasons to fully understand what is necessary to be successful. With high turnover, you lose that gained knowledge and must start over with training. Some labs also have trouble finding funding to hire ample staff (Fig. 9).

Figure 9. Rearing lab staff.

Figure 10. Infested hemlock used for predator rearing.

Equipment/Rearing Space

Because biocontrol agents of HWA are winter active, this presents a unique challenge for rearing in terms of temperatures. Some predators require temperatures as low as 4 °C (39 °F) for extended periods of time. Temperatures of roughly 12 °C (54 °F) must be available in large spaces for rearing of *L. nigrinus* larvae. Most rearing facilities have faced the challenge of retrofitting old buildings and equipment to create these large spaces. Even with the best equipment, there are failures, which can cause temperature spikes or drops. These fluctuations can be devastating to colony health. Several labs have seen high mortality due to this reason.

Food Quality

As the HWA infestation front moves westward and trees continue their rapid decline, it has become increasingly difficult to find heavy adelgid infestations on relatively healthy trees with which to feed colonies. Significant travel time is involved for some labs to find the best infestations, often requiring overnight trips. Other labs must have food shipped in, which presents problems in terms of quality of prey health. Since such a high volume of food is necessary for rearing, especially in the spring, this becomes the primary challenge for most labs (Fig. 10).

Pathogens

As touched upon in other chapters of this publication, pathogens such as microsporidia are a significant issue for rearing predators in the lab. Colonies are routinely tested for the presence of microsporidia since this organism is found occurring naturally in wild populations. Since most *L. nigrinus* rearing begins with yearly introductions of insects collected in the wild, it is important to do these tests. Infections in the lab can be devastating to fecundity levels and very difficult to eradicate.

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Chapter 12: Microsporidian Disease in Predatory Beetles

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INSECT PATHOGENS IN THE FIELD AND LABORATORY

Like all other living organisms, insects continually interact with a variety of natural enemies that include predators, parasites and pathogens. These interactions stabilize insect populations over the long term (Anderson and May 1981), but immediate effects on individuals or populations can be severe when the prevalence of natural enemies in the host population increases. Pathogens typically persist in insect populations at low, enzootic levels, but may increase exponentially, often when host population density increases, causing local or large-scale and, typically, cyclic host population declines (Fuxa and Tanada 1987).

Insects reared in laboratory colonies are usually protected from most of their naturally occurring predators and parasites, but pathogens are more insidious because often they are not detectable or easily identifiable without the use of specialized microscopic and molecular tools. Pathogens that initially occur in colonies at low prevalence or at low infection intensity in the hosts may not immediately produce noticeable effects, and a low level of host mortality is generally accepted as "normal" in a colony. Nevertheless, a single insect harboring a highly infectious pathogen can, when introduced into the colony or is used to found a colony, initiate an epizootic. The vast majority of pathogens are orally transmitted via feces, oral exudates and/or decomposing tissues, and some are additionally transmitted from infected females to their offspring. The laboratory colony provides a highly favorable environment for transmission of pathogens because of the high densities of insects being reared in confined spaces, and build-up of disease can quickly compromise expensive, long-term efforts to establish and maintain insect colonies.

IMPORTANCE OF PATHOGEN-FREE COLONIES FOR BIOLOGICAL CONTROL PROGRAMS

Under the authority of the Plant Protection Act of 2000, natural enemies of pest insects that are collected for use outside their native environments in biological control programs are regulated by USDA APHIS Plant Protection and Quarantine. A PPQ 526 permit is required and natural enemies are held in quarantine for a period of time after arrival in the U.S. to assess their specificity to the host and determine whether they carry diseases. Any unusual mortality is investigated but infections may be latent in the host at the time of collection, or produce chronic effects that are difficult to observe, allowing diseased individuals to escape detection and resulting in much higher levels of prevalence and infection intensity than typically occurs in the field. Because biological control programs usually require mass rearing to increase numbers of biological control agents for release, it is critical that costly and time-consuming efforts are not destroyed by the build-up of pathogens in laboratory colonies. Early detection and mitigation can avoid debilitation or complete loss of the colony (reviewed by Etzel and Legner 1999), as well as introduction of exotic diseases into new environments where other natural enemies of the targeted pest may be compromised.

It is nearly impossible to ensure that insect colonies are completely pathogen-free, particularly because chronic submicroscopic viruses are extremely difficult to detect and may not cause acute mortality, but many pathogens are detectable and, if prevalence levels are initially low, it is sometimes possible to "cure" the colony.

MICROSPORIDIAN INFECTIONS

Some of the most commonly detected pathogens in laboratory insect colonies are the Microsporidia. Single-cell organisms related to Fungi, microsporidia are obligate pathogens—they can only reproduce within the cells of their hosts (Tanada and Kaya 1991) (Fig. 1). Of the > 1,200 species that have been described from vertebrate and invertebrate animals, over half are pathogens of insects (Becnel and Andreadis 1999). They are typically chronic pathogens causing slow larval development, increased larval mortality, decreased adult lifespan and reduced fecundity (Brooks 1988, Becnel and Andreadis 1999). These effects, while they may not be immediately devastating, eventually reduce colony growth and prevent production of sufficient numbers of natural enemies to support a biological control program.

Microsporidia typically infect the gut tissues or are systemic, and mature infective spores are passed in the feces and/or when dead infected insects decompose, contaminating the food source. Many species are also transmitted to the offspring of infected females in or on the surface of the egg, and mortality is typically high for larvae that are infected as embryos or neonates (Solter 2006). Infections are rarely detectable via visual inspection of the host and the chronic nature of the disease may allow the pathogen to build up to very high levels before noticeable mortality occurs. Several generations may appear to be healthy and then, quite suddenly, the adults fail to reproduce and the colony declines or is lost.

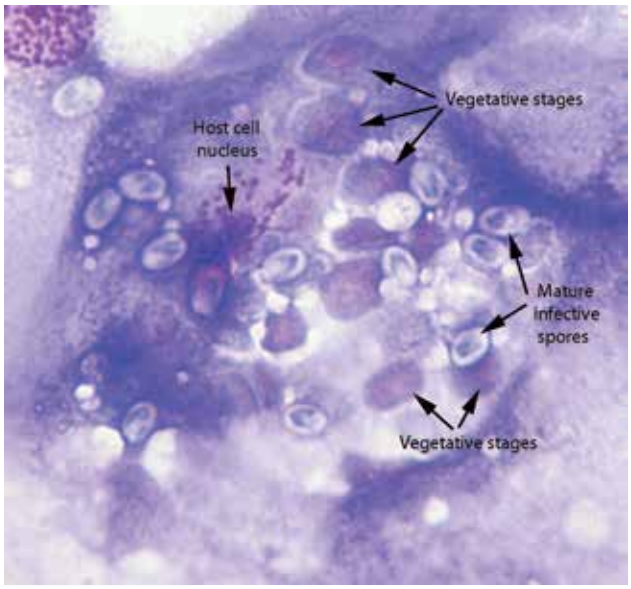

Figure 1. Developing microsporidian stages in midgut epithelial tissues of Sasajiscymnus tsugae.

Microsporidian disease is detectable using light microscopy at 250x-500x. The oval or "jellybean" shaped infective spores are typically 3-5 microns long and are brightly refractive, particularly when viewed with a phase contrast microscope (Fig. 2). Detection usually requires dissection of the host and examination of tissues, but if prevalence is high in the colony, the spores can sometimes be detected in the feces.

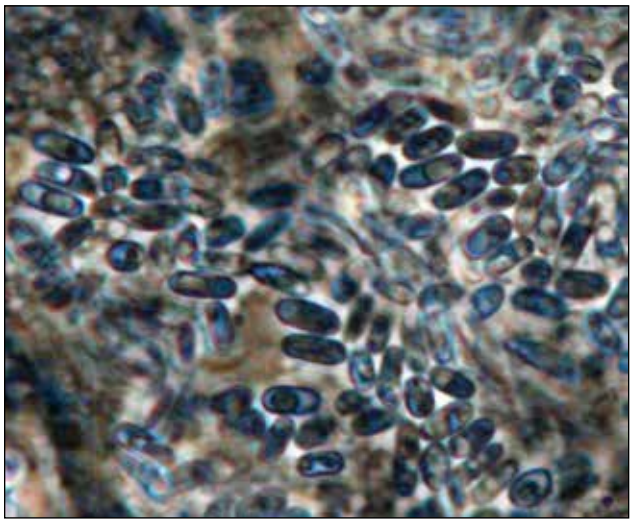

Figure 2. Mature spores of a microsporidium isolated from Laricobius nigrinus.

MICROSPORIDIA IN SASAJISCYMNUS TSUGAE AND LARICOBIUS NIGRINUS

In 2002, an unprecedented high level of mortality occurred in a laboratory colony of the HWA predator *Sasajiscymnus tsugae* and was attributed to a microsporidian infection. The infection prevalence increased over one year from 12% to approximately 50% of the colony beetles. Subsequent examinations of other laboratory-reared *S. tsugae* colonies revealed that microsporidia were common and were associated with high levels of mortality. As investigations intensified, microsporidia were isolated from several additional laboratory colonies of *S. tsugae* and were epizootic in one of the colonies. Screening of the F_1 generation of field collected *S. tsugae* adult beetles from Japan resulted in one detectable infection. A microsporidian species was also isolated from the HWA predator, *Laricobius nigrinus*, collected from western hemlock near Seattle, Washington in 2005. Microsporidia were not observed again in the field or in laboratory colonies of *L. nigrinus* until late 2010 when isolates were found in another site near Seattle, Washington and in a laboratory colony of beetles originating from Idaho. Prevalence of infection in the *L. nigrinus* laboratory colony exceeded 50%. Because the 2005 observations suggested a field prevalence of 20%, microsporidia may also be important in field populations of *L. nigrinus*.

There are several reasons to be concerned about microsporidian infection in the HWA predators. These typically chronic pathogens have significant deleterious effects on their hosts. In the laboratory, infected beetles easily transmit the pathogen due to high density rearing, and infection levels and prevalence increase quickly. Infected adults may appear to be "normal" and carry on their usual activities, but oviposition may be reduced and infected larvae fail to mature. Infections in *L. nigrinus* were systemic (Fig. 2) and male testes were filled with spores. The colony failed to reproduce and mortality was high. In the field, infection may result in high winter mortality, compromising the release project. A preliminary semi-field experiment in one laboratory resulted in 90% winter mortality of infected *S. tsugae*. Laboratory host specificity testing suggested that one *S. tsugae* microsporidium can infect several species of predatory beetles that are either being reared for release or are under study for use in the HWA biological control program. Whether this physiological susceptibility is important in the field is unknown; studies are in progress to determine whether the microsporidia infect and are persistent in reproducing populations of released beetles.

SCREENING OF PATHOGENS IN COLONIES OF HWA PREDATORY BEETLES

Molecular studies are underway to determine the identity of microsporidia infecting HWA predatory beetles. Current data suggest that several microsporidian species infect beetles in mass rearing programs and those under study for use in the HWA biological control program. These include at least two distinct *Nosema*-type species and a *Tubilinosema* sp. in *S. tsugae* laboratory colonies; a different microsporidian species yet to be characterized in an F_1 generation *S. tsugae* specimen reared from adults collected in Japan; a *Nosema*-type species in a field-collected *Scymnus coniferarum* from Washington; a *Nosema* similar to one of the *S. tsugae* isolates in *S. sinuanodulus* reared in two laboratories; and at least one species in field and laboratory-reared *L. nigrinus*. Three of these microsporidian species produced laboratory epizootics in three different colonies and compromised the rearing programs.

True entomopathogenic fungi have not been observed frequently in the HWA program, although some *S. tsugae* individuals in one colony were apparently infected with a *Beauveria bassiana*-like fungus (Fig. 3). No entomopathogenic fungi have been reported to cause serious epizootics in laboratory colonies. Occasionally, saprophytic fungi have apparently overgrown some of the cultures, resulting in higher than usual mortality. Parasites such as mites and nematodes have not been reported, nor have less common pathogens such as protozoans (e.g. trypanosomes, amoebae

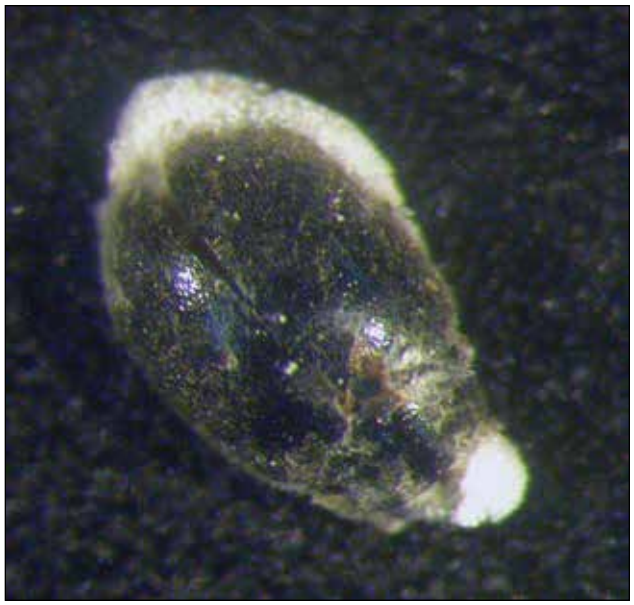

Figure 3. Entomopathogenic fungus infecting Sasajiscymnus tsugae.

and gregarines). Rearing lab managers should be watchful for unexplained mortality; viral diseases of insects, for example, are very difficult to recognize because most are not detectable under light microscopy and some, like the microsporidia, may cause chronic, relatively low intensity infections, or occur at low prevalence levels until laboratory rearing conditions stress the hosts and allow for unusual levels of transmission.

MITIGATION OF MICROSPORIDIAN DISEASE IN LABORATORY COLONIES

To reduce the possibility of contaminating laboratory colonies with infectious organisms and, thus, compromising mass rearing and field releases, a series of protocols for handling and evaluating colonies should be developed and carefully followed. Broadly considered, individual insects should not be crowded into cages during field collection. Insects collected from different populations should be held in separate collecting cages and equipment such as aspirators, vials and cages should be cleaned with a microbiocide (5% bleach is adequate) and rinsed well before collecting from different populations. If possible, different field populations should be reared in isolated groups until screened for pathogens. Field collected insects also should be isolated from the main laboratory colonies until they are screened for pathogens and parasites, and for at least one generation before being added to the main colony. Screening of field collected insects to be introduced to the colony should include evaluation of all beetles that die during transport or in the laboratory, adults after mating and oviposition, and all larvae reared from field collected adults that die during development. Random samples of the offspring of field collected insects, even if apparently healthy, should be screened before being added to the main colony. In addition, the main colony should be regularly screened by evaluating dead and post-ovipositional adults, and larvae that die during development. Cages should be cleaned and sterilized with diluted bleach after each rearing. Hygienic practices and reducing the overall stress on the beetles during the collection, handling and rearing of beetles in the laboratory will greatly reduce the risk of contamination and spread of many pathogens.

While individual insects infected with microsporidia are seldom curable, colonies that are infected at relatively low prevalence levels sometimes can be saved. There are few treatments for most insect diseases; microsporidia may or may not respond to Fumidil (fumagillin, an anti-microsporidia treatment sometimes used in honeybee colonies) and effects on the host species would need to be evaluated. This chemical typically suppresses development of the microsporidia (the response of microsporidia to the drug is species-specific) but it rarely, if ever, cures the colony. Removal of the treatment usually results in a resurgence of the pathogen that can occur very quickly, a situation that could have negative implications for success of field releases. Heat treatment of eggs is sometimes successful for some species of microsporidia, but may not completely eliminate the pathogen and often causes unacceptable levels of mortality in the hosts.

It is possible to mitigate microsporidian infection in insect colonies by initiating new colonies from uninfected females, a technique employed in the 1800's by Louis Pasteur to control "pebrine disease" (*Nosema bombycis*) in silkworm colonies (Undeen and Vavra 1997). It requires a very labor-intensive process but is frequently successful when initial microsporidian prevalence is relatively low. It is best utilized when first establishing a colony or when adding field collected insects into an established colony. This is a useful protocol even if pathogens are not initially observed in the field collected insects. For this procedure, males and females are mated in isolated single pairs (or sometimes one female and two males to ensure mating) and, after oviposition is completed or at death, the adults are evaluated for infection by destructive sampling. If any adult is infected, all eggs produced by the pair (or mating group) are destroyed. If the adults are uninfected, the offspring are reared in isolation from those of other pairs and other colony insects, and are evaluated by random sampling during development. In addition, all larvae that die are checked for disease. If any one F_1 insect from the rearing group is infected, the entire F_1 stock produced by the breeding pair or group is destroyed. (This procedure also can be used for single gravid females collected from the field.) If no infections are observed, the F_1 generation can be combined with other putatively uninfected F_1 offspring. Ideally, an F_2 generation is produced from these insects and is evaluated for infection prior to addition to the main colony, but this may not be possible due to time constraints, particularly with univoltine insects. The "Pasteur Technique" can also be used when microsporidia are found in low prevalence in a colony by pairing newly eclosed adults. Use of this technique should be accompanied by scrupulous cleaning and sterilization of the rearing facility and of all rearing cages and materials.

Modern biotechnology can support use of the Pasteur Technique, especially for early and rapid diagnosis and detection of microsporidian infection in large numbers of adults or dead larvae, and also for detection of infection in colonies after single pair matings have produced offspring. This group of predatory beetles, however, appears to harbor a complex of microsporidia similar in diversity to those found in Lepidoptera, so to use PCR detection to its full extent, pathogens must be identified and stored properly, DNA extraction methods need to be refined, and primer construction is needed when "universal" primers for microsporidia do not amplify DNA.

SUMMARY

Pathogens are perhaps the most serious of the issues associated with mass rearing of insects in biological control programs. Because of the chronic nature of some of the more insidious disease organisms, they are often not noticed until the colony is in danger of being completely decimated. Field collected insects that will be used to found colonies or will be added to existing laboratory colonies should always be isolated and evaluated for pathogens so that extant rearing programs are not compromised. Frequent assessments of colony health that include presence and prevalence of pathogens should be routine activities for establishment and maintenance of mass-reared insects.

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Chapter 13: Defining PC/QC Standards for Mass-Rearing HWA Predators

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ABSTRACT

We have defined guidelines to help mass-rearing laboratories establish programs of process control (PC) and quality control (QC) in the production systems where *Sasajiscymnus tsugae* and *Laricobius nigrinus* are reared. We have included process and quality characteristics that span a range of technological difficulty and organizational levels. The tests for measuring these characteristics include biomass, morphology, biochemistry, and behavior. We emphasize the importance of using statistically-based QC and PC systems according to the protocols established by the industrial engineering community.

INTRODUCTION

Systems of process control (PC), quality assessment (QA) and quality control (QC) have been in use in industry for nearly 100 years, and they have been more recently applied to insect rearing over the past several decades. Using PC/QA/QC systems properly greatly adds to the value of the products in question and to the economy of their production and to consumer/user satisfaction. Chambers' (1977) excellent review of quality control explains the biological aspects of quality and how they can be applied in mass rearing systems.

MASS-REARING FRAMEWORK

Importantly, Chambers points out that numbers of insects produced in true mass-rearing systems over time must exceed 10,000 → 1,000,000 times the average fecundity for an individual female over the cycle of one generation. Along with the numerical constraints (quantities) that Chambers sets forth, he also treats the quality assessments that result in successful programs. These quality considerations have served as the basis of many mass-rearing systems and include biological features such as weight, mobility measurements, search capacity, fecundity, fertility, development rate, longevity, and Chambers also reviews other biological aspects such as pheromone production and response, sound production, and other features that reflect the health and vigor of insect to be used in large scale programs such as biological control or genetic pest management. We note that the rearing systems for HWA predators do not produce the numbers to qualify as mass-rearing by the standards of Chambers; however, the tenets of process control and quality control can still be applied profitably to these smaller-scale rearing programs.

Therefore, in this chapter, we focus on development of a practical system of process control and quality control. We distinguish these concepts as follows: 1) process is the series of events, procedures, and materials involved in the production of the product (in this case, the HWA predators); 2) the quality of the end-product (*S. tsugae* and *L. nigrinus*)

means the relative ability (or capability) of the product to do the job for which it is intended. In this chapter and in the PC/QC program that we are developing, we try to present a systematic analysis of the processes involved in mass-rearing these HWA predators to allow early detection of flaws in the process. We further try to define the standards that characterize the end product as capable of controlling HWA in hemlock forests. We also try to make the PC/QC system one that harmoniously fits the current efforts, minimizes the efforts of production teams, and adds to the value of the product without adding substantially to the cost. In accord with other PC/QC systems in industry, all these goals can be achieved by application of appropriate techniques that are based on careful study of the existing system of production. Several authors have treated quality control including reviews by Boller (1979), Boller and Chambers (1977), Chambers (1977), Calkins et al. (1996), and a comprehensive work that updates modern QC standards, Dyck et al (2005). Reviews of the genetics of mass-production of insects are provided by Bartlett (1984, 1985) and Mackauer (1976). Finally, an excellent introductory statement about the need for quality control and process control is provided by Bigler (1989). He expressed the treatment of quality control and process control in earlier applications to insects:

> Boller and Chambers (1977) divided the overall quality of fruit flies reared for sterile insect release programmes into major quality components, traits and measurable parameters. The question remains whether laboratory assessed traits or attributes have a predictive value for the performance of an insect in the field. Mackauer and Van Den Bosch (1973) and Messenger et al. (1976) concluded that it is hardly possible to identify attributes which will "precisely" characterize an effective biocontrol agent for a particular situation. The first problem is the clear definition of *what* attributes are to be measured.

PRODUCTION PROCESS FOR SASAJISCYMNUS TSUGAE

An analysis of the rearing process must be performed, starting with a listing of all the elements of the rearing system. In the case of *S. tsugae*, the components of the process are as follows:
1) collection of insects to start colonization;
2) holding P generation adults in containers;
3) feeding them adelgid prey presented as infestations on hemlock; 4) supplementing natural diet with honey, Wheast, or other supplements;
5) adding water as a spray or in some other manner;
6) allowing oviposition and either collecting eggs or allowing them to remain in adult cages;
7) harvesting F_1 generation to start new cage; and
8) continuing process by repeating steps 1)-8) to produce subsequent generations (F_2, F_3…F_n). At some point in the process $P \rightarrow F_n$, a harvesting step is added where some stage (usually adults) are removed from the colony and prepared for release. As Chambers (1977) points out, the harvesting/preparation/release step is very important and can be the point of great losses in quality and failure of the system. However, the scope of this chapter is confined to the production steps.

PRODUCTION OF LARICOBIUS NIGRINUS

The steps in production of *L. nigrinus* are similar to those involved in *S. tsugae* production, except that the former species includes a step that involves a complex life stage where *L. nigrinus* larvae enter the soil to pupate, aestivate for several months, then emerge as adults in the fall.

In Figure 1, we see the five major factors that can contribute to the loss of quality in a production program for an HWA predator: 1) microbial factors, 2) containers, 3) soil factors, 4) diet quality, and 5) environment. For example, if the production process allows microbial contaminants or pathogens to enter the target insects, these microbial components can either kill or sicken the incipient products (Cohen 2003). One microbe that has gained considerable attention in HWA predators is a species of protozoan known as a microsporidia. Although viruses, bacteria, and fungi have received lesser attention in HWA predators than microsporidia, they can be equally destructive.

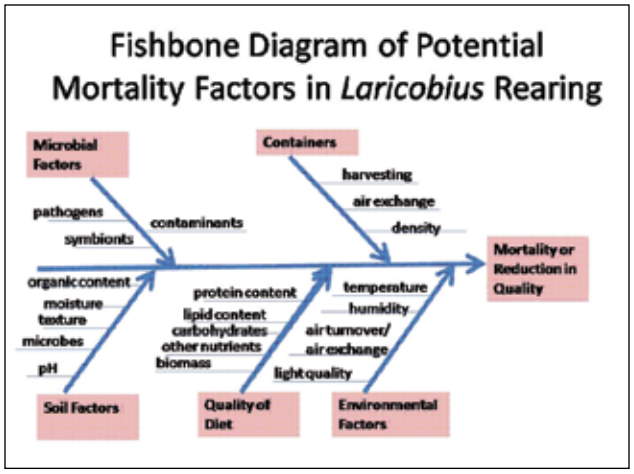

Figure 1. A fishbone diagram that covers five major facets of rearing of Laricobius nigrinus.

Although containers seem to be a simple component, their design can lead to many problems in rearing HWA predators (and other insects as well). Frequently, in rearing situations, improvements in cages can influence gas exchange, heat exchange, moisture retention, and many other factors that can make the difference between a highly successful, economically-sound program and a fail.

For soil-dwelling insects, including *L. nigrinus*, soil features, including texture, moisture content, pH, microbial profile, etc. can be of huge significance in survival and health of the insects (Johnson et al. 2007), and this includes the predators that are products of our rearing systems.

In Figure 2, we extended the hypothesis that over-watering could be a substantial cause of mortality by causing the larvae to drown. Conversely, desiccation could also be a source of mortality, but in our measurements of rearing soils provided by the PABIL laboratory, all soil samples tested had a water activity of close to 1.00 (equivalent to 100% relative humidity). The sphagnum moss in the artificial soil is known to hold moisture to help keep soil air spaces humid. We also had input from rearing labs that they felt that larval nutrition had a strong impact on survival of larvae in the soil.

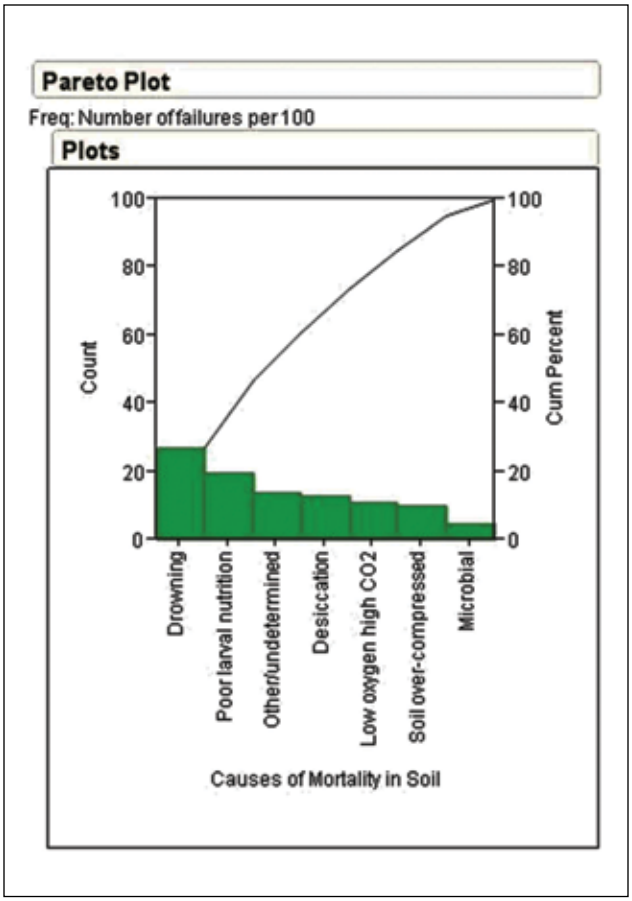

Figure 2. A hypothetical Pareto plot that "dissects" or demonstrates the various potential causes of mortality (failure) in the soil phase of rearing L. nigrinus. The possible causes of failure or mortality were derived from discussions with workers in L. nigrinus rearing labs and from the literature such as Johnson et al. (2007).

In the soil choice pupation experiment, soil sizes represented by coarse and fine hemlock soil, approximated coarse sand and larger particles vs. fine sand, silt, and clay-sized particles, sifted out from soil collected under a planting of urban hemlock trees. Results indicated that when *L. nigrinus* had a choice of different soil textures to pupate in, all pupae were located in coarse hemlock soil, and none in either the standard soil mixture used in laboratory rearing of this species or fine soil (Fig. 3). In all cases of successful pupation, new adults emerged from pupal chambers in the fall.

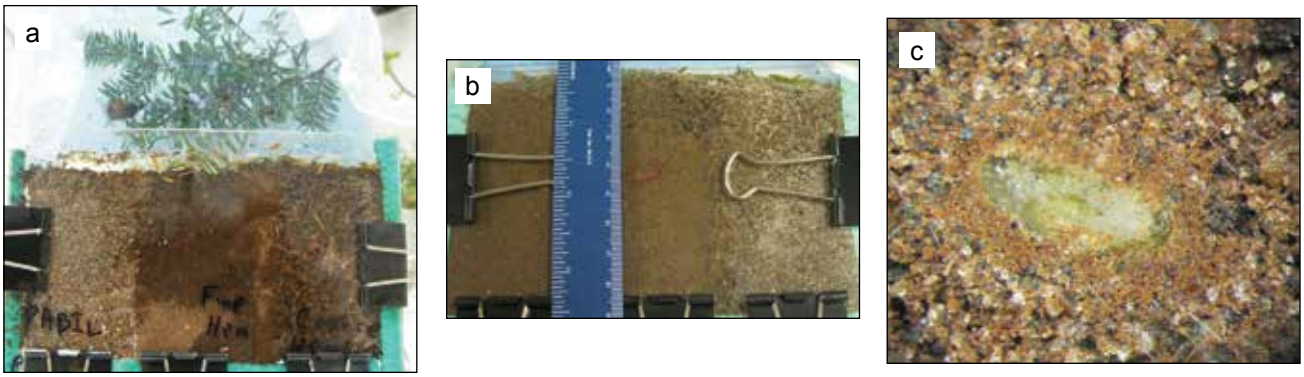

Figure 3. Plexiglass soil sandwiches used to test choices of Laricobius nigrinus mature larval responses to soil moisture, soil texture, organic content, pH, or other soil parameters. The dimensions of the sandwiches are about 9 cm x 10 cm and about 1 cm in width. (a) The soils used in these chambers are, from left to right, standard sand and milled sphagnum mix used in rearing L. nigrinus, fine and coarse hemlock soils sifted to particle sizes of ≤ 0.60 mm and > 0.60 mm, respectively; (b) depth at which a pupal chamber of L. nigrinus (circled in red) was detected in coarse hemlock soil; (c) close-up of a newly formed pupa of L. nigrinus in pupal chamber in coarse hemlock soil (photos by C. Cheah).

As we see in Figure 4, the collection and presentation of data allows us to treat quality in an objective and quantifiable way. The control chart in Figure 4 shows the weights measured in female *S. tsugae* produced over a 45 week period, where collections of 100 beetles were weighed, and average weights calculated. It is evident from this chart that during two periods over the whole rearing interval were notable for weights dipping below the lower control limit (LCL). These declines in weight during Week 24 and Week 38 must be considered indications that the process was out of control and that the products were of inferior and unacceptable quality during these periods.

It is important to note that the biomass or weights of *S. tsugae* have not definitively been shown to be indicators of quality, i.e. to be related to the desirable characteristics of voracity, large search capacity, longevity, and high fecundity, but we are assuming that the correlations exist. Therefore, the underlying hypothesis of this portion of our study is that a certain, minimal mean body weight is correlated with the biological characteristics stated above.

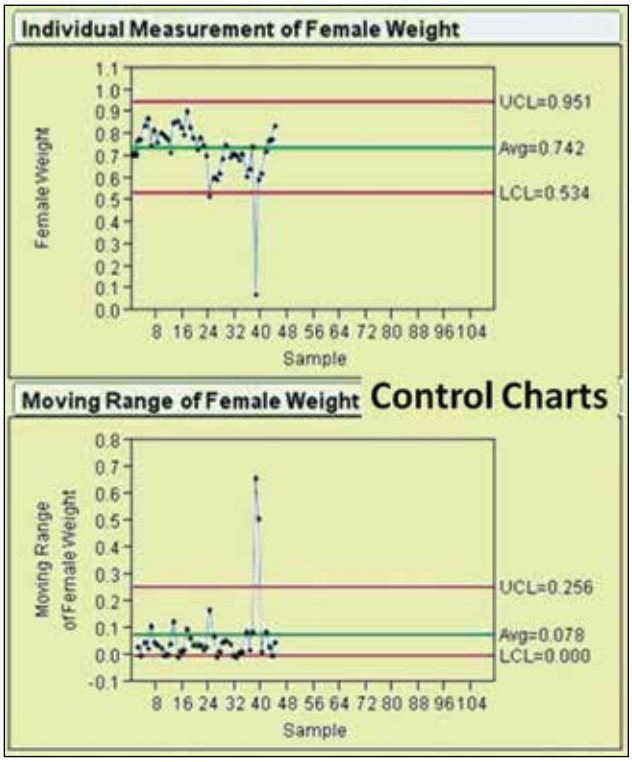

Figure 4. Range charts based on data from Sasajiscymnus tsugae program at the North Carolina Department of Agriculture's Biological Control Laboratory.

This leads to the question of "upstream" issues having potential effects on production. Clearly, in light of the high cost of the HWA predators, it would be advantageous to catch the problems that cause defects before they are manifested in the final product. This is especially important in predators such as *S. tsugae* and *L. nigrinus*, which have very long life cycles with a large input of materials (hemlocks infested with HWA, cages, soil) and labor. The entire rearing process from adults in one generation to adults in the next generation takes months (nearly a year, in the case of *L. nigrinus*). Figure 5 and the ensuing discussion explain the distinctions between product control and production control.

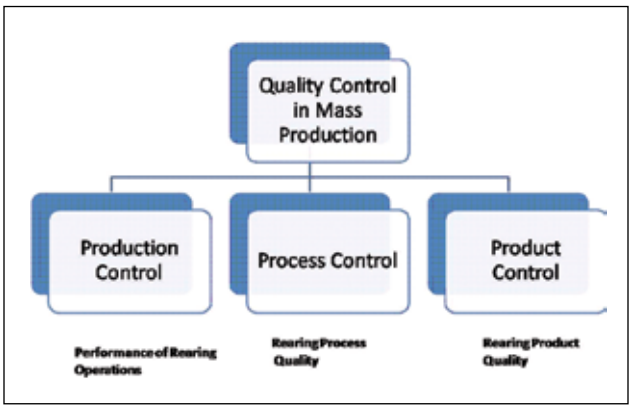

Figure 5. The components of a quality control system for mass-reared natural enemies, adopted from Leppla and Fischer (1989) and from discussions by Penn et al. (1998).

Strictly speaking, when we discuss product quality, we are focusing on the characteristics of the end-product and how they meet the needs for which the product was intended. In our case, this product must have the characteristics that lead to the control of HWA to reduce pest populations below a biological threshold, which translates into preventing HWA from killing hemlock trees. In the discussions by Leppla and Fischer (1989) and Penn et al. (1998), there is a separation of "process" and "production" control where the process is analyzed by measurement of the materials, including the biological materials such as the immature stages of the insects being produced, are examined and evaluated. We suggest that the diagram could be simplified for convenience, with Production Control and Process Control fused into one category. In our model, overall quality control would be divided into Process Control (where all elements of the production are potential elements of scrutiny) and Quality Control (where the final product is measured in a context of standards developed to assure product capability to perform as expected).

RECOMMENDED PROCESS CONTROL AND QUALITY CONTROL SYSTEM FOR HWA PREDATORS

This leads to the specifics of the process control measures in rearing *Laricobius* and *Sasajiscymnus*.

The areas that we have identified for Process Control are 1) environment, 2) diet materials, 3) microbial factors, 4) soil factors, 5) container factors, and 6) genetics. We note that genetics would be important to assess, but there has been little background established for assessing genetic factors. Therefore, we summarize our recommendations for the further development of measurement of the five components mentioned here and illustrated in Figure 1.

1) Environmental Factors can be evaluated by the use of a temperature, humidity, and light measurement data logger (or similar device). We have found that Hobo-type data loggers are small enough to fit into rearing containers, and they can be programmed to report temperature, humidity, and light intensity over a one-week period. The accumulated data can be downloaded to a computer so that deviations from standard conditions can be detected by examination of the graphs that the data loggers and their software support. This technique is discussed in detail by Cohen (2003). It is important to mention that for under $500, a laboratory can be equipped with four data loggers and the software required to read the loggers. We

further suggest that the nearly continuous output of the data loggers gives insectary workers a much more comprehensive sense of deviations in the rearing system. Other kinds of sensing systems can be used to detect other parameters such as CO_2 or O_2 concentrations.

2) Diet Factors: There is common agreement that predators' diets are among the most important determinants of quality. Yet, there is no clear definition of what is meant by high or low quality diets (Cohen, personal observation). Basing the following discussion on interviews with rearing personnel, judgments of prey quality are based on a) the condition of the tree, b) the number of woolly masses present, c) the size of the woolly masses, and d) the numbers of eggs present in egg masses. We have tried to elaborate on these factors to include biochemical/biomass factors including: a) weight of excised woolly masses, b) protein content, c) lipid content, d) carbohydrate content, and e) antioxidant content of woolly masses. We developed or modified tests for measuring 1 to 10 HWA for each test. We found that the presence of HWA "wool" complicated the analysis of the nutritional factors, especially lipid analysis.

   Protein: To determine protein content, we refined a dye-binding test according to modifications of the method of Heller and Sherbon (1976), (see Udy website). This method involves the homogenization of HWA tissue in a solution of Acid Orange 12 Dye, then centrifuging and measuring with a spectrophotometer at 480 nm. When the disappearance of color is compared with a standard curve established with authentic proteins of known concentrations, the protein content of individual HWA woolly masses can be determined.

   Lipids: The total lipid concentration of egg masses is determined by the vanillin method, which is a colorimetric procedure performed similarly to the protein test, using a spectrophotometer. As with the protein determination, authentic standards are used to establish a standard curve. The method is explained by van Handel (1988) as is the following analysis of carbohydrate concentrations. In the vanillin method, the materials to be tested, such as insects, are homogenized in concentrated sulfuric acid and phosphoric acid, then reacted with the vanillin reagent.

   Carbohydrates: Both soluble (free) sugars and glycogen can be determined by using the anthrone test with samples of HWA and comparison with standard curves as described by van Handel (1988). Like the vanillin/lipid test, the anthrone test is performed with sulfuric acid, which breaks down (hydrolyzes) the organic components, including all kinds of carbohydrates, which then react with the anthrone molecules to form a colored product whose optical density can be read colorimetrically and compared with known carbohydrate standards.

   Free-Radical Scavengers: We have determined that the most simple and comprehensive test of free radical scavengers (anti-oxidants) in diet materials and in the insect products is the colorimetric DPPH method described by Cohen (2003) and Cohen and Crittenden (2004).

3) Microbial Factors: Although there is a potential that any of several taxa of pathogens may adversely affect the HWA predators in our production systems, Dr. Lee Solter (personal communication) has stated that the most common and serious microbial threats to HWA predator-quality are the protozoan pathogens known as microsporidia (Phylum: Microspora, by the classification of Undeen and Vavra 1997). In light of Solter's findings, we strongly recommend that HWA production facilities include a search for pathogens. A trained technician can perform tests of predators by making wet-mounts or Giemsa or Gram stains of dry mounts (Undeen and Vavra 1997). With these stains and a phase-

contrast microscope, microsporidia-infected individuals can be efficiently recognized. We must add, however, that the number of specimens that must be examined expressed as a percentage of the population that is to be released has yet to be determined.

4) Soil Factors: Predators belonging to the genus *Laricobius* spend more than half of their life-cycle (4-5 months) in the soil as pre-pupal larvae, pupae, and newly-eclosed adults, which must dig their way out of the soil to seek populations of HWA prey and mates. We found that *L. nigrinus* larvae burrow about 4-5 cm into the soil (Figure 3b). Once larvae reach the appropriate depth, they pupate and remain in their pupal cocoons (Figure 3c) until they are ready to emerge as adults. In Figure 1, we list several soil-related factors that we hypothesize as related to biological fitness and survival of *Laricobius*.

5) Container Factors: The environmental factors can be major forces in determining the quality or loss of quality in any insect, but researchers who study HWA predators have discovered that these insects are especially attuned to temperatures and light/dark cycles that signal seasons and potential prey availability. Given the importance of environmental conditions, we recommend a rigorous attention to light, temperature and humidity conditions in insectaries and especially within cages. The technology of the cages, including sites of foliage placement, degree of crowding of foliage and beetles, and mechanisms for harvesting can greatly affect numbers of predators being produced and also the quality of these predators. When we consider the architecture of a hemlock tree in nature, it becomes clear that the arrangement of shorn branches in cages can become a maze, rather than a natural series of corridors for beetles to discover their prey. As far as predator density is concerned, the derodontids and the coccinellids in the HWA predator programs are not strongly cannibalistic as are some predators, but they clearly can become competitors when resources such as high quality prey are scarce or inadequate.

6) Genetic Factors: Too often, declines in production and/or quality of insects is attributed to genetic truncation or deterioration, but too seldom has the genetic deterioration hypothesis been confirmed as being causative (Bartlett 1985). In fact, Cohen (2003) has summarized a number of failings in diet or environment, or simply personnel errors that were documented to cause declines in quality or production numbers. Hopefully, with molecular methods having become readily available, insect rearing programs will be able to develop a clearer understanding of causes and effects in genetic truncation or genetic shifts that are clearly inherent in mass-rearing.

THE FINAL PRODUCT: QUALITY CONTROL OF PREDATORS THAT ARE TO BE RELEASED

The tests of quality that we recommend for both species are 1) weights of adult beetles, 2) sex ratios, 3) internal morphology/development, 4) protein content, 5) carbohydrate content, and 6) voracity.

1) Weights of individual adults can be determined with a balance sensitive enough to read to 0.01 mg (10 µg), or if a less sensitive balance must be used (such as analytical balances that read to 0.10 mg (100 µg), collections of either 10 individuals or 100 can be weighed in pools. Weights can be evaluated for their fit to process control charts (Figure 4).
2) Sex Ratio: For *S. tsugae*, sex ratios can be determined externally, using live beetles. For *L. nigrinus*, sexing would have to be done by examining internal morphology. Normal sex ratios of both species are approximately 1:1 (males: females).
3) Internal morphology/development: These tests must be performed with dissected insects, and we have determined that a sampling of 6-10 insects is adequate to reflect the condition of the population as a whole (Fig. 6).

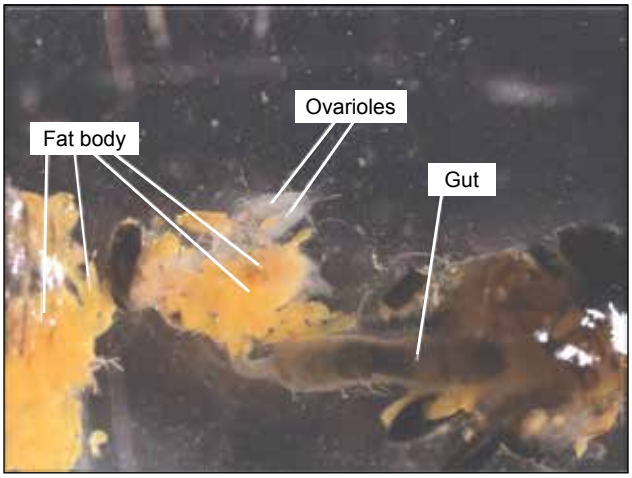

Figure 6. The internal structures of a female Sasajiscymnus tsugae, showing the poorly developed ovaries and fat body. It is evident from this image that the insect had recently fed, but the internal organs are not developed to a point where the insect could soon reproduce. On a scale of 0-10, with 10 being fully developed and ready to lay eggs, this insect would be rated as a 2-3 (photo by A. Cohen).

4) Protein content: We recommend use of the dye-binding test known as the Acid Orange Test, which is outlined above under "Process Control." In Figure 7, we present a standard curve for authentic proteins measured as a comparison with the proteins from either diet materials (HWA) or predators.

5) Carbohydrate content: We suggest the anthrone test, which is same type of analysis used for HWA (above in section on Process Control).

6) Voracity: A voracity or feeding vigor test of a sub-sample of beetles that are to be released is important. Each beetle should be given a twig with 30 HWA adults with eggs confined in a 9 cm diameter Petri dish at optimal temperatures and light cycles for each species. After 72h, the number of prey consumed should be measured by visual observation of disturbed woolly masses and/or consumed adelgid stages. The numbers consumed should be compared with a control chart to determine whether or not the voracity is comparable to the established mean.

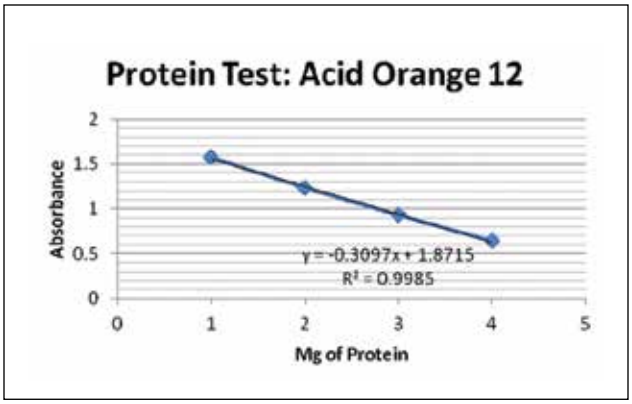

Figure 7. A graph of absorbance at 482 nm vs. protein concentration (1-4 mg).

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Chapter 14: Development of Artificial Diets for Predators of Hemlock Woolly Adelgids

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ABSTRACT

We report the results of the process that we undertook to develop a rearing system based on artificial diets or factitious prey for two species of specialist predators of hemlock woolly adelgid, *Adelges tsugae* (HWA). The predators in these studies were beetles (Coleoptera), *Sasajiscymnus tsugae* (Coccinellidae) and *Laricobius nigrinus* (Derodontidae). Besides testing more than 50 different artificial diets, we also attempted to use factitious prey, including insect eggs, insect larvae, and annelid worms. We also experimented with a variety of diet presentation systems that were designed to fulfill the feeding requirements of the two beetle species and to meet the needs to preserve the diets to prevent desiccation and deterioration. Although we had little success with most of the artificial diets and none of the factitious prey, we succeeded in developing several forms of a hen's egg-based diet and a diet presentation system that involved both gels made from alginate and slurry diets that were made from adhering liquid materials to a proprietary solid/capture medium. The most successful diets and diet-presentation systems allowed adults of both species of predators to stay alive and active for several months and to return to egg production after being returned to natural hosts (HWA) for a few days. Larvae fed readily on these chicken egg-based diets, but they failed to develop on any of the diets.

INTRODUCTION

Biological control of invasive adelgids remains one of the most promising means of control for these threatening pests. Predators have been the most emphasized biological control agents (Grenier et al 1994). Currently, rearing programs for two widely-used predators (*Sasajiscymnus tsugae* Sasaji and McClure, Coleoptera: Coccinellidae, and *Laricobius nigrinus* Fender, Coleoptera: Derodontidae) use hemlock woolly adelgids, *Adelges tsugae* Annand (Hemiptera: Adelgidae), collected from eastern hemlock, *Tsuga canadensis*. The collection of branches infested with adelgids is a costly process in terms of labor, travel, and destruction of large portions of the trees. Clearly, this method of rearing predators imposes severe limitations to the scale of production. A further complicating factor is that the complexity of the HWA life cycle (Figure 1) imposes further limitations on how many predatory beetles can be produced and even more constraints on the quality of the predators. Therefore, an artificial means of supplying high quality nutrition to HWA predators would be a tremendous advantage to HWA control programs.

When we specify "artificial nutrition", we imply either 1) insect prey that are not natural hosts to the predators, known also as factitious hosts, or 2) artificial diet that is composed partially or entirely of non-insect derived materials. Several species of predators (ladybeetles, pirate bugs and lacewings)

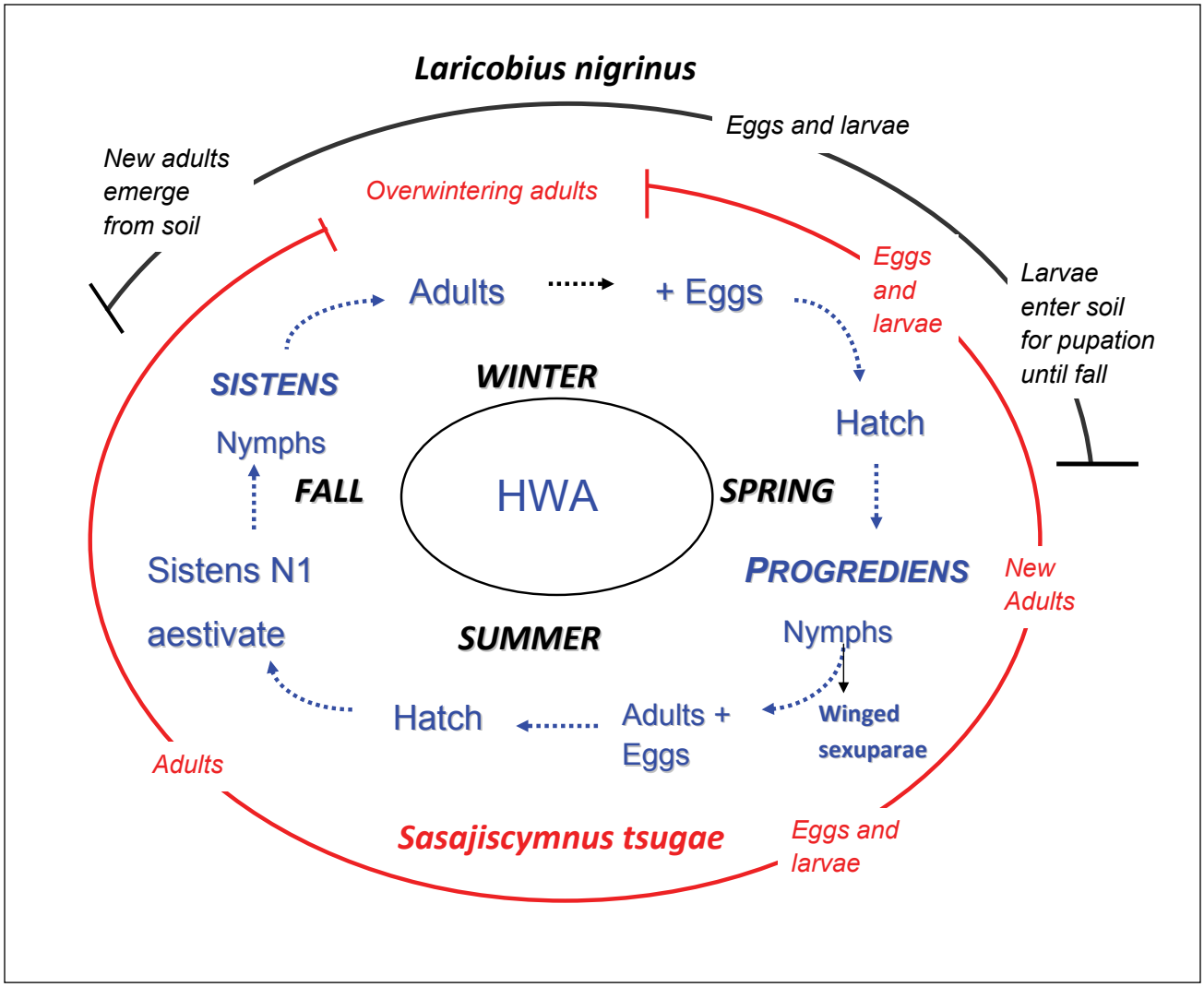

Figure 1. Life cycles of the hemlock woolly adelgid (------) and its introduced predators, Sasajiscymnus tsugae and Laricobius nigrinus in eastern North America. Solid lines indicate relative active periods of adult and larval predator feeding on the adelgid (——— S. tsugae; ——— L. nigrinus); Cheah 2011.

and parasitoids (such as *Trichogramma*) have been reared successfully on factitious hosts such as the eggs of brine shrimp (Arijs and de Clercq 2001, Castane et al. 2006) and the eggs of various grain moths such as the Mediterranean grain moth *Ephestia kuehniella* (Pyralidae) (Bonte et al. 2010) and the Angoumois grain moth, *Sitotroga cerealella* (Gelechiidae) (Abdel-Salam et al. 2001). The advantages of using factitious hosts are that they are more conveniently available and generally less costly than natural hosts (Cohen 2003), but the disadvantages are that they are not always the most nutritious prey, and they are more expensive than artificial diets. Besides nutritional value and palatability, a further consideration in various foods used in mass-rearing is the packaging of predators' foods. The cuticle of natural and factitious hosts is a rather incredible packaging material, being made of chitin. While the cuticle is strong and water-proof, it can be as thin as 5-10 μM. This means that even predators with very small, short mouthparts can penetrate the cuticle and gain access to the foods. Furthermore, it is important to understand that many predators (including those in the current study) feed by extra-oral digestion where they inject digestive enzymes into the host to pre-digest

it (Cohen 1990). The highly nutritious slurry that exists as a digestive product is then ingested, leaving behind the nearly intact cuticle empty of its previous contents. Also relevant to this discussion of packaging is the fact that the adelgids as prey are very small "packages" of nutrients (about 1-10 µg) which the specialist predators are fully adapted to feed on and thrive. Likewise, factitious prey, such as *Ephestia* eggs, are small (ca 20 µg), and they resist microbial contamination and chemical deterioration, in part by being so small and separate from one another, that contaminating micro-organisms do not spread through an egg mass. All this being said, it is clear that a mass rearing system for *S. tsugae* and *L. nigrinus* would be well-served if it could utilize factitious prey instead of natural hosts. In Figure 2 (a-d), *S. tsugae* are pictured feeding both on the natural prey (HWA) and artificial diet. In this case, the diet presentation is either in the form of natural eggs of HWA or the chicken egg diet offered as an alginate gel whose surface was made into a film by allowing the diet to interact with a calcium compound used as a cross-linking agent.

The description of natural and factitious prey leads to a comparison of the issues with artificial diets and the requirements of a diet presentation system for *S. tsugae* and *L. nigrinus*. As explained by Cohen 2003, for an artificial diet and diet-presentation system (called "diet system" for this chapter) to be considered completely successful it must:

1. Stimulate robust feeding
2. Support survival
3. Support growth and development
4. Support reproduction
5. Allow production of continuous generations indefinitely
6. Support high quality insects that are fully useful for their intended purpose (biological control, genetic pest management, food for other species, conservation, education, research, etc.)

Relatively few diet systems have been developed to meet all these specification, with a rough estimate of about 20 basic diets that have been shown to support about 300 species (Cohen 2003, Singh 1977). The accepted diets developed for *S. tsugae* and *L. nigrinus*, have supported survival in the absence of adelgids for several months (*S. tsugae*) and one month (*L. nigrinus*), with negligible mortality. Adults of *S. tsugae* fed exclusively on diet were able to commence normal reproduction on return to adelgid-infested foliage, although eggs were not laid while on diet only. Use of the most successful diets as supplements have improved laboratory survival of adult predators when quality and or quantity of adelgid-infested foliage was degraded or depleted.

Even fewer diets have been developed successfully for predaceous lady beetles (Kariluoto et al. 1976, Racioppi et al. 1981, Hodek 1996), the most notable success being that of Attalah and Newsom (1966), who reared eight successive generations of *Coleomegilla maculata* De Geer on a diet void of insect materials. Several authors reported using factitious diets such as formulations with powder honey bee brood (reported in Singh 1977). The most successful factitious host diet for a coccinellid in our experience was the use of pink bollworm eggs to rear multiple generations of

Figure 2. Sasajiscymnus tsugae (a) adult feeding on HWA egg sac, (b) larva feeding on artificial diet, and (c & d) adults feeding on artificial diet (photos by C. Cheah).

Serangium parcesetosum in the USDA, APHIS Pink Bollworm Facility in Phoenix, AZ in the 1990s in a program developed by Dr. Robert T. Staten.

SPECIALISTS VS. GENERALISTS

In most programs and research efforts to control forest pests, specialist predators or parasitoids have been used in classical biological control systems (Pschorn-Walcher 1977). Because hemlock woolly adelgid is an exotic pest, only host specific natural enemies have been selected, such as the HWA specialists, *Laricobius* and *Sasajiscymnus*. In accord with the complex life cycle of HWA in the Northeast (Fig. 1), both species of predators have complex life cycles (Cheah and McClure 1998, 2000; Zilahi-Balogh et al. 2003), which track the prey's periods of dormancy, or reductions or surges in nutritional availability (Fig. 1). This "tracking" and "meshing" of the predators' life cycle with that of the prey is an indication of the degree of specialization of the predator. A further indication of the high degree of specialization is the fact that *L. nigrinus* has been shown to be restricted either nutritionally or in terms of feeding stimulation to require HWA to complete its life cycle. This fastidious feeding response, which excludes acceptance of substitute (factitious) prey, raises the question about whether rearing *L. nigrinus* can be possible on foods other than HWA.

The life cycle of the hemlock woolly adelgid is complex, with progrediens and sistens generations, along with phases where the insects actively feed, develop, and reproduce, and other periods when it is inactive, e.g., during summer aestivation. Along with the morphological and behavioral differences in HWA during these different phases, there are also biochemical differences. Our preliminary work on lipid, carbohydrate, and protein content indicates that during certain periods, HWA nutritional value greatly drops, especially during the late phases of their torpor and early stages of feeding activity onset. We have found the nutritional composition of HWA in their inactive phases drops to less than half of what is present in actively feeding individuals. This helps explain the periods of dormancy in predators that are specially adapted to feed on HWA, where their nutritional needs must mesh with their hosts. This concept of predator/prey ecological meshing is depicted in Figure 1.

We have divided our experiments and observations into three major categories to cover all aspects of our research on development of artificial diets and rearing systems for predators of HWA:

I. Feeding on Natural Hosts (Prey)
II. Feeding on Factitious Hosts
III. Feeding on Artificial Diet
    a. Various artificial diets
    b. Diet presentation techniques
    c. Factors in diet stability

Natural Hosts

The different life stages of HWA offer different nutrient rewards to predators, especially with respect to 1) overall biomass, 2) protein content, 3) lipid content, 4) carbohydrate content, and 5) vitamins and minerals. We have begun analysis of these factors in several of the life-stages, but because these studies are preliminary, we can provide only partial results. Using the analytical techniques outlined in the chapter (Chapter 13 in this volume), we have found that eggs that have been oviposited, as well as eggs that are inside females, have a high lipid content, with approximately 50% of the dry weight of an egg being lipid. The protein content ranges from about 30-40% of dry weight and the carbohydrate content is less than 10%, leaving about 4-5% ash (minerals) and a small biomass (less than 3%) composed of other components such as nucleic acids and components derived from host plants. These findings are in accord with Cohen and Patana (1985) regarding the nutritional composition of eggs. However, adelgid eggs and neonate larvae have a higher lipid content than comparable lepidopteran eggs and neonates.

It is evident from Figure 3 that a great deal of lipid material, especially oil, is stored in the eggs and remains present in the neonate crawlers. The oil is present as a storage material providing

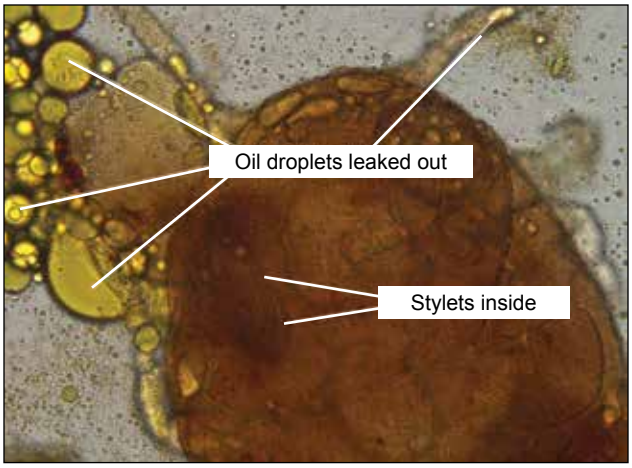

Figure 3. A pre-hatched 1st instar adelgid nymph that had a gentle pressure applied to express the droplets of oil that are present inside the insect (photo by A. Cohen).

energy and bio-materials for crawlers as they settle on host plants and begin their feeding forays. The lesson that we learn from analysis of natural prey is that HWA predators, especially those that feed on adelgid adults with eggs, derive a large lipid component from their diet.

Factitious Prey

The use of factitious prey or hosts has a long history in rearing entomophagous insects. For example, the Mediterranean grain moth *Ephestia kuehniella* (Pyralidae) and the Angoumois grain moth *Sitotroga cerealella* (Gelechiidae) have been used extensively in commercial predator and parasitoid production of green lacewings and egg-parasites such as *Trichogramma* spp. (Cohen and Debolt 1983; Cohen and Smith 1998).

In Figure 4a, an egg that had been fed on by *S. tsugae* is seen with its characteristic depletion of materials removed by the predator. The egg is also dark as a result of the polyphenol oxidase action that takes place during the extra-oral digestion process (Cohen 1995). Because *S. tsugae* fed minimally on *Ephestia* eggs, we also tried to "disguise" the *Ephestia* eggs by placing fresh adelgid wool and exudates around the eggs, to determine whether or not the HWA materials would enhance predation. All uses of factitious prey met with little success and a very limited amount of feeding on such prey.

Feeding on Artificial Diet:

a. Various artificial diets

Starting with the Cohen and Smith 1998 diet, we tested more than 100 different diets. The formulations that we have deemed most successful are proprietary combinations of cooked chicken egg mixture with functional diet components that are suspended in a freeze-dried (proprietary) carrier material. The formulation is again freeze-dried and stored until used with appropriate re-hydrating agents, which are discussed below (under "diet presentation techniques" and "diet preservation or stability techniques").

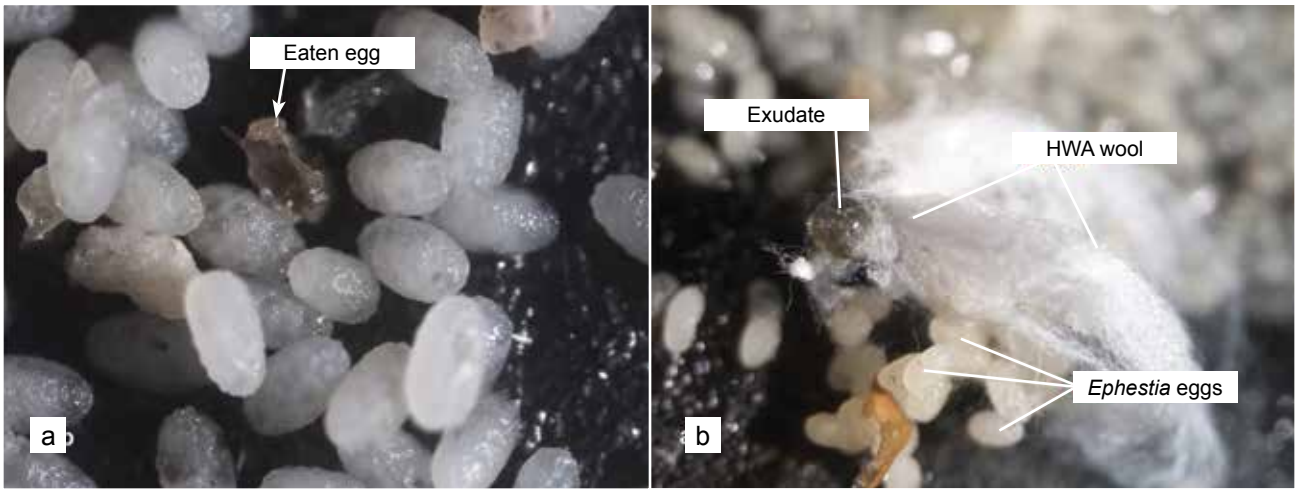

Figure 4. Eggs of Ephestia kuehniella that were exposed to Sasajiscymnus and Laricobius, indicating (a) minimal feeding and (b) eggs wrapped in adelgid wool (photos by A. Cohen).

b. Diet presentation techniques

It has long been realized that diet texture and presentation techniques can be as equally important as the nutritional composition of the diet itself (Singh 1977, Cohen 2003). Diet researchers have often resorted to the "missing nutrient hypothesis" (Cohen 2003) to explain the reasons for a diet's failure. This hypothesis was advanced in early studies of insect nutrition by such eminent researchers as G. Fraenkel and S. Beck, who had discovered cryptic nutrients that were essential factors for insects as a whole or target species. For example, the discovery that sterols were essential to insects went far to explain why earlier formulations that lacked sterols failed to support insect growth. Similarly, the discoveries of carnitine and choline as essential to some insect species further supported the "missing nutrient hypothesis". However, extensive analysis and empirical trials during many diet-development studies did not reveal hidden nutrients that could result in successful artificial diets for a large number of insect species. Such failures may very well be explained by alternative hypotheses such as texture failure or packaging (or presentation) failure. For example, if an insect uses extra-oral digestion, presentation of a complete diet that is in liquid form can be unsuitable (Cohen 1985, Cohen 1995, Cohen and Smith 1998).

Other issues in diet presentation include whether 1) the diet is covered with a material that can be penetrated by the target insect's mouthparts, 2) the diet covering is capable of preventing excessive water loss or other degradation factors such as contamination with microbes. In response, diet researchers have used films such as Parafilm™ or Whatman Laboratory Film, the dipping of diet in molten wax, or encapsulation techniques such as those illustrated in Figure 5.

Some feeding responses of *S. tsugae* are shown in Figures 6 through 8. *Laricobius nigrinus* adults also appear to accept similar diet formulations originally developed for *S. tsugae* (Fig. 9).

c. Factors in diet stability

Once a palatable and nutritious diet has been developed and the presentation system (encapsulation, film, uncovered diet, etc.) has been established, the next stage concerns the preservation or stability of the diet. In addition to using coverings that protect the diet from contamination, techniques that employ heat, water activity, extremely low or high pH, and/or chemical prophylaxis are conventionally employed.

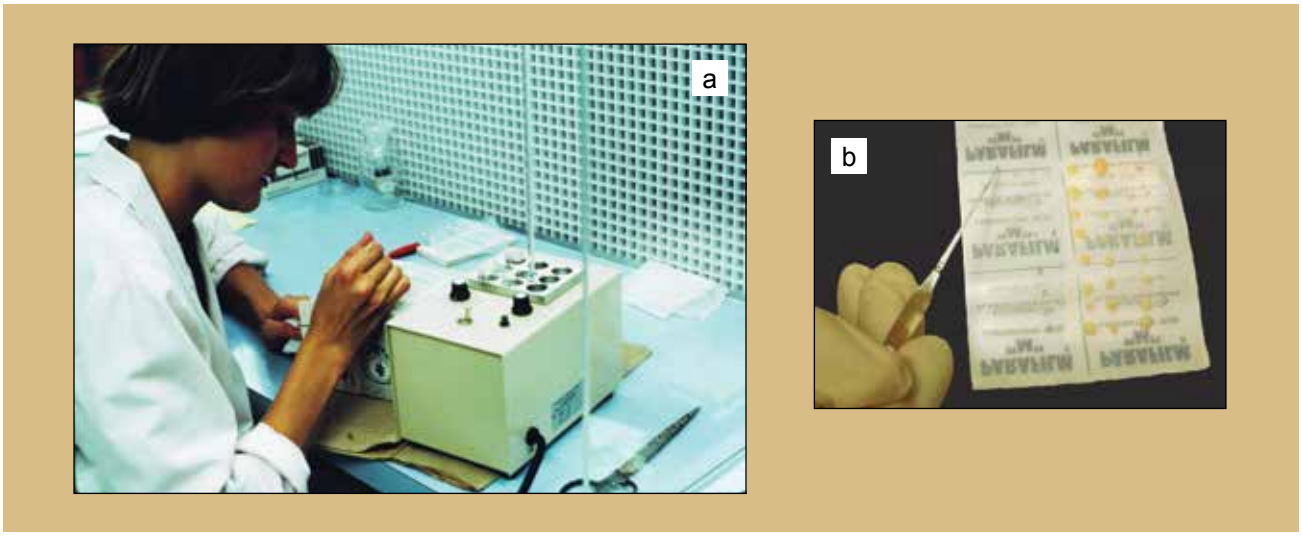

Figure 5. A technician (a) making wax-coated capsules (b) with liquid/slurry centers (according to the method of Cohen 1983; photos by A. Cohen).

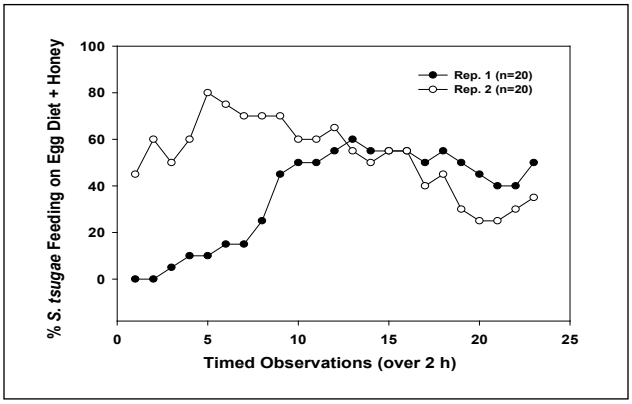

Figure 6. Mature adult S. tsugae feeding response to egg diet and honey presented on filter paper.

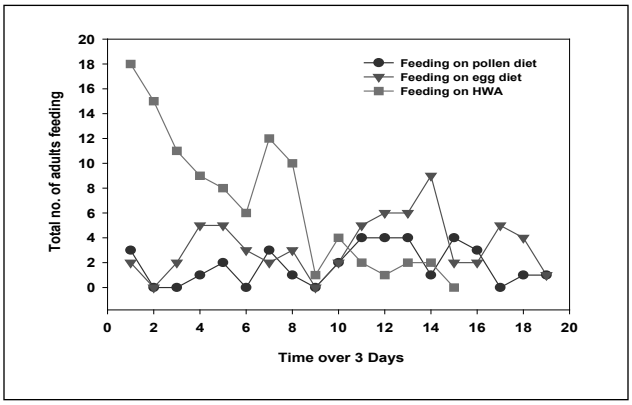

Figure 7. The changes in adult S. tsugae feeding preferences over time when presented simultaneously with artificial diets and HWA.

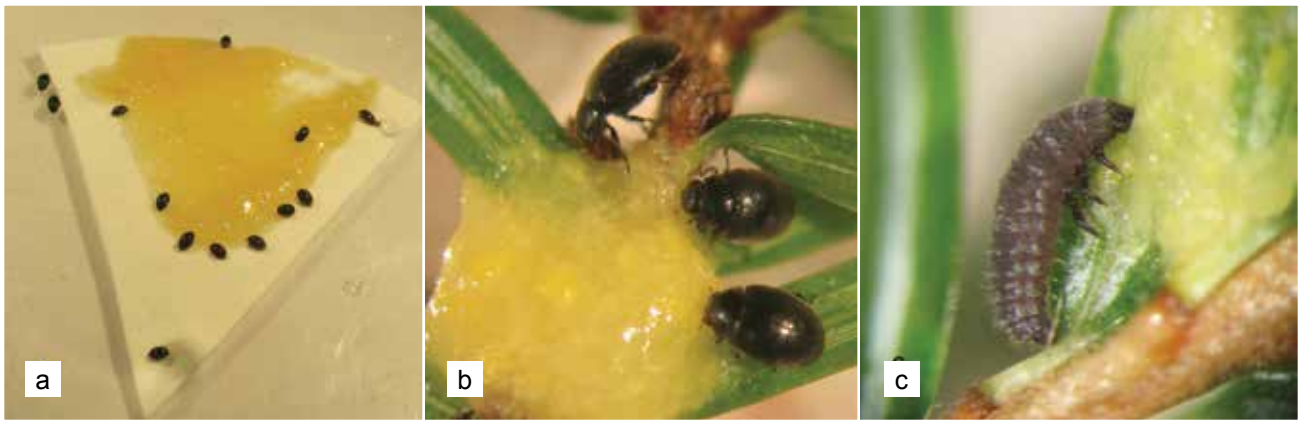

Figure 8. Sasajiscymnus tsugae adult and larval feeding on an egg-based diet which has been mixed with honey or an alginate/calcium base. The diet was presented on (a) filter paper with honey, or as an alginate formulation on hemlock twigs for (b) adults & (c) late instar larva (photos by C. Cheah).

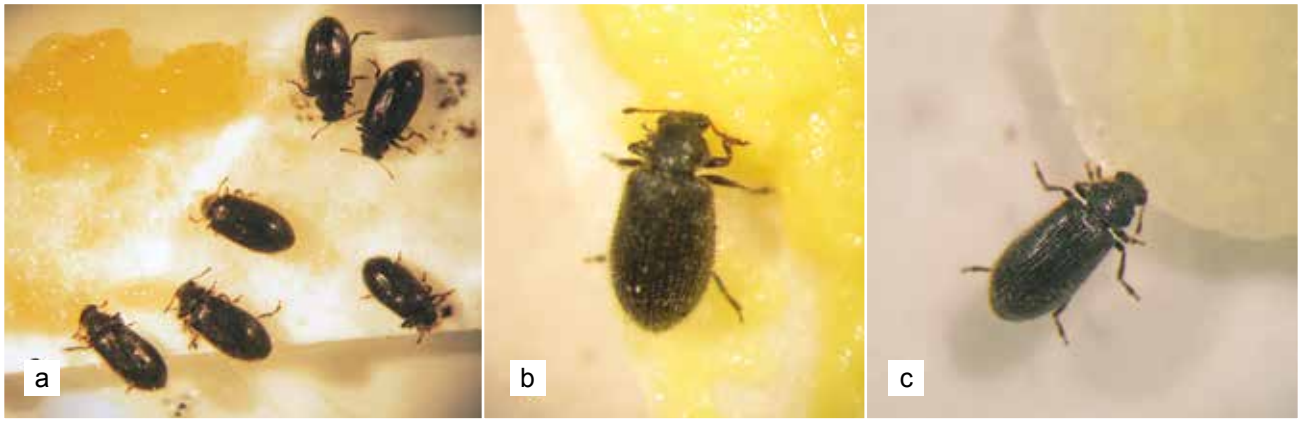

Figure 9. Laricobius nigrinus adults feeding on (a) egg diet mixed with honey, (b & c) egg diet-alginate formulations (photos by C. Cheah).

The use of heat is nearly universal in diet synthesis where diets are heated to the point where all microbes are killed (by autoclaving or other temperature/pressure treatments) or where vegetative stages of microbial contaminants have been killed (Pasteurization). We have found that either method produces diets that can be made acceptable to the predators in terms of maintaining palatability. We have demonstrated this fact with the egg-based formulation that we call FDFE3 diet. However, because the predators can inoculate the diet with microbes while they are feeding, heat treatment alone cannot continuously protect the diet from microbial contamination. Therefore, we resorted to lowered water activity and chemical preservatives.

The concept of water activity (a_w) is a useful way of expressing the tendency of a diet to suffer microbial deterioration and other degradation processes such as oxidation. Water activity is expressed as a unitless ratio of the actual vapor pressure divided by the potential vapor pressure at a given temperature. This can be conveniently thought of as relative humidity divided by 100. Common examples of foods with naturally low water activity are honey and molasses. Although both of these are liquids at room temperature, and although both contain ample nutrients, neither supports microbial growth. Water activity below 0.60 does not support microbial growth of any of the common environmental microbes that habitually contaminate our diets. The range of water activity is between 0 and 1.0, with the lower value being associated with concentrated sulfuric acid and the higher number being associated with distilled water. Most common diets for insects, including most of those that we have tested, have a water activity of 0.98-0.99 (Cohen 2003). This means that the diets are well above the minimal threshold for support of microbial activity, and unless we used special measures to lower water activity, we must expect the diets to be subject to unimpeded microbial growth. In contrast with common insect diets, honey has a water activity of about 0.50-0.55; therefore, it is very uncommon for microbial growth to take place in honey.

Therefore, we have incorporated several measures to lower the water activity of our diets, including the use of freeze drying diets for preservation, then hydrating the diets with honey or similarly low water activity hydrating sources (such as glycerol or sorbitol solutions). We have found honey to be an especially suitable medium for presentation of diets to adult *S. tsugae*. In keeping with our efforts to lower water activity of test diets, we have used freeze drying (lyophilization or cryodesiccation) to prepare diets for storage, transport, then re-hydration with appropriate mixtures to help retain low water activity. We have adopted freeze drying extensively because it not only preserves diet components that are highly perishable (such as eggs), but it also lends itself to diet presentation techniques that maintain low water activity. To clarify this concept, we offer this example. If we lower the water activity of our egg diet from 0.98 to 0.10 by freeze drying, then if we re-hydrate the diet with honey (water activity of 0.50), the overall diet/honey mixture has a water activity of 0.50 or less. This mixture will not support microbial growth, and it has an extremely long shelf life (well over 6 months without refrigeration). We must add that the concept of lowering water activity with materials that have high concentrations of dissolved small molecules (such as sugars, sugar alcohols, or salts) is known as the "***humectant***" strategy.

However, the two drawbacks of the use of humectants is that 1) the material making up the humectant/water mixture may be unpalatable to the target insects (for example, glycerol is not palatable to the predators thus far tested), 2) the mixture can be very sticky and cause insects to become trapped (we have found this with *S. tsugae* larvae), and 3) the humectant/diet mixture can become hydrated (for example, by watering the cage contents to provide free water to the predators or to raise the humidity), and this hydration can raise water activity to above the minimal threshold to prevent microbial growth.

This leads to the 3rd strategy: the use of low pH. We have used various pH lowering agents

such as acetic acid, propionic acid, phosphoric acid, and citric acid (all fairly well-established in insect diets) to lower the diets' pH below 5.0. We have found that acetic acid and citric acid are fairly well-tolerated by the predators, and they are fairly, but not totally, efficient at lowering microbial growth. For example, we have found little problem with bacterial contamination and deterioration of our diets, but fungal contamination remains a problem in our formulations.

Therefore, we have had to resort to the 4th strategy of using chemical agents to prevent fungal growth. We have experimented with several anti-fungal chemicals (benzoic acid, nystatin, methyl paraben, sodium propionate, and potassium sorbate, to mention a few), and we have discovered that the most well-tolerated agents are propionate and sorbate in their salt forms, which makes them soluble in our diets. We also have found that the combination of the acids and the antimicrobial chemicals have added to the efficiency of mold-prevention and the duration of the time frame that diets can be kept in cages. However, we must add that because of the highly nutritious character of the diets, they still attract mold growth after several days or several weeks' exposure to the predators. Therefore, mold prevention remains one of the barriers to completely successful diets for HWA predators.

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SECTION IV ESTABLISHMENT AND MONITORING

Chapter 15: Whole-Tree Enclosures: A Tool to Assess Introduced Predators of Hemlock Woolly Adelgid, Adelges tsugae

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WHY CAGE A TREE?

Many experimental (laboratory and field) methods exist to evaluate the effectiveness of natural enemies against insect pests (Grant and Shepard 1985). Several of these methods (such as Petri dishes, small arenas, growth chambers, greenhouse studies, and sleeve cages) have been used to assess predators of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), since its introduction into North America (Cheah and McClure 2000; Cheah et al. 2004; Flowers et al. 2005; Grant et al. 2005; Lamb et al. 2005, 2006; McClure 2001; Zilahi-Balogh et al. 2002, 2005).

These types of evaluative approaches provide important and essential information on the predatory capabilities of introduced predators, as well as better define their biology, life history, predator/prey interactions, etc. However, the small size, extremely confined spatial resources, and reduced scale of these arenas relative to naturally-occurring hemlock systems limit their use in fully characterizing the impact of introduced natural enemies on field-established populations of HWA on eastern hemlock, *Tsuga canadensis* (L.) Carrière. New tools to complement the existing methods would further enhance and strengthen our abilities to assess predatory capabilities of introduced predators of HWA. Can existing methods be improved or modified to further evaluate natural enemies of HWA? Can a whole-tree assessment approach be developed to better define predatory performance in the field? To address these questions, a pilot study was conducted to evaluate the feasibility of using whole-tree canopy enclosures (i.e., cages) to assess: 1) survival, colonization, interactions, and establishment of introduced predators on HWA on eastern hemlock, and 2) impact of introduced predators on population densities of HWA and on tree health.

This pilot study focused on the use of large cages to qualitatively and quantitatively assess the successful field application (colonization and impact) of three introduced biological control agents (*Laricobius nigrinus* Fender, *Sasajiscymnus tsugae* Sasaji and McClure, and *Scymnus sinuanodulus* Yu and Yao) on populations of HWA in the southeastern United States (Tennessee). This chapter provides a general overview of this project and describes how these cages could enhance our biological control activities against HWA.

HOW DID WE DO IT?

This study was conducted at Blackberry Farm in Walland, Tennessee, near the boundary of the Great Smoky Mountains National Park. HWA was first documented in the Park in 2002 (Lambdin et al. 2006). Cages were designed in 2007 through cooperation of personnel from the University of Tennessee, U.S. Forest Service, and Camel Manufacturing (Pioneer, Tennessee). The resulting nylon-screened cages (ca. 8 m tall;

Fig. 1) were constructed by Camel Manufacturing and erected in the field from October through December 2007. Fifteen HWA-infested eastern hemlock trees (ca. 7-8 m tall) were selected, and 12 cages were erected over HWA-infested hemlock trees (one cage/tree). The following treatments (three replications per treatment) were applied beginning in December 2007:

a) releases of *L. nigrinus* (190 adults/caged tree; January 2008),
b) releases of *S. tsugae* (300 adults/caged tree; March 2008),
c) releases of *S. sinuanodulus* (90 adults/tree; March 2008), and
d) control (no releases of predatory beetles).

In addition, three non-caged, HWA-infested trees also were included as control trees. Adults of *L. nigrinus* and *S. tsugae* were obtained from Lindsay Young Beneficial Insects Laboratory, University of Tennessee, and adults of *S. sinuanodulus* were obtained from the University of Georgia and the U.S. Forest Service Northern Research Station, Connecticut.

Cages remained over the trees for more than 1½ years and were removed in July 2009. Trees were sampled for beetles and HWA densities were assessed every three to four months after cages were removed.

WHAT DID WE LEARN?

Survival and Colonization

Following their releases, all three species of introduced beetles survived, colonized, and reproduced inside the cages, as larvae of each species were recovered in 2008 (Grant et al. 2010b). Adult *L. nigrinus* were found inside the cages in March and November 2008 (about one year after initial placement in cages), adult *S. sinuanodulus* were found in April, June, and July

Figure 1. Nylon-screened cages erected over eastern hemlock, Blackberry Farm, Walland, Tennessee, 2007.

2008, and adult *S. tsugae* were found in April, May, June, July, and November 2008 (about 8 months after initial placement in cages). No *S. sinuanodulus* was found during the November 2008 sampling period. In 2009, prior to cage removal, adult and larval *L. nigrinus* and *S. tsugae* were recovered from trees inside the cages, but no *S. sinuanodulus* was recovered (Grant et al. 2010b).

Impact on HWA Densities

In July 2009, HWA densities had declined about 62% on trees caged with *S. tsugae* and about 85% on trees caged with *L. nigrinus*, suggesting that both of these predatory species reduced HWA populations during this 1½-year period (Grant et al. 2010b).

Establishment and Interactions

In 2010, on previously caged trees, adult and larval *S. tsugae* (F3 to F5 generations) were recovered from February to November (Wiggins et al. 2010a). In 2010, adult and larval *L. nigrinus* (F2 to F3 generations) also were recovered. Following removal of the cages, *L. nigrinus* and *S. tsugae* dispersed throughout the site (Hakeem et al. 2010b, Hakeem et al. 2011). The lack of recovery of *S. sinuanodulus* may be due to the low numbers of adults released inside each cage and/or the low vigor of many of the released adults. These results also suggested that predation of HWA by *L. nigrinus* and *S. tsugae* benefited tree health (Wiggins et al. 2010b).

WHAT CONDITIONS ARE APPROPRIATE?

Several conditions are necessary and certain needs must be considered in using whole-tree canopy enclosures in forest settings (Fig. 2). These include: the availability of a suitable location for 2+ years (the location must be easily available for use, generally secure, few activities in area, etc.), trees of appropriate height (7-9 m tall) for canopy enclosures, trees that are healthy and consistently shaped, trees infested with appropriate densities of HWA (a relatively new infestation is best), trees with new growth, trees that are easily accessible using a bucket truck or lift, trees that are "solitary" (none intermingled/side-by-side), a ground surface that is relatively flat or slightly slanted, and whole-tree canopy enclosures. Once these conditions and needs are satisfied and a design has been developed, canopy enclosures can be constructed and deployed into the field for use in biological assessments.

WHAT ARE THE BENEFITS AND CHALLENGES OF USING TREE CAGES?

Once deployed, researchers must consider the potential benefits and challenges associated with the use of these enclosures. Benefits include:

- a more realistic and complementary field assessment of introduced biological control agents (than other methods)
- long-term monitoring of the impact of natural enemies on an invasive pest and on tree health, as well as predator performance and survival
- a tool to assess single species or combination of species of natural enemies
- a better understanding of compatibility and interactions of introduced and/or native predatory species
- a better understanding of actual predatory expectations in the field

Figure 2. Whole-tree canopy cage in understory of forest/urban interface, Walland, Tennessee, 2007.

The benefits, however, are accompanied by several challenges to the use of these whole-tree enclosures. These challenges include:

- enclosures are still a "controlled" or artificial environment (how similar is "inside" to "outside"? In our cages, however, temperature and humidity levels varied little between open and caged trees.)
- nontarget organisms cannot be removed thoroughly from the trees before they are "caged"
- environmental stresses (e.g., high winds, snow, rain, hail, animals, humans, etc.) that can damage cages are difficult to control
- installation of tree cages may require specialized equipment and/or be labor intensive/time consuming
- high costs associated with initial construction and deployment of cages

WHAT DOES IT ALL MEAN?

The use of whole-tree canopy enclosures provides a viable technique to assess single or multiple species of indigenous or introduced predators against HWA on eastern hemlock. Populations of *L. nigrinus* and *S. tsugae* were successfully established using whole-tree canopy enclosures and their impact on HWA was documented. Several implications can be drawn from the results of this study. First, these enclosures allow the use of whole trees in natural, field situations and provide a better understanding of actual predatory expectations in the field. Similar to sleeve cages, tree cages limit the dispersal of predators to other trees, while limiting influx/colonization of pests from outside sources. Unlike sleeve cages, tree cages allow the evaluation of predators on a whole-tree scale and allow predators and prey to move freely throughout the entire tree canopy. Second, whole-tree canopy enclosures provide a tool to acclimate introduced natural enemies to field situations and conditions, as well as a mechanism to establish natural enemies in the field. Recoveries of established populations of *S. tsugae* using standard release protocols generally occurred five to seven years after release in the Great Smoky Mountains National Park and, in some areas, these predators have not yet been recovered (Grant 2008, Grant et al. 2010a, Hakeem et al. 2010a); however, introduced predators were recovered from hemlock trees after a single release in only two to three years using whole-tree canopy enclosures. Finally, in addition to providing an assessment of predatory performance and survival, this technique facilitates the monitoring of long-term impacts of these predators on HWA and tree health. By confining predators to specific trees for an extended amount of time, tree cages allow the comparison of direct and indirect impacts of predators on the short- and long-term tree health between caged trees and those in the open environment. The use of whole-tree canopy enclosures should enhance our knowledge of the establishment and effectiveness of introduced predators of HWA to improve their use in management efforts.

WHAT DOES THE FUTURE HOLD?

Whole-tree canopy enclosures also could be used to assess other types of tree/organismal interactions, and cages can be modified for other predators or for other tree pests or tree species. For example, a new, more durable tree cage for use in further research of predators of HWA has been designed and constructed (Fig. 3), and these cages will be used in future studies to assess complexes of predatory species and to rear large numbers of natural enemies for re-release. In summary, whole-tree canopy enclosures are a new and innovative approach to assessing natural enemies for release against HWA, they complement other evaluative methods, and can be a vital tool in the management of this invasive pest in forest systems.

Figure 3. Newly modified field cage erected over eastern hemlock, Great Smoky Mountains National Park, 2011.

ACKNOWLEDGEMENTS

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Chapter 16: A Case Study of a Release of the Predator Laricobius nigrinus Fender against Hemlock Woolly Adelgid, Adelges tsugae, Annand, at the Urban Community Forest Interface: Hemlock Hill, Lees-McRae College, Banner Elk, North Carolina

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WHY RELEASE AT THE URBAN COMMUNITY FOREST INTERFACE?

For the purpose of efficient collection and redistribution of large numbers of predators of the hemlock woolly adelgid, *Adelges tsugae*, Annand (HWA), the urban community forest interface has many advantages: easy access by auto and by foot; urban hemlock trees have skirts with lower branches, making them more accessible for collecting/monitoring; hemlocks in parks, golf courses, schools, and cemeteries are sometimes fertilized and irrigated; urban hemlocks can occur singly in open areas exposed to winter sun, which favors predator presence; there is convenient nearby overnight shipping in urban areas, and easy access to collecting/shipping resources (boxes, blue ice, pint cartons, excelsior, etc).

BACKGROUND OF HIGH COUNTRY REGION OF NC

The High Country of northwestern North Carolina is one of the most biologically diverse areas in the world. This area includes Alleghany, Ashe, Avery, Mitchell, Watauga, Wilkes and Yancey Counties in northwestern North Carolina. The headwaters of five major river systems begin in this mountainous seven-county area, including the New (both North and South Forks of the New), Watauga, Yadkin, Catawba, and French Broad Rivers. Grandfather Mountain was the first privately owned property that has been designated as a biosphere by the U.N.; it is now owned by the state of North Carolina. There are extensive native stands of Carolina and eastern hemlock, as well as Eastern White Pine (*Pinus strobus*), Fraser Fir (*Abies fraseri*), and Red Spruce (*Picea rubens*) in this region. Abundant landscape plantings of Blue Spruce (*Picea pungens*) and other landscape conifers like Mugo Pine (*Pinus mugo*) are also present. The diversity of conifers gives this area abundant alternate adelgid hosts for predators as well. The HWA was first found in Avery County in 2002. The entire northwestern corner of North Carolina's "High Country"—Avery, Ashe, Alleghany, Mitchell, Watauga, Wilkes, and Yancy counties—is now considered generally infested with HWA. The HWA is causing region-wide loss of hemlock populations, resulting in an environmental disaster.

BACKGROUND OF RELEASE SITE

Hemlock Hill is a 25-acre old-growth hemlock forest remnant area that is part of Lees-McRae College and is next to the town of Banner Elk, NC (elev. 1100 meters) (Fig. 1). It had moderate to heavy HWA infestations in 2003 (Mausel 2007).

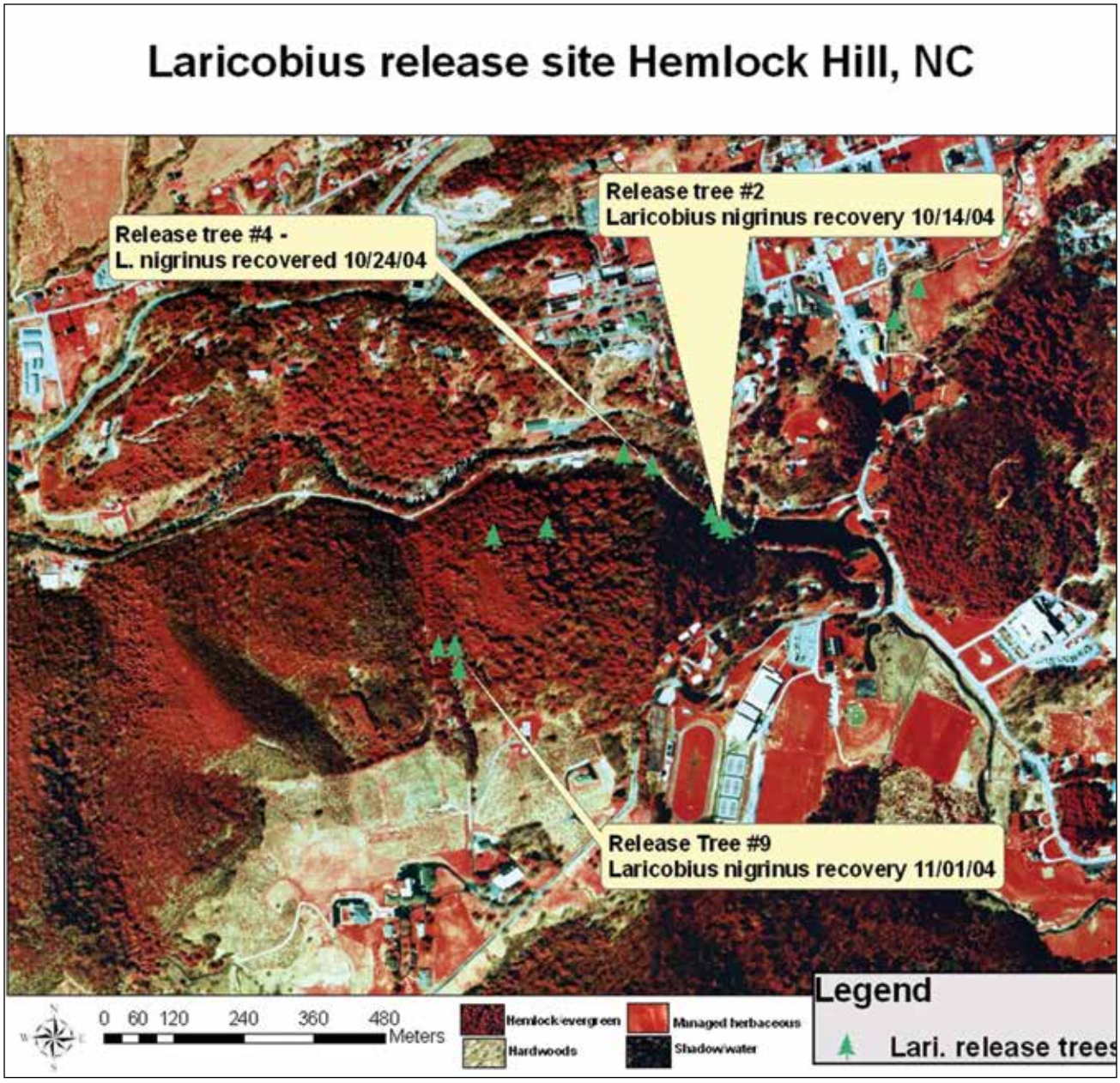


Figure 1. Location of Laricobius nigrinus release trees (in green - 10 trees, 30 beetles per tree) at Hemlock Hill in 2003. The yellow boxes are F1 recoveries during the 2004 season. Note that releases one through five were in a shaded area during winter, while releases six through ten were in areas that received abundant winter sun.

RELEASE SITE - HEMLOCK HILL

300 L. nigrinus adults released 31 December 2003, plus 300 adults augmented near release tree #9 in late March 2006

In late December 2003, 300 adult *L. nigrinus* reared in the laboratory at Virginia Tech University (British Columbia strain) were released at Hemlock Hill, at Lees-McRae College, Banner Elk, NC, into a stand of old growth hemlock infested with HWA. The release, first- and second-year monitoring of the site was done in conjunction with David Mausel's research for his Ph.D., with guidance from Brad Onken and Rusty Rhea, U.S. Forest Service, and Professors Scott Salom and Loke Kok (Mausel 2007, McDonald et al. 2005). One hundred fifty beetles were released along the Elk River, a shady, cool, flood-prone area (5 trees with 30 beetles each), and the remaining 150 beetles were released near or at the top of the ridge on 5 trees, 30 per tree in an area which received winter sunshine (Fig. 1). This gave us two release areas to compare: a shaded, cool, wet river area, and a sunny, drier, ridge area.

Initial Recovery and Dispersal Patterns of L. nigrinus from Hemlock Hill

We sampled the release trees and nearby trees twice monthly from October until April, using 1 meter squared beat sheets. We sampled approximately 30 trees (10 release trees and 20 nearby trees) each sample date. We were initially fortunate in that we divided the release of 300 beetles into two halves. The following fall/winter after the release (fall 2004), we found a total of 3 adults (F1 generation) and 10 F2 larvae (April 2004 sample). The next year (Fall 2005), 12 *L. nigrinus* adults were found, along with 314 F3 larvae from branch samples by Mausel during April 2005 (Table 1) (Mausel 2007). During years one and two, we only found beetles on release trees; by the third year, beetles began dispersing to nearby trees.

Determining Predation Rates of L. nigrinus on HWA ovisacs at Hemlock Hill

HWA-infested branches were collected at Hemlock Hill in February and April 2006 and 31 March 2007. Branch samples were taken along the river and on the ridge above. We focused our samples on hemlocks in an area with prior high larval and adult beetle recoveries (trees 9 & 10) from branch sampling work done by Mausel (2007). HWA-infested hemlock branches were bagged into gallon Ziploc bags and brought back into the laboratory, where we dissected ovisacs in order to determine presence of a *L. nigrinus* egg/larva. From samples taken between release trees 9 and 10, we found 10% of HWA ovisacs had a *L. nigrinus* egg or larva during April of 2006. We found an increase to 31% predation rate of ovisacs in the same area a year later, during late March 2007 (Figs. 2, 3, and 4). After this date, we began to sample using beat sheet method only to determine the presence of adult beetles at various areas distant to the original release site.

Table 1. Recovery of *L. nigrinus* adults by season and place at Hemlock Hill 2004-2008. "River" is a shaded area during winter; "ridge" is a sunny area at the top of the Hemlock Hill (see Fig. 1). Larval numbers are from Mausel (2007).

Season or place	F1 Adults ('04/'05)	F2 Larvae (April '05)	F2 Adults ('05/'06)	F3 Larvae (April '06)	F3 Adults ('06/'07)	F4 Adults ('07/'08)
Fall	3		12		93	80
Winter	0	10	0	314	109	23
River	2		1		4	41
Ridge (fall)	1		11		89	39

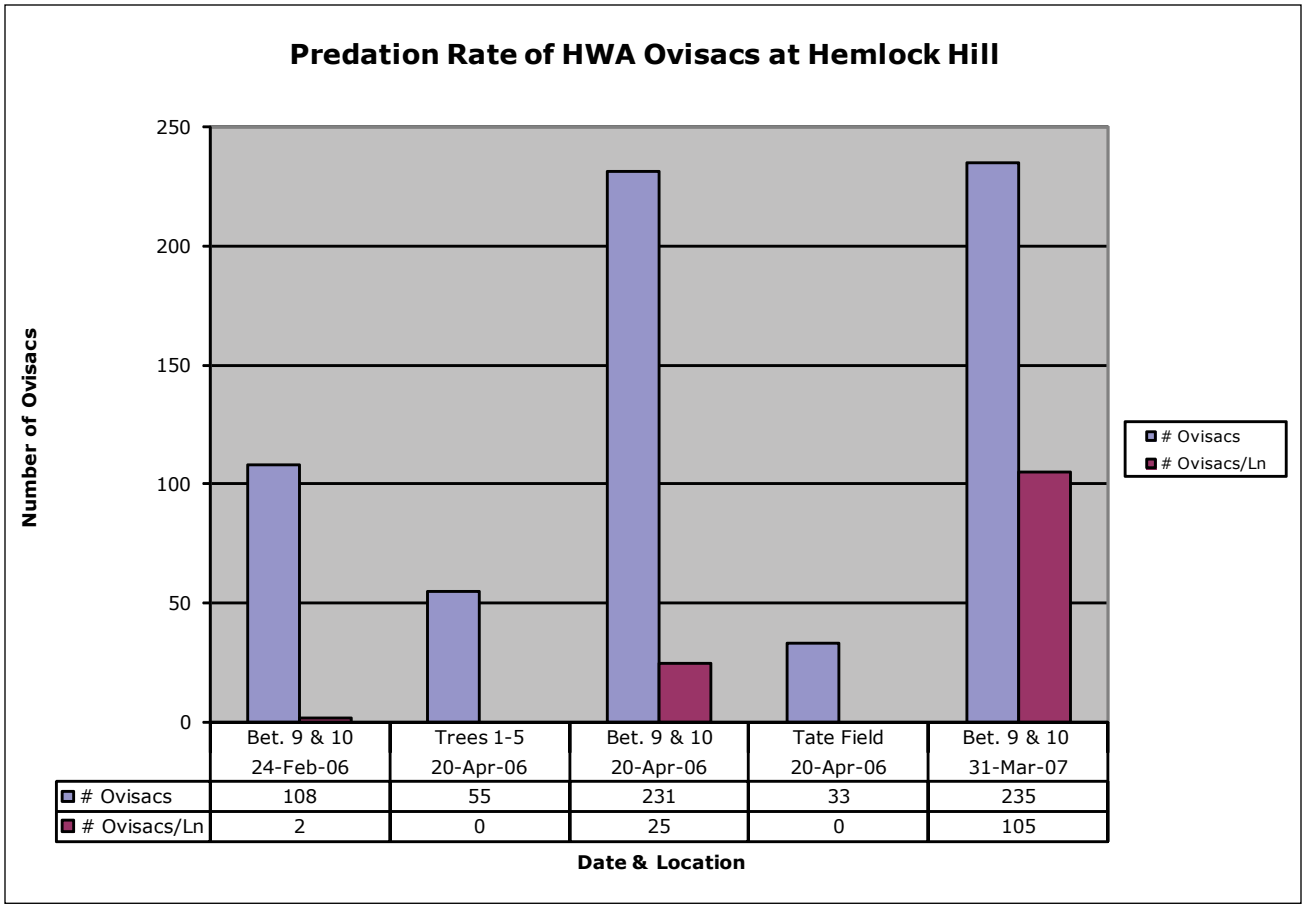


Figure 2. Predation rate of HWA ovisacs at the Hemlock Hill L. nigrinus release site during 2006 and 2007. During 2006, we found 10% of ovisacs with a Laricobius egg or larva present; during 2007 we found 31% of ovisacs with a Laricobius egg or larva. (Bet. 9 & 10 = sample taken between release trees 9 and 10.)

Figure 3. Laricobius nigrinus egg (yellow) above a cluster of HWA eggs. The distinct size, shape, and yellow color of the L. nigrinus egg versus HWA eggs are evident (photo by David Mausel).

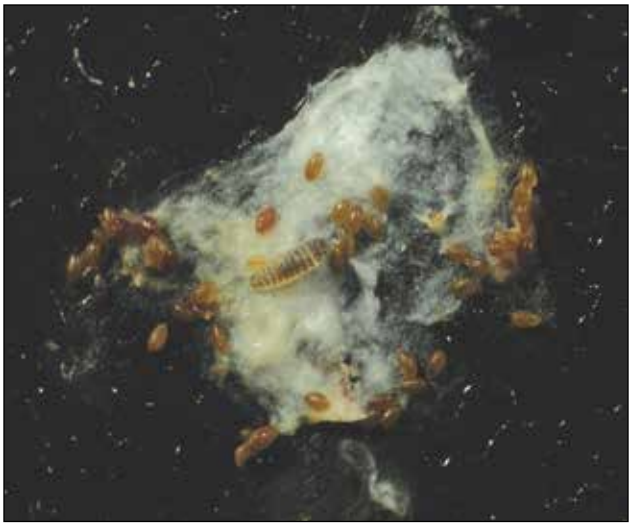


Figure 4. Laricobius nigrinus larva inside a dissected HWA ovisac; a single larva consumes 200-250 HWA eggs to complete development (photo by David Mausel).

Establishment and spread of Laricobius nigrinus at Hemlock Hill, Banner Elk, North Carolina

As we began to recover beetles over the first three years post-release (2004-2007), we noticed a pattern of distribution by the beetles during the late fall and winter months. We initially recovered more beetles in sunny areas with HWA (trees 8, 9 and 10), compared to cooler, shaded areas with HWA. For example, we recovered 101 beetles over the first three years in the upper ridge area, which received winter sun, versus only 7 beetles in the release trees 1 through 5 along the river, which did not receive winter sun (Table 1). From beat sheet samples taken during 2004 to 2008, we began to find that more beetles were dispersing in a southerly direction (Fig. 5, blue boxes), following the presence of winter sun on HWA-infested hemlocks. This pattern of beetles dispersing into areas having winter sun continued for the next 2 years (Fig. 6); with beetles now (2011) present more than 3 kilometers in every direction from the original release site. During fall 2006-April 2007, we recovered over 200 F3 adults, giving us establishment of *L. nigrinus* at the Hemlock Hill release site and over 100 F4 adults (Fall 2007) from an area of over ½ square mile.

Collection numbers of *L. nigrinus* adults that were taken from the Hemlock Hill area and redistributed to other sites are as follows: 2007 – 46; 2008 – 189; 2009 – 581; 2010 - 1,838 (Figure 7). These beetle recovery data were entered into the Forest Service's HWA database, maintained by Virginia Tech, at http://hwa.ento.vt.edu/hwa/hwa.cgi.

SUMMARY

We can see three distinct phases (early, middle, and mature phases) of colonization, establishment and dispersal of *L. nigrinus* at the Hemlock Hill release site. During the "early phase" of colonization (Years 1 (2004) and 2 (2005)) by *L. nigrinus*, beat samples showed that beetles stayed on the release hemlocks trees with HWA. During the "middle phase" of establishment and dispersal (Years 3 to 5, 2006-2008) we had establishment of populations of *L. nigrinus* (recovery 3 years in a row), significant predation rates on sampled ovisacs near release trees 9 & 10 (31% ovisacs with *Ln*), and limited dispersal to the south, into sunlit areas. During the "mature phase"—5 to 7 years (2009-onward)—we were able to collect and move hundreds to thousands of 6th and 7th generation beetles to new areas, both

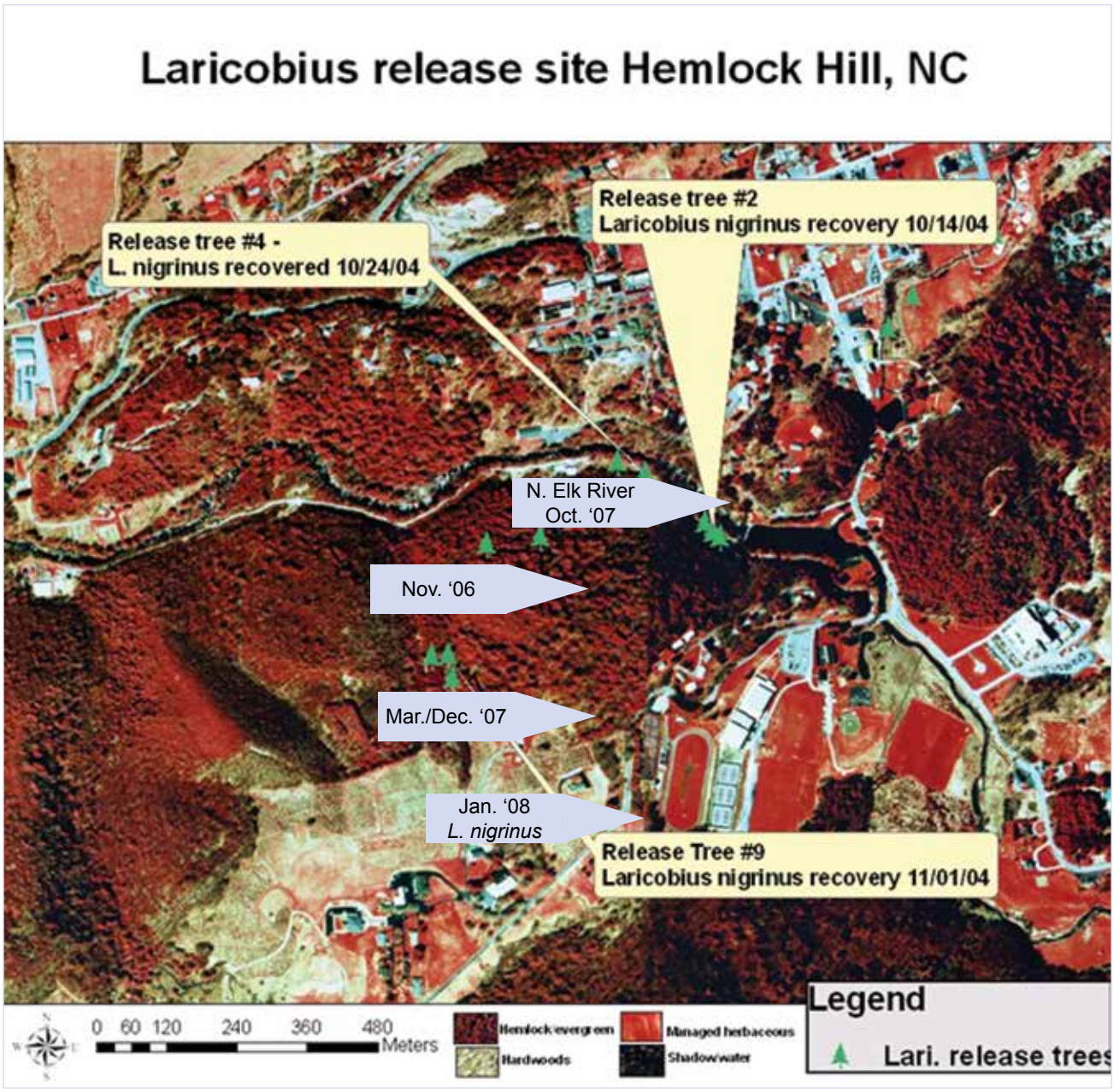


Figure 5. Pattern of dispersal of L. nigrinus adults from 2004 through January 2008 (light blue boxes). Adults appear to be more prevalent on south facing slopes during the coldest times of their season (Dec.-Feb.). Dispersal recoveries shown in 4 blue boxes: Mid-November of 2006: recovery of a single L. nigrinus adult on the main ridge of Hemlock Hill, 300+ meters from the nearest release tree. March/December 2007: recovery of a single L. nigrinus adult more than 3/8ths mile from the closest release site during March; December 2007: 2 adults in same area. January 2008: 1 L. nigrinus adult below Tate Field scoreboard. This was 200 yards beyond prior known dispersal at that time, in a southerly direction.

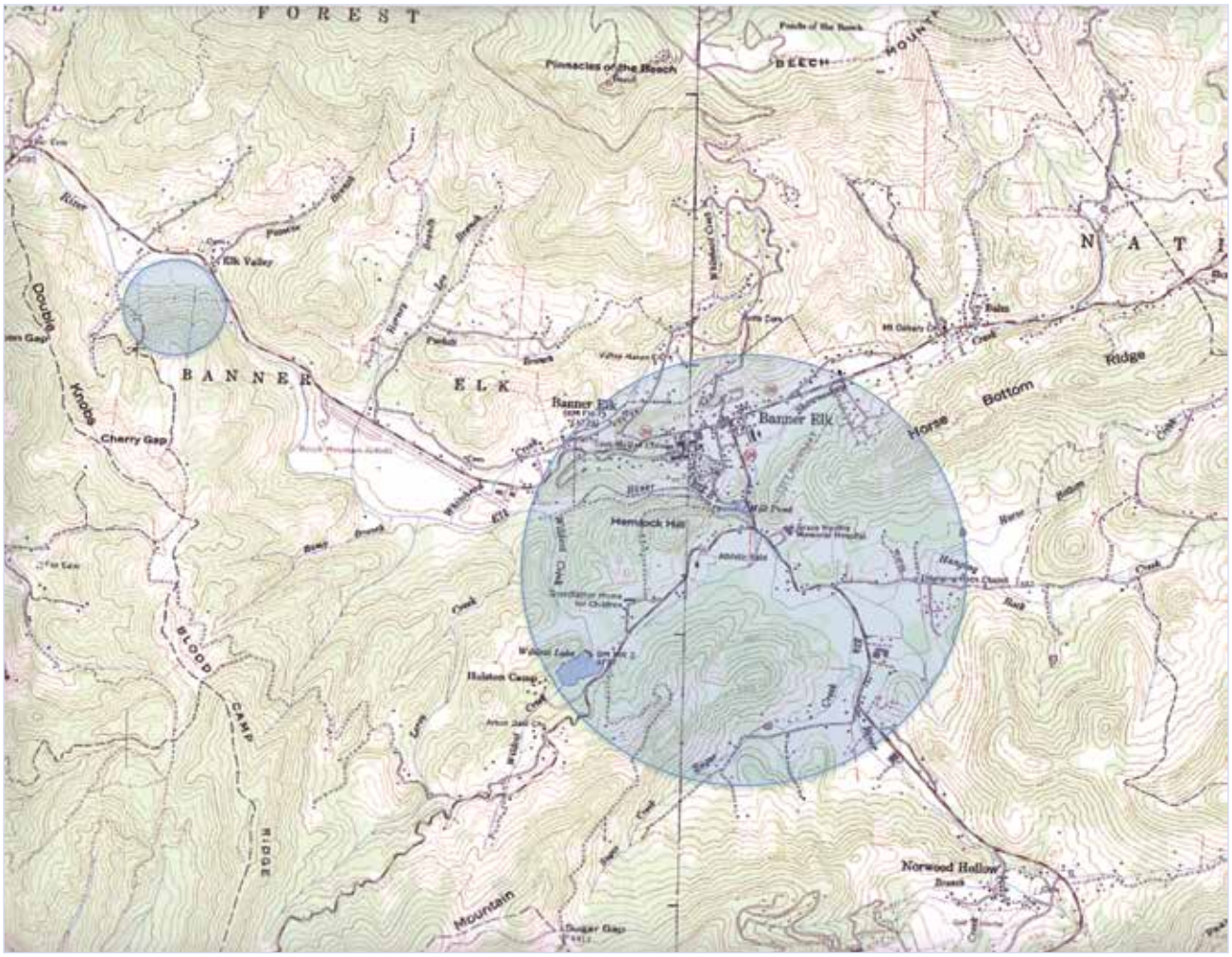
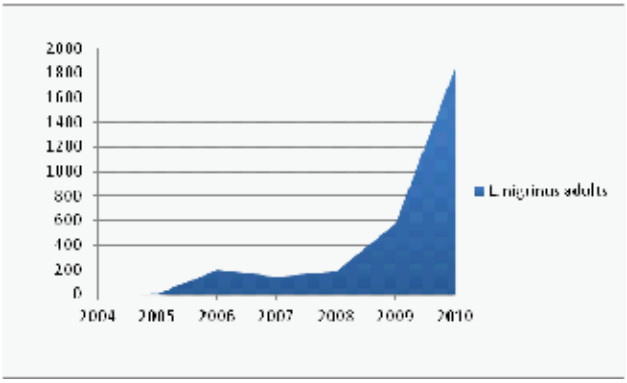


Figure 6. Current distribution of L. nigrinus adults in the Hemlock Hill area as of December 2010; compare to Figure 2. Adults are now present in a 3-kilometer radius from the original release. Beetles appear to have moved mainly to the south and west. We have collected a total of 2,757 L. nigrinus adults since 2007 in the Banner Elk area. Beetles have dispersed westward and are now found in the Pisgah National Forest.

Figure 7. Sum of beetles caught by beat sheet method at Hemlock Hill, Banner Elk, NC each year since 2004 (x axis years post release). Collection numbers of L. nigrinus adults that were redistributed to other areas: 2007 – 46; 2008 – 189; 2009 – 581; 2010 - 1,838.



locally and regionally as need drives the system. For example, we collected and redistributed 1,838 beetles during fall of 2010 & spring of 2011 (Fig. 7). We are also seeing sustained hemlock tree regrowth evident in the release areas. Beetles are now common on hemlocks with HWA throughout the Banner Elk, NC area. *L. nigrinus* is extinguishing populations of HWA locally and fragmentation of HWA populations and outbreaks is beginning—similar to patterns we see at the field insectary at Virginia Tech and in the field in Seattle. From our field studies and collections, we have found that *L. nigrinus* beetles do best in the following areas:

1) Release beetles in an area with good levels of HWA;
2) Release site must have hemlocks that receive abundant winter sun;
3) Hemlocks need to have needle duff under tree for *L. nigrinus* beetles to pupate;
4) Site must be undisturbed (no logging, mowing, pesticide spraying, etc.); and
5) More conifer species in the area is desirable; the increased biodiversity and alternate adelgid species as food.

Two thirds of the hemlock forest in the eastern USA is privately held. Thus for the future, we need to interface with the public by publishing a guide sheet for *L. nigrinus* focusing on landowner interest in the program. The urban community forest interface has the potential to produce high numbers of HWA predatory beetles that can be released into forest ecosystems to assist in bringing the hemlock woolly adelgid into balance much more rapidly than if we had to rely on natural dispersal alone.

RECOMMENDATIONS BASED ON THIS STUDY

To accelerate the establishment of HWA predators through the use of field insectaries, we suggest using abandoned hemlock nurseries or other favored areas in the urban/forest community interface with hemlocks: parks, cemeteries, schools, golf courses, playgrounds, colleges, universities, and similar areas that have hemlocks trees or hedges. These areas must also have resource managers that are willing to protect beetle release areas from spraying, cutting, disturbance of needle duff, etc.

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Chapter 17: The HWA Predator Release and Recovery Database

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INTRODUCTION

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) is an introduced insect that attacks and kills eastern hemlock, *Tsuga canadensis* (L.) Carriere, and Carolina hemlock, *T. caroliniana* Engelmann, trees in the eastern United States (Cheah et al. 2004). Importation of its natural enemies is the most promising option for controlling HWA on a landscape level (Wallace and Hain 2000). Since the late 1990s, state and federal agencies have released lab reared or wild predatory beetles of HWA in an effort to control this pest. Until recently, data pertaining to the release, monitoring, and recovery of the predators *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), *Scymnus sinuanodulus* Yu et Yao (Coleoptera: Coccinellidae), and *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) have been maintained on paper data forms or in small local databases and as a result have been inaccessible to HWA scientists and managers at regional and national levels. The management, accessibility, and usability of these data are made more cumbersome because of differences in how various agencies performed releases and monitoring and in how data were collected and stored, with multiple data forms and protocols among agencies being the norm. In 2007 we initiated the development and implementation of the HWA Predator Release and Recovery Database (PDB) which is housed in the Department of Entomology at Virginia Tech in Blacksburg, VA. The goal of the project is to include all historic release and monitoring information as well as provide a mechanism for field personnel to enter and update current and future records. Inherent in this program is an attempt to standardize field protocols as well as data forms. The PDB will impose an organizational structure on the data and serve as a central repository for information collected on release and recovery efforts. Implementing the PDB will facilitate improved access to and use of the data; provide project-wide reporting, mapping, and analysis; ensure that data from all cooperators are maintained, archived and available; and improve decision-making for future actions.

DATABASE STRUCTURE

The database is developed in Oracle® 10g database management system installed on a Windows 2003 Server platform in the Department of Entomology at Virginia Tech. Each release site record contains approximately 85 data fields distributed among four predator related database tables plus an additional table containing information on database users (Fig. 1). These data are obtained from the forms used when beetles are released (Appendix 17-1) or monitored (Appendix 17-2). In addition to information on HWA predator release sites the database contains data on the individual and organization performing the activity, site and stand conditions, weather, origin of beetles released, release tree information, post-release survey, and predators recovered.

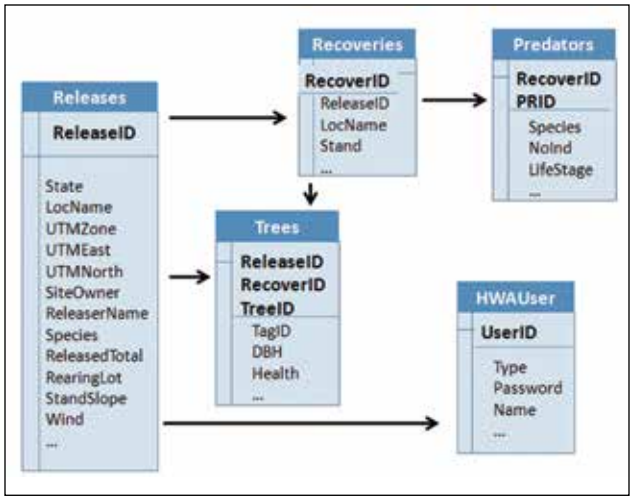


Figure 1. Database table structure and relationships in the HWA PDB.

The database is organized around release sites. Each site is geographically referenced and contains from one to several release trees. Data for post-release monitoring, sometimes referred to as recoveries, are in almost all cases associated with release sites. A subsequent beetle release at an initial site is called an augmentation, and the database includes a mechanism to attach an augmentation to an existing site.

Each record in the PDB must have a unique identifier. This unique ID is assigned by the database at time of data entry and consists of the species (LN, SS, or ST), state abbreviation, year (2 digits), and a sequential number. Thus, LNTN06007 is the ID for the seventh recorded *L. nigrinus* release in Tennessee in 2006. Post-release survey records are associated with their initial release record; their unique ID, assigned at data entry, consists of the state abbreviation, year, and a sequential number.

Currently the standard release form specifies that geographic coordinates be recorded in the Universal Transverse Mercator (UTM) coordinate system. Recently, however, we altered the database to include data entry in any of the three standard formats for latitude and longitude (decimal-degrees, degrees minutes seconds, degrees decimal-minutes). We found this necessary because of the large amount of historical information collected in lat/long as well as the fact that this is the preferred format for many agencies conducting release and survey work. Conversion from lat/long to UTM can be convoluted and tedious on a user's desktop computer but is straightforward in the database.

The PDB is relatively unconstrained with regard to restrictions placed on data fields. Because there have been no standard protocols and forms to which different agencies adhered, there is variability in many of the data values as well as the absence of data in many fields. This is especially true when integrating older data with the most recently collected information. To address this looseness in the data, it was necessary to construct the database with a minimum of data rules and restrictions. For example, of the roughly 85 possible data fields, only nine are required at data entry. This results in a somewhat messy database, but the decision was to capture as much data as possible, including historical records, rather than expend resources on cleaning up data which easily could be over a decade old. It is hoped that consistency and clarity will emerge as the database matures and begins to consist primarily of current records.

DATABASE FUNCTIONALITY

The PDB is accessed through a web portal (http://hwa.ento.vt.edu)(Fig. 2).

Two types of user accounts are available. The "VT User" account is reserved for persons who require both read and write privileges for the entire database. This includes database managers and technicians (usually at Virginia Tech) who are responsible for entering or correcting data from multiple agencies. A "Remote User" account is assigned to non-VT personnel whose responsibility is to enter and maintain data for a specific agency. Currently, the site is publicly available and anyone can log in as "guest" without a password. Guest logins may view all site content but have no editing capability. Presently there are approximately 48 registered users, about 30 of whom are actively involved with data entry and management.

Figure 2. The login screen to the PDB web portal.

The functionality of the database and associated web site is fairly standard and consists of data entry and editing for both initial release data, post-release monitoring and predator recovery, augmentative release, database query and reporting, map query and display, and data download. In addition, there is access to documents addressing field protocols, data entry protocols, and user account administration. The main page for the remote user interface is shown in Figure 3.

The VT User main page is similar to the Remote User page except it includes access to data entry screens for earlier versions of the release data forms and access to database user statistics. It also allows someone with VT user privileges to review and approve data submitted by a remote user. Records submitted by a remote user are marked as pending until they are reviewed and approved by a VT user. These records enter the database, are marked for review, and are available for most database functions and products, including reports and summaries.

Data entry is straightforward (Fig. 4). One area where we have imposed quality control at the data entry stage is in geographic coordinates. The database accepts coordinates in either Universal Transverse Mercator (UTM) or in latitude/longitude, which may be in any of the most common formats: decimal-degrees, degrees decimal-minutes, or degrees minutes seconds (Fig. 4). Constraints have been placed on these coordinates so that values out of range are not allowed. The database stores only UTM coordinates (and the UTM zone), but all web display and output includes both latitude/longitude and UTM. The

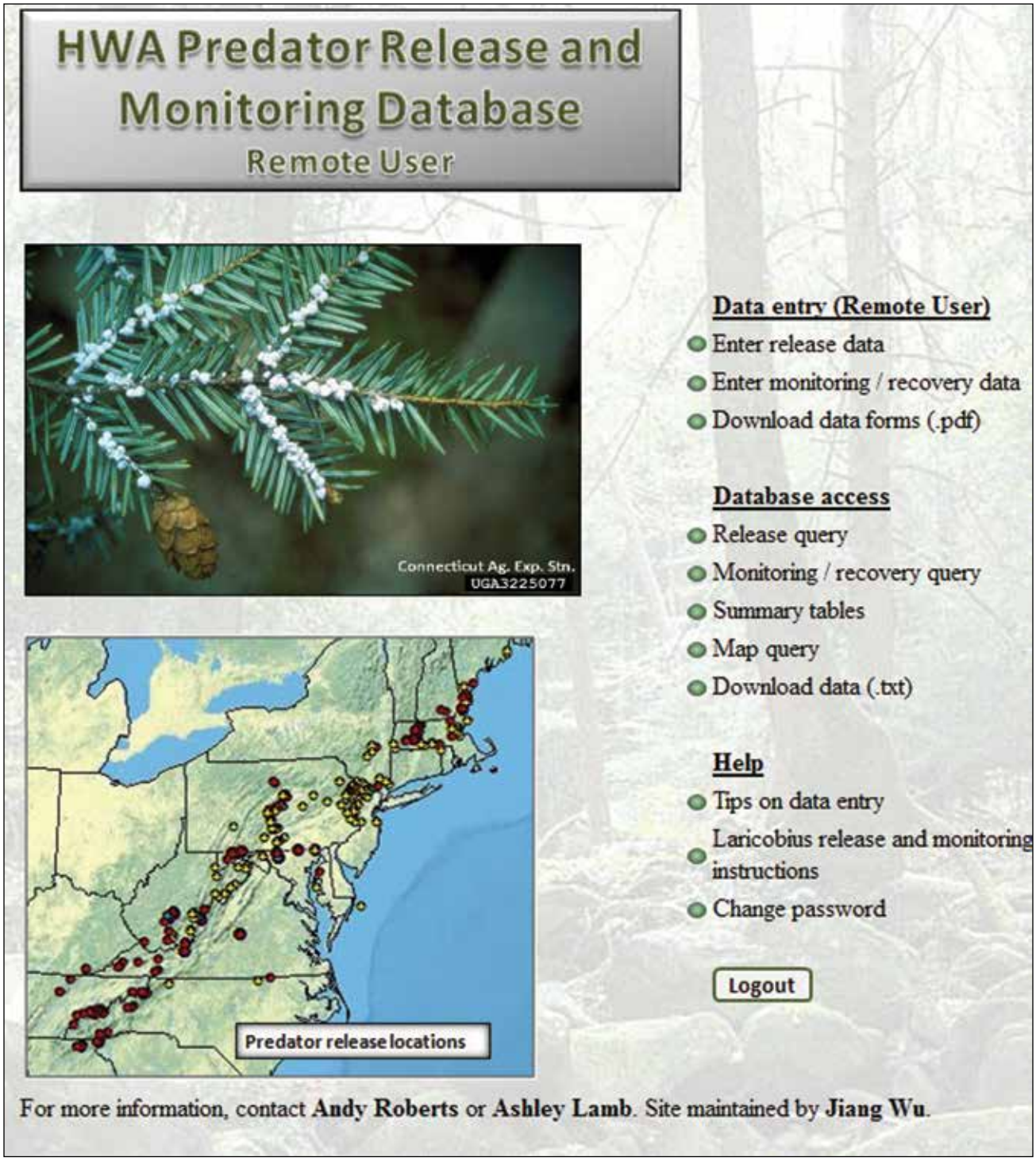


Figure 3. The primary page on the Remote User database site.

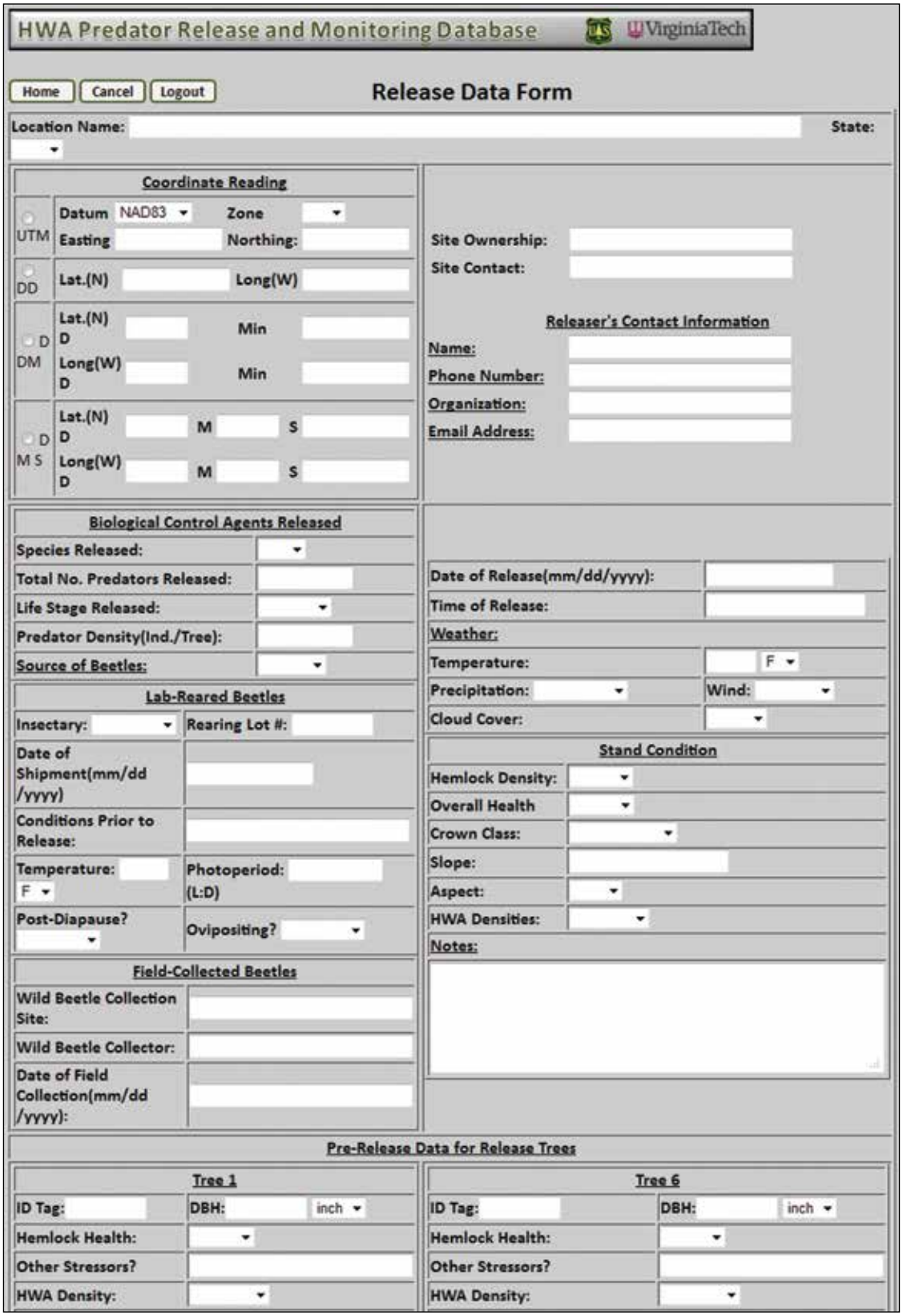


Figure 4. The predator release data entry screen. Entry fields for trees 2 through 5 and 6 through 10 are not shown.

conversion between UTM and lat/long is performed in database memory, and to accommodate different protocols, latitude and longitude are displayed in the three formats mentioned above.

Database actions which may occur on a site record subsequent to the initial beetle release include editing release information, adding post-release monitoring data, or adding augmentative release data. To perform these tasks, the user must first access the initial release data through a database query and then select the appropriate action, either Update, AddRecovery, or AddAugmentative (Fig. 5). When post-release data are entered in this way, the database populates the online data forms with pertinent information from the release records and thus expedites data entry. In addition, this reduces possible omissions or data entry errors by the user. An example is shown in Figure 6 where relevant release information for the Gauley River site has been pre-loaded into the post-release monitoring data form.

DATABASE CONTENT

Reliable estimates place current HWA predator releases at above 2.5 million for *S. tsugae*, 30,000 *S. sinuanodulus*, and over 150,000 *L. nigrinus* (B. Onken, pers. comm.), and only a fraction of the estimated field releases have been recorded in the PDB.

Adding previously existing data can be more time-consuming than adding newly collected data; it can appear daunting to consider adding hundreds of historical records especially if data formats and/or collection methodologies do not fit nicely with the data entry structure of the PDB. It is for this very reason that we relaxed data entry constraints

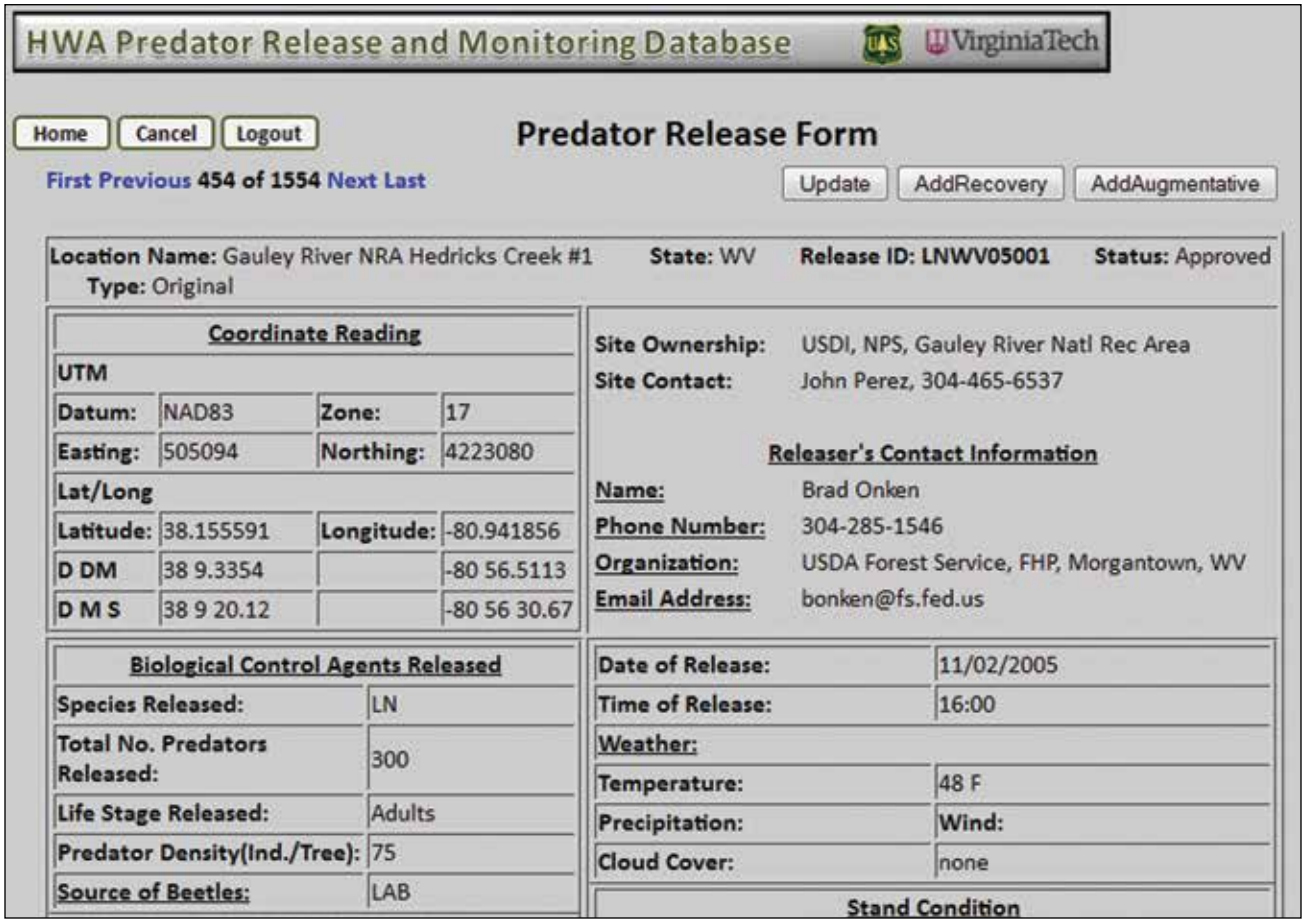


Figure 5. The results returned by a query of the release data. This template is used to access data entry for subsequent actions at the site. Only the top portion of the form is shown.

as well as incorporated the ability to enter either UTM or lat/long coordinates. It is possible to enter a large amount of data in one batch process if those data currently exist in a digital format, such as an Excel spreadsheet. In this case, the data may require some manipulations to accommodate the new database structure, but these efforts are minor compared to entering records manually. For example, with only a modicum of effort we recently batch loaded 236 historical records from Great Smokey Mountains National Park. Efforts are ongoing to remedy the discrepancy between the contents of the database and existing information.

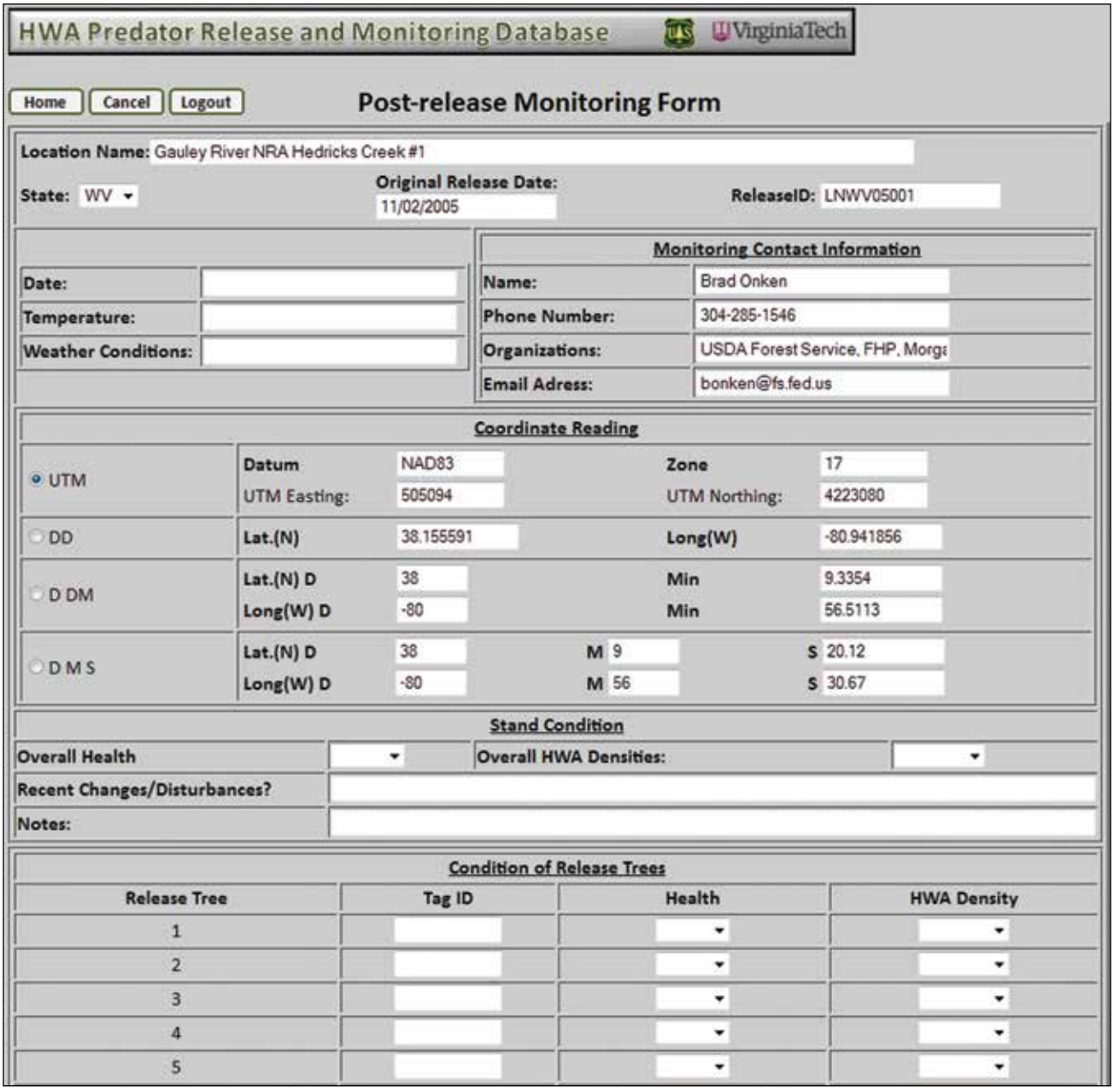


Figure 6. The post-release monitoring and predator recovery data entry form. Only the top portion of the form is shown.

Accessing data through the web portal allows more refined searches than a simple export from the database. One can perform queries based on the values of specific variables, such as Location Name, State, Species, and Year. The default Summary Report generates a table by species of the number of unique locations and beetles released to date and a yearly time series of post-release monitoring results (Fig. 7). Numbers reported for the total number of release locations in the Summary Report differ from those in Table 1 because the Summary Report does not include augmentative releases in this statistic. Of the 164,381 *L. nigrinus* released at 345 locations, there were 51 post-release surveys in the following year, 24 of which were positive for beetle recoveries (Fig.7). To date, there were a total of 109 post-release surveys at these sites and beetles were recovered at 54 of those. Because some locations have been surveyed multiple times over the years the grand total does not equal the sum of each yearly total.

The PDB has the ability to "drill down" through the data to reveal more detail. For example, clicking on the 54 sites with positive recoveries will bring up a list of those sites with associated data as well as the number of beetles recovered at each site (Fig. 8).

Further, selecting the 1067 individuals recovered for the Rocky Gap release site from the previous query (Fig. 8) will return a list of the results for each individual survey (Fig. 9). In the returned data table, selecting the value in the Date field will return the original survey record for that activity.

MAP QUERY

The PDB incorporates a simple map display developed in a Google Maps® application which serves as a front end to the database. As with the reporting section of the web portal, the mapping function supports parameterized database queries using several data fields (Fig. 10). Symbology is simplified to differentiate among species and between release and post-release monitoring sites. Data in the map are grouped by unique geographic coordinates, and these unique locations

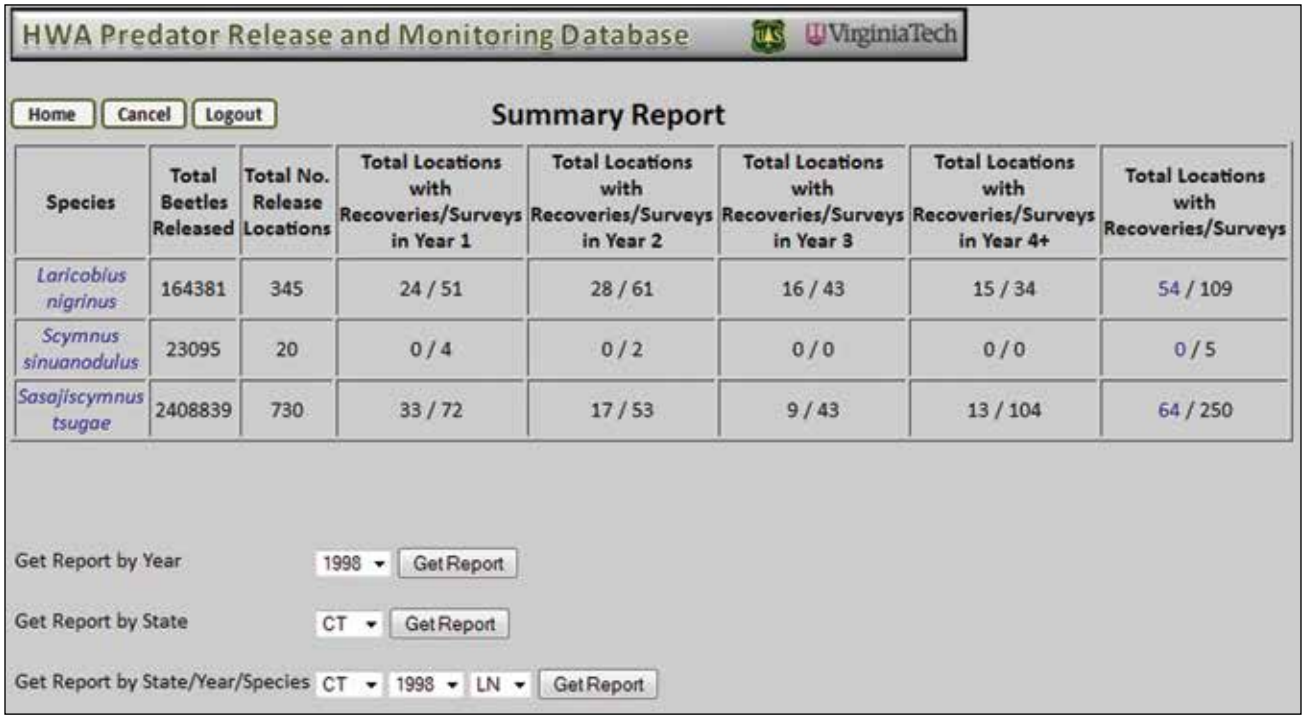
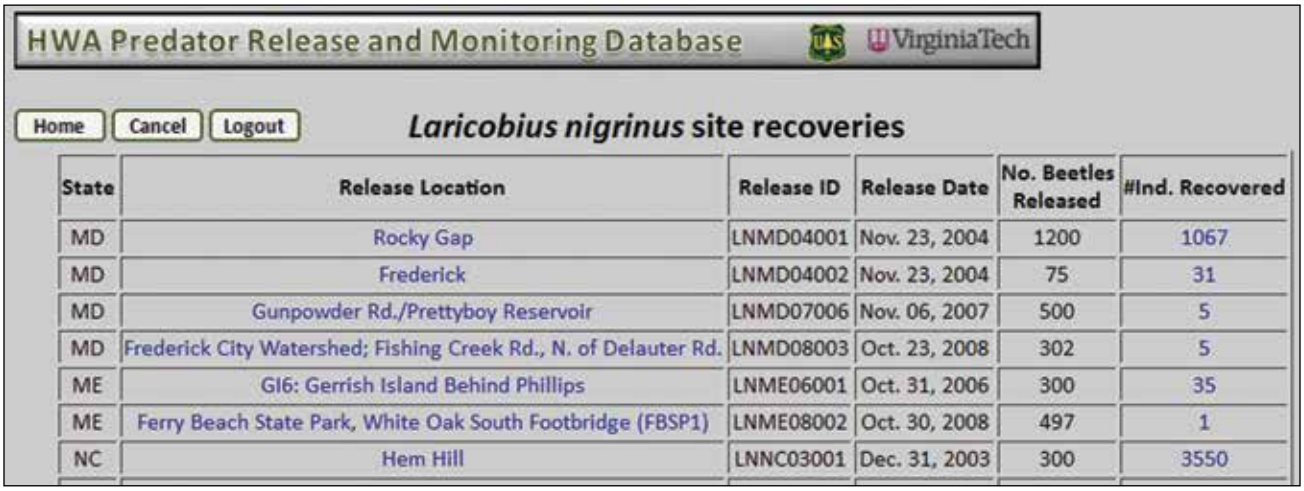


Figure 7. The default Summary Report from the database.

Table 1. Numbers of predator release sites and beetles released in the database by species and state.

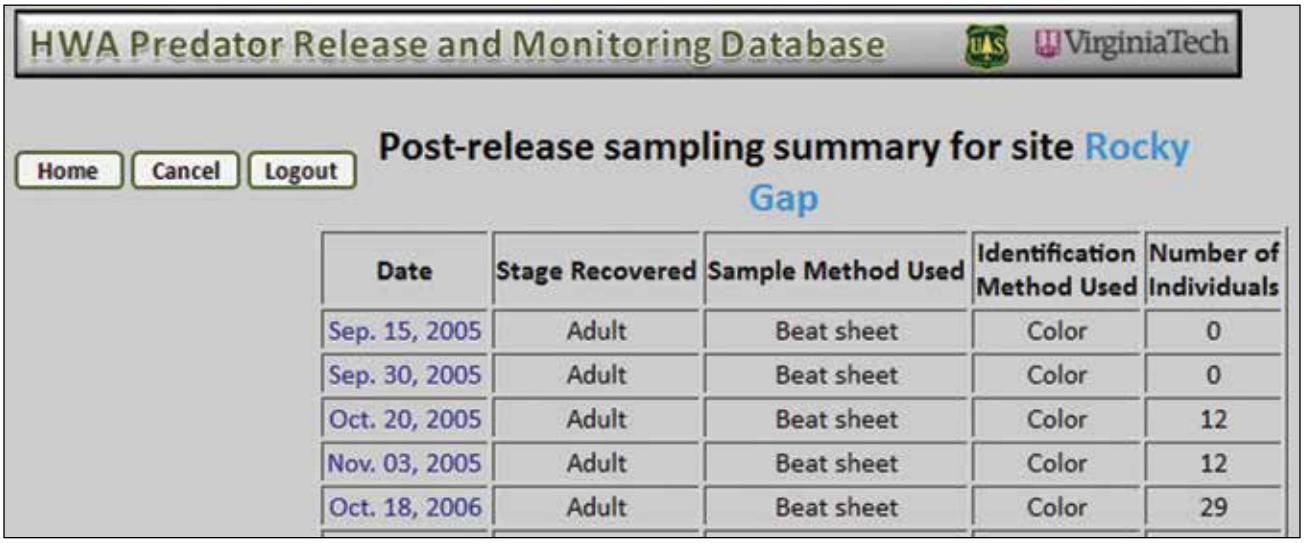
# Releases																	
Species	GA	KY	MA	MD	ME	NC	NH	NJ	NY	PA	RI	SC	TN	VA	WV	VT	Total
L. nigrinus	214	9	32	33	16	24	7	18	6	13		1	87	23	36	2	521
S. sinuanodulus	154			2				5		2		2	1		5		171
S. tsugae	286	17	31	6	28	62	8	147	20	51	1		183	8	61		909
Total	**654**	**26**	**63**	**41**	**44**	**86**	**15**	**170**	**26**	**66**	**1**	**3**	**271**	**31**	**102**	**2**	**1601**

# Beetles																	
Species	GA	KY	MA	MD	ME	NC	NH	NJ	NY	PA	RI	SC	TN	VA	WV	VT	Total
L. nigrinus	49,486	5,739	7,159	14,549	5,268	5,760	2,700	10,597	1,800	4,407		69	26,196	11,627	18,620	404	164,381
S. sinuanodulus	10,548			945				6,305		2,200		42	55		3,000		23,095
S. tsugae	403,023	27,500	144,756	40,410	54,818	167,792	38,052	594,500	47,500	171,900	5,000		501,307	20,000	192,281		2,408,839
Total	**463,057**	**33,239**	**151,915**	**55,904**	**60,086**	**173,552**	**40,752**	**611,402**	**49,300**	**178,507**	**5,000**	**111**	**527,558**	**31,627**	**213,901**	**404**	**2,596,315**



State	Release Location	Release ID	Release Date	No. Beetles Released	#Ind. Recovered
MD	Rocky Gap	LNMD04001	Nov. 23, 2004	1200	1067
MD	Frederick	LNMD04002	Nov. 23, 2004	75	31
MD	Gunpowder Rd./Prettyboy Reservoir	LNMD07006	Nov. 06, 2007	500	5
MD	Frederick City Watershed; Fishing Creek Rd., N. of Delauter Rd.	LNMD08003	Oct. 23, 2008	302	5
ME	GI6: Gerrish Island Behind Phillips	LNME06001	Oct. 31, 2006	300	35
ME	Ferry Beach State Park, White Oak South Footbridge (FBSP1)	LNME08002	Oct. 30, 2008	497	1
NC	Hem Hill	LNNC03001	Dec. 31, 2003	300	3550

Figure 8. Example of records returned through the site query (top of table only).



Date	Stage Recovered	Sample Method Used	Identification Method Used	Number of Individuals
Sep. 15, 2005	Adult	Beat sheet	Color	0
Sep. 30, 2005	Adult	Beat sheet	Color	0
Oct. 20, 2005	Adult	Beat sheet	Color	12
Nov. 03, 2005	Adult	Beat sheet	Color	12
Oct. 18, 2006	Adult	Beat sheet	Color	29

Figure 9. Example of monitoring records returned from the database for a specific site (top of table only).

are numbered and listed in the panel to the left of the map. Release sites as well as post-release monitoring sites are grouped under each unique location. For example, site 3 in Figure 10 references four *S. tsugae* release records from TN that may or may not occur on the same date. Obviously, only data for which geographic coordinates are available are displayed on the maps.

As with all Google® maps, the base layer can be either a street layer, a terrain layer (Fig.11) or remotely sensed imagery. Each location has a hyperlinked reference to all activities at that site. For example, in Figure 11 data for an initial release of 1000 *L. nigrinus* (ID=LNWV08005) and a subsequent survey (ID=WV090002) are referenced by the point in the center of the map. Clicking the ID of the one of the referenced events will open up the database record for that event.

DATA EXPORT

All data in the PDB can be exported in text file format. Information on releases, release trees, recoveries, recovery trees, and predators are downloaded in separate files which will easily load into spreadsheets such as Excel® and GIS software such as ArcGIS®. One planned enhancement is to implement .kmz file formats so PDB output can be exported directly to Google Earth®.

DATABASE ISSUES

There are a number of important issues to address in the continued development of the PDB:

Completeness. The necessity to incorporate past survey data which exists on paper and/or local data files has been discussed above. This is one of the most pressing issues facing the PDB.

Cooperator buy-in. Equally important is to obtain buy-in from groups that are releasing and monitoring beetles. Cooperators who are not invested in the project will find it more difficult to take the additional step of updating the database as they collect field data. This is especially pertinent to the task of submitting historical data.

Data Correction. The unconstrained nature of the PDB facilitates data entry from disparate sources, but it allows null or erroneous data to pass unchallenged into the database. Detecting and correcting these data after the fact is challenging from both a technical, practical, and human nature aspect. In its current state, the PDB has a large amount of missing or erroneous data including geographic coordinates. Since inception, we have added or corrected coordinates for over 200 release sites, but there still remain dozens of records with no geographic information.

Location name standards. Standardizing the naming procedures of release sites such that location names provide more consistent location information is one methodological change that would dramatically improve database value. Not surprisingly, releases at identical or nearby locations performed by different persons or at different times appear in the database with different Location Names. These often are due to spelling or abbreviation inconsistencies, but the result is that it reduces the accuracy and effectiveness of database queries.

Map display. There are more advanced options for the mapping component of the PDB which would provide more flexibility and power. Development in this area is contingent on user demand and response.

In conclusion, the HWA predator release and recovery database is an ongoing project to add meaning to historical, current, and future biocontrol efforts directed toward this pest. Hopefully, increased knowledge regarding release activity numbers and locations will supplement associated research and control activities as well as increase access to data and awareness of the extent of these activities in the country.

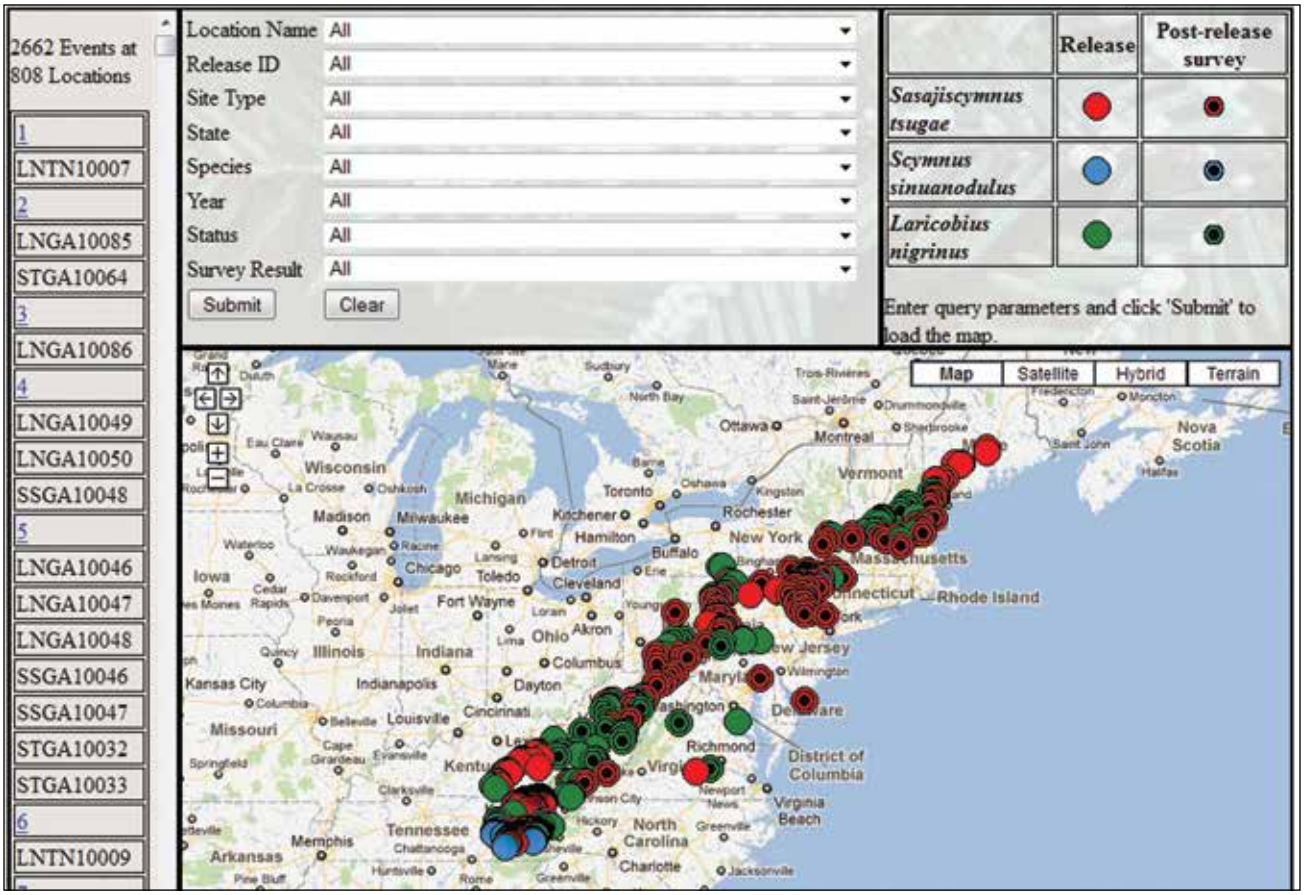


Figure 10. Results of map query displaying all georeferenced records in the database.

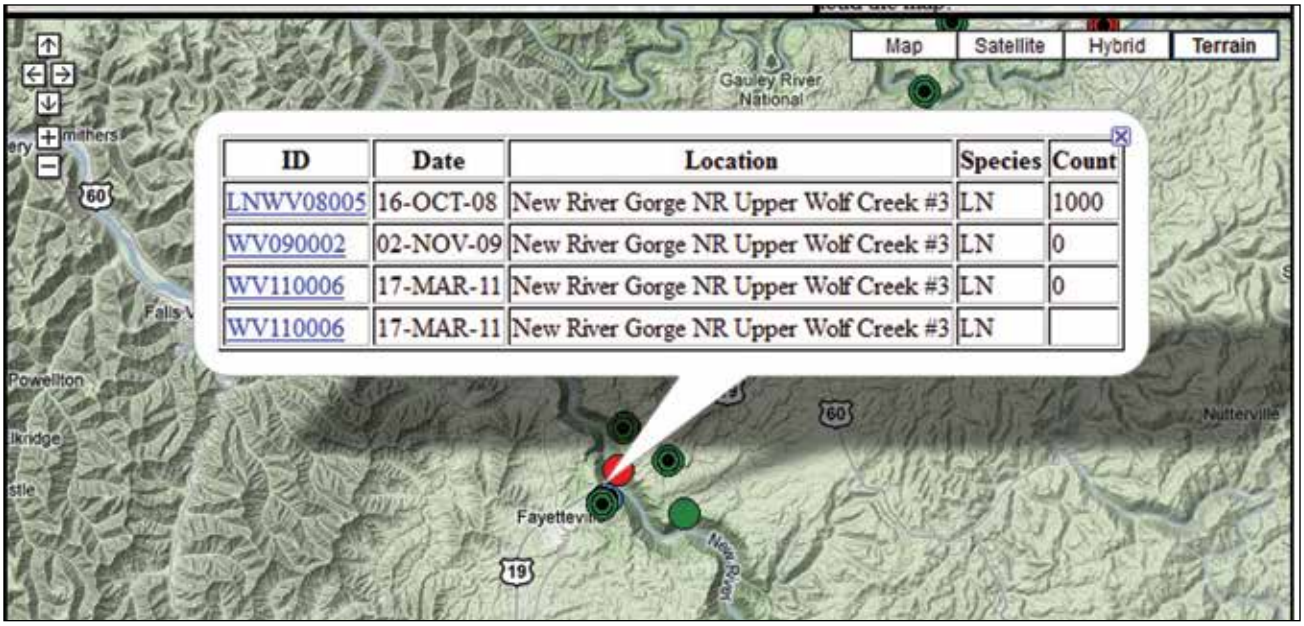


Figure 11. Map display with linked data records and terrain background.

ACKNOWLEDGMENTS

In addition to those listed above as contributors, Jiang Wu and Ian Firkin, both at Virginia Tech, have devoted much time to the database and mapping applications. Karen Felton with the U.S. Forest Service in Morgantown greatly assisted in developing the database structure and schema. Funding for this project is supplied by the U.S. Forest Service HWA Initiative.

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

Appendix 17-1. Data collection forms and instructions for predator release.

Pre-Release Data Form for HWA Predators

Release Location Name:

UTM Reading (Datum NAD83 is preferred)	Releaser's Contact Information
Datum: Zone:	Name:
Easting: Northing:	Phone Number:
Site Ownership:	Organization:
Site Contact:	Email Address:

Biological Control Agents Released	Date of Release (m/d/y):
Species Released:	Time of Release:
Total No. Predators Released:	Weather: Temperature: (°C / °F)
Life Stage Released: Eggs Larvae Adults	Precipitation: Wind:
Predator Density (Ind./Tree):	Cloud Cover: none low high
Source of Beetles: LAB or FIELD	Stand Condition

Lab-Reared Beetles	Hemlock Density: > 50% or < 50%
Insectary: Rearing Lot #:	Overall Health: G F P
Date of Shipment (m/d/y):	Crown Class (circle one):
Conditions Prior to Release:	dominant codominant intermediate suppressed
Temperature: (°C / °F) Photoperiod: (L:D)	Slope:
Post-Diapause? Ovipositing?	Aspect: NE N NW W SW S SE E
Field-Collected Beetles	HWA Densities: L M H
Wild Beetle Collection Site:	Notes:
Wild Beetle Collector:	
Date of Field Collection:	

Pre-Release Data for Release Trees

Tree 1	Tree 6
ID Tag: DBH: (inch / cm)	ID Tag: DBH: (inch / cm)
Hemlock Health: G F P	Hemlock Health: G F P
Other Stressors?	Other Stressors?
HWA Density: L M H	HWA Density: L M H
Tree 2	**Tree 7**
ID Tag: DBH: (inch / cm)	ID Tag: DBH: (inch / cm)
Hemlock Health: G F P	Hemlock Health: G F P
Other Stressors?	Other Stressors?
HWA Density: L M H	HWA Density: L M H
Tree 3	**Tree 8**
ID Tag: DBH: (inch / cm)	ID Tag: DBH: (inch / cm)
Hemlock Health: G F P	Hemlock Health: G F P
Other Stressors?	Other Stressors?
HWA Density: L M H	HWA Density: L M H
Tree 4	**Tree 9**
ID Tag: DBH: (inch / cm)	ID Tag: DBH: (inch / cm)
Hemlock Health: G F P	Hemlock Health: G F P
Other Stressors?	Other Stressors?
HWA Density: L M H	HWA Density: L M H
Tree 5	**Tree 10**
ID Tag: DBH: (inch / cm)	ID Tag: DBH: (inch / cm)
Hemlock Health: G F P	Hemlock Health: G F P
Other Stressors?	Other Stressors?
HWA Density: L M H	HWA Density: L M H

Appendix 17-1 (continued). Data collection forms and instructions for predator release.

Pre-Release Data Collection: Instructions for Data Form
UTM Reading: Set the Datum to NAD83 (setting in GPS unit), then record zone, easting, and northing (reading on GPS unit)
Predator Data Source of Predators (circle one): were the predators to be released reared in a lab or collected from the wild? Insectary: if the predators to be released were lab-reared, in which insectary were they reared? What generation or lot # are they? Wild: if the predators released are wild, from where were they collected? (State/County, UTM if possible) Total No. Predators Released - life stage and total number of predators released at site Predator Density (Ind./Tree): the number of predators being placed on each release tree
Release Stand
Hemlock Density(circle one): Hemlock comprises more or less than 50% of the stand
Hemlock Health G - Foliage has normal color and density and general overall appearance is good F - Foliage somewhat off color and/or some trees have thinning crowns, overall appearance is fair P - Most trees look stressed, foliage color chlorotic and/or thinning crowns common overall appearance is poor
Crown Class Dominant: largest trees in stand, receive full light from above and crown extend beyond general crown cover Co-dominupant: tree crowns comprise general crown cover, receive full light from above Intermediate: trees are shorter than crown cover, receive little light from above Suppressed: tree crowns well beneath general crown cover, receive no direct light from above
Slope: use measure on clinometer (percent) Aspect (circle one): cardinal direction in which the slope is facing
Stand Level HWA Density L - Most trees uninfested and/or most infested trees have <10% infested branches. M - Number of infested trees is 50% or more and most infested trees have 10-50% infested branches H - Most trees infested and often >50% of branches are infested
Release Trees
Hemlock Health: use the hemlock health scale shown above Other Stessors: list if known (eg. Drought, scale, mite, damage, fungal needle disease, defoliators, etc. Diameter at Breast Height (DBH): inches around diameter of tree
Release Tree HWA Density L - Less than 10% of the branches on the tree are infested with HWA M - 10-50% of the branches on the tree are infested with HWA H - More than 50% of the branches on the tree are infested with HWA

Appendix 17-2. Data collection forms and instructions for post-release monitoring.

Post-Release Monitoring Data Form for HWA Predators

Release Location Name:

Sample Date (m/d/y) : **Original Release Date:**

Site Location: (UTM)	Weather	Monitoring Contact Information
Datum: NAD83 preferred	Temperature: (°C / °F)	**Name:**
Zone:	Wind:	**Phone Number:**
Northing:	Precipitation:	**Organization:**
Easting:	Cloud Cover: none low high	**Email Address:**

Condition of Stand

Overall Health: Good Fair Poor Dead

Overall HWA Densities: Low Medium High

Recent Changes/Disturbances?

Notes:

Condition of Release Trees

Release Tree	Tag ID	Health (GFP)	HWA Density (LMH
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

Predator Recovery

Species	No. Ind.	Life Stage	Generation	Host	Dist. (m)	Time	UTM (E)	UTM (N)	Sample Method	Id Method

Appendix 17-2 (continued). Data collection forms and instructions for post-release monitoring.

Instructions for Completing Post-Release Monitoring Data Form
Site Information
Record Location Name, Date of Orginal Release (m/d/y), today's date, temperature, wind speed, precipitation (none/rain/snow), & cloud
UTM Readings: make reading in central area among release trees and at location of each predator recovery
For each UTM readingset the datum to NAD83 (setting in GPS unit), then record zone, easting, and northing (reading on GPS unit)
Stand Condition
Hemlock Health G - Foliage has normal color and density and general overall appearance is good F - Foliage somewhat off color and/or some trees have thinning crowns, overall appearance is fair P - Most trees look stressed, foliage color chlorotic and/or thinning crowns common overall appearance is poor D - Trees are not displaying any green needles
Stand Level HWA Density L - Most trees uninfested and/or most infested trees have <10% infested branches. M - Number of infested trees is 50% or more and most infested trees have 10-50% infested branches H - Most trees infested and often >50% of branches are infested
Condition of Release Trees
Hemlock Health: follow instuctions shown above Release Tree HWA Density L - Less than 10% of the branches on the tree are infested with HWA M - 10-50% of the branches on the tree are infested with HWA H - More than 50% of the branches on the tree are infested with HWA
Predator Recovery Data
Species: record identification of insect collected No. Ind.: record the number of individuals per sample Life Stage: record the life stage of the predators collected Generation: record the generation being collected (ie F1, F2, F3 etc.) Host: record if the tree was a release or non-release hemlock tree? Dist. (m): for each recovery, record the distance to the nearest release tree Time: record the time of day each recovery is made UTM (N): record the northing reading from the GPS unit UTM (E): record the easting reading from the GPS unit Sampling Method:record whether predators were recovered using beat-sheeting, branch collection sampling, or other methods Id Method: identification by DNA or Visual and the name of person that confirms the identity of predators recovered

SECTION V ADDITIONAL TOPICS

Chapter 18: Field Insectary: Concept for Future Predator Production

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INTRODUCTION

Predators for use in biological control can be obtained one of three ways: 1. collect them from their native habitat and ship them to their destination (lab or field); 2. rear them in a laboratory or other type of controlled setting (Lamb et al. 2005); and 3. use a natural or planted setting, in an area close to the targeted release locations, to encourage prey production to build up the predator populations for harvesting and redistribution on a consistent basis. This is called a field insectary. For hemlock woolly adelgid biological control, importing the predator *Laricobius nigrinus* and rearing them in labs have been the most common approaches used. But it is understood that both approaches are expensive. The concept of employing field insectaries are especially helpful after a biological control program has matured and the knowledge of how to rear successful agents has been obtained.

Reports of use of field insectaries/nurseries for rearing of predators, parasitoids, and weed biological control agents have been well documented, but few have been in the forest ecosystem (Kok and Salom 2002). The biological control lab at Virginia Tech created a hemlock plantation with the long-term objective of developing a sustainable field insectary. What follows is a description of the effort to date.

ESTABLISHMENT OF A FIELD INSECTARY

An eastern hemlock plantation was established at Virginia Tech's Kentland Farm, McCoy, Virginia in October 2001 (Kok and Salom 2002, Mausel et al. 2008). A field that was left fallow with naturally occurring wild grass on a northeast-facing slope (10 to 15%) was selected for the location of a 0.4 ha field insectary. Adjacent to the field insectary was a young (10-12 yr old) white pine, *Pinus strobus* L., plantation that was naturally infested with pine bark adelgid. For the hemlock plantation, twelve 12 × 20 m blocks were marked in a 4 × 3 block rectangle and spaced 5 m apart. Trees were spaced 2.4 m within and 3.7 m between rows that were oriented northeast to southwest. Six rows with five tree locations were marked in each block (30 trees per block) (Fig. 1).

Figure 1. Eastern hemlock field insectary soon after its establishment in Kentland Farms, McCoy, VA.

Three hundred 1.2 to 2.4 m tall hemlock trees with 0.6 m diameter root balls wrapped in burlap were purchased from a local nursery. Ten of the 12 blocks were planted with these large hemlocks. The other two blocks were planted at the same spacing but with 60 potted 0.6 m tall hemlocks purchased from a nursery in Pennsylvania, for a total of 360 trees. Augers (60 and 15 cm diameter) were used to make holes for the root balls of the large and small hemlocks, respectively. Bark mulch was applied 10 to 15 cm deep and 4 to 8 liters of water were poured to the drip line of each tree immediately after planting. They were watered again at 3 and 6 wk after planting.

The trees were inoculated with HWA three times in March 2002 and again in April 2003 to supplement existing populations. Two hemlock clippings (~30 cm long) infested with progrediens eggs from local forests were implanted in the mid-crown of each living tree every 2 wk until the onset of egg hatch (i.e. on the 1st, 15th, and 30th day of each month).

We estimated the density of HWA on 193 trees greater than 1.0 m tall to determine which trees had enough HWA for a predator release. On 12 November 2003, the number of new shoots infested with one or more HWA sistens and total number of new shoots were counted on 30 cm segments at the middle third of a branch's length. One branch was measured at each cardinal point and the percentage of infested shoots from the four branches was averaged for each tree. On 18 November 2003, predators were released on each tree with the numbers released based on the estimated density of HWA as follows: six adult *L. nigrinus* were released on each of four heavily-infested trees (≥ 76% of new shoots with at least one HWA), four were released on 42 moderately-infested trees (25 ≤ x ≤ 75%) and one was released on 66 lightly-infested trees (≤ 25%). This open release amounted to 258 *L. nigrinus* adults (unknown sex ratio) on 112 hemlocks that ranged in height from 1.8 to 3.0 m.

Population estimates of *L. nigrinus* and *L. rubidus* were conducted in winter of 2005 through 2009 on the hemlocks using canvas (71 cm^2) beat sheets (Bio-quip, Rancho Dominguez, CA). We sampled the most heavily HWA-infested branches of a tree first (typically at the top of each tree's crown) followed by lower branches. We spent ~1 min. per tree hitting branches with a 1.0 m long bamboo stick in the afternoon (from 13:00-17:00) and counting dislodged *L. nigrinus* and *L. rubidus* adults on the sheet. The species were differentiated on the basis of color and then carefully returned to the trees. In 2007, both species were collected from the beat sheets with an aspirator, transferred to plastic containers with HWA-infested foliage and transported to the insectary for processing. They were sorted and *L. nigrinus* adults were packaged in ventilated plastic containers for release in HWA-infested natural forests.

In winter of 2004 through 2007, HWA infestation level was categorized on each tree as heavy, moderate, light, or absent (after ~1 min. of searching). These estimates were based on the quantitative estimates taken in 2003, when the number of *L. nigrinus* to release per tree was initially determined (described previously). Data were summarized as the total number of trees in each infestation category and presented graphically. In 2007, tree decline was classified subjectively into categories as healthy (<10% HWA damage symptoms), light (10-25%), moderate (26-50%), severe (51-100%), or dead (no green foliage). Damage symptoms included no new shoot growth, twig dieback, foliage discoloration, and needle loss (Young et al. 1995).

MONITORING THE FIELD INSECTARY

After collecting over 200 F3 *L. nigrinus* in 2007, the number of *L. nigrinus* and *L. rubidus* dropped in 2008 and 2009. *L. rubidus* is present because the hemlock plantation supporting HWA is located next to a 20-yr old white pine plantation that supports pine bark adelgid, the primary host of *L. rubidus*. The decrease in predators collected (Fig. 2) coincides with a reduction in HWA found on hemlock trees (Fig. 3). Such a high percentage of trees exhibiting none to light infestation provides some circumstantial evidence that the predators are playing a role in suppressing HWA populations.

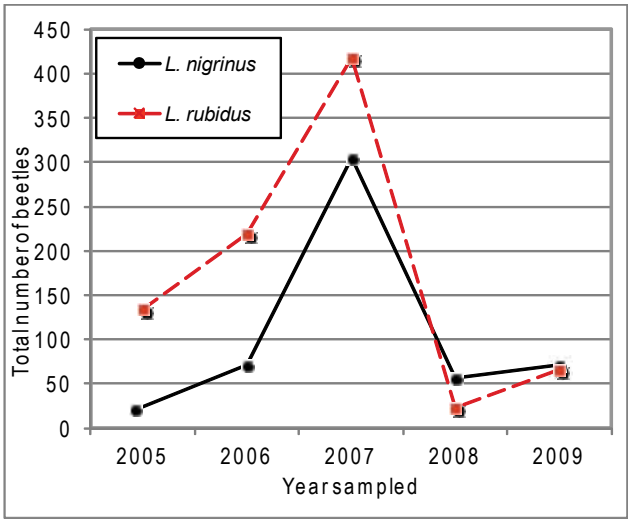

Figure 2. Population trends of Laricobius nigrinus and L. rubidus at the field insectary at Kentland Research Farm, in Montgomery County, VA, 2005-2009.

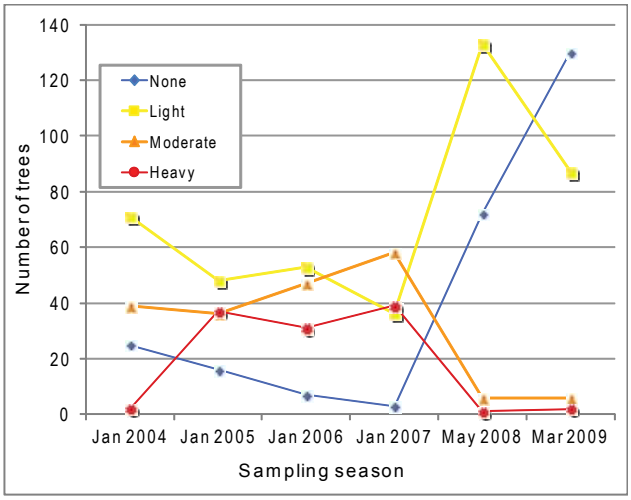

Figure 3. HWA infestation levels at Kentland Research Farm, in Montgomery County, VA, 2004-2009.

REINFESTING AND EXPANDING THE INSECTARY

In spring 2010, hemlocks were reinfested with HWA sistens and progrediens when each stage was ready to hatch from eggs. In October, 2010, hemlocks were sampled for *L. nigrinus*. Eighty-two *L. nigrinus* were collected from 17 trees identified as modestly infested. Thirteen additional beetles were collected from 10 trees considered uninfested. In fall 2010, the insectary was doubled in size to 24 blocks with 1.5 m tall trees. Additionally, 1 m tall trees were added to the old blocks where trees had died early on due to drought.

NATURAL INSECTARIES

In northwestern North Carolina, where *L. nigrinus* was first released in 2003 and has been established since 2006, healthy hemlocks remain. Beetles (F7) from our 2003 release have dispersed more than 1.5 miles in every direction from the original site in Banner Elk, NC. Collections of *L. nigrinus* adult beetles have been made each year since 2007, when 46 F4 beetles were collected and released in Sugar Grove, NC. Collections of beetles have increased each year; 2008 – 189 beetles (collected by Salom, Story, and McDonald; all beetles returned to Virginia Tech); 2009 – 581 beetles (100 to Mausel, 381 released behind Mast Store, 42 beetles to Mountain Aire near Celo and 58 beetles to Stewart Skeate); 2010 – 1,094 beetles during fall of 2010 (468 to Brad Onken, 200 beetles to Mausel, 320 beetles to Calloway Ridge road, Foscoe, NC and 106 to Blowing Rock, NC). We are now able to collect *L. nigrinus* beetles in significant numbers from our 3 oldest release sites. Collection data show that we are able to recover more beetles in the urban community forest interface because of ease of access, edge factors, consistently high HWA pest populations, and tree characteristics in open grown sites. For more detailed information on natural insectaries, please see the Case Study, Chapter 16 in this publication.

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Chapter 19: Integrating Chemical and Biological Control

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INTRODUCTION

Research and management efforts to establish an effective biological control program against HWA has received significant support by the U.S. Forest Service over the past 17 years. Other federal and state agencies, universities, and private entities have also contributed to this overall research and management effort. Although a number of HWA-specific predator species from Asia and western North America have been studied in quarantine, mass reared, and released, the work discussed here will focus on *Laricobius nigrinus* (Coleoptera: Derodontidae). This predator, from western North American hemlock forests, has become established throughout the mid-Atlantic region (Mausel et al. 2010). Also current studies by G. Davis (Ph.D student at Virginia Tech) show that the beetle does not disperse very far the year they are released and only about 300 m 5 years after release. Long-term impact studies of the predator are ongoing, but it is apparent that at many of the release locations where *L. nigrinus* has established, older mature trees have succumbed to HWA. The younger, more vigorous understory trees do not decline as quickly, and appear to sustain growing populations of *L. nigrinus*.

Imidacloprid has been the standard insecticide for application against HWA in urban and other settings where individual trees are highly valued. Merit 75WP and, more recently, Advance Tree and Shrub (Bayer) for homeowners have been used effectively in soil applications. Stem injections of various imidacloprid formulations have also found a niche for treating high value hemlocks. Recent formulation advances by Bayer have included CoreTect®, slow-release tablets placed under the organic layer around the root collar of trees. This recently registered product allows for a much easier application of imidacloprid and makes treatment of trees in remote areas more feasible.

Laricobius nigrinus susceptibility to imidacloprid was recently studied by Eisenback (2008). While acute toxicity was demonstrated in the laboratory from topical application and from feeding on poisoned prey (Eisenback et al. 2009), results were much less conclusive in the field. At sub-lethal dosage applications, predator mortality and fitness impacts from feeding on HWA settled on previously treated trees were minimal. Furthermore, HWA is extremely sensitive to imidacloprid (Cowles et al. 2006) and the presence of HWA on previously treated trees should indicate that imidacloprid concentrations in those branches are low or absent. A greater source of negative effects of imidacloprid on HWA predators was therefore predicted to be a result of reduced prey quality and density (Eisenback, 2008). Although imidacloprid exposure through feeding on adelgids on treated trees is possible, most HWA available to predators should be located on untreated trees or trees with little risk of exposing predators to toxicity.

Therefore, the new chemical technologies, the limited dispersal ability of the predator, and the predicted limited impact that systemically applied insecticides may have on predators in the field all lead toward the idea of developing a strategy that uses both chemical and biological tactics in the same stands. One integration scenario is to maintain the health of a select number of large hemlocks with insecticide applications, and at the same time

release and allow the biological control agents to become established on understory trees, increase, and serve as long-term suppressers of HWA. We hypothesize that this integrated approach could save more hemlock trees over time in a given area than the use of either control treatment (biological or chemical) in isolation. If shown to be an improvement over current strategies, it can become the standard approach to area-wide IPM for HWA.

TESTING THE HYPOTHESIS

A study has been initiated at Kentucky Ridge State Forest, near Middlesboro, KY. Three blocks, each containing one replicate of four treatments, have been established (Fig. 1). The treatments are: 1. treat a cohort of co-dominant to dominant trees with imidacloprid; 2. release *L. nigrinus* on a cohort of understory hemlock trees; 3. combine insecticide and beetle release treatments as in 1 and 2; and 4. do nothing (control).

In plots assigned chemical-only and chemical plus predator treatments, 6 dominant or co-dominant eastern hemlock trees were chosen for chemical treatment. Chemical treatments consist of soil injection of imidacloprid (Merit 2F) applied with a Kiortz soil injector at a recommended rate of 0.2 ounces of product per inch dbh (0.5 g [AI] /cm dbh). Insecticide treatments were applied

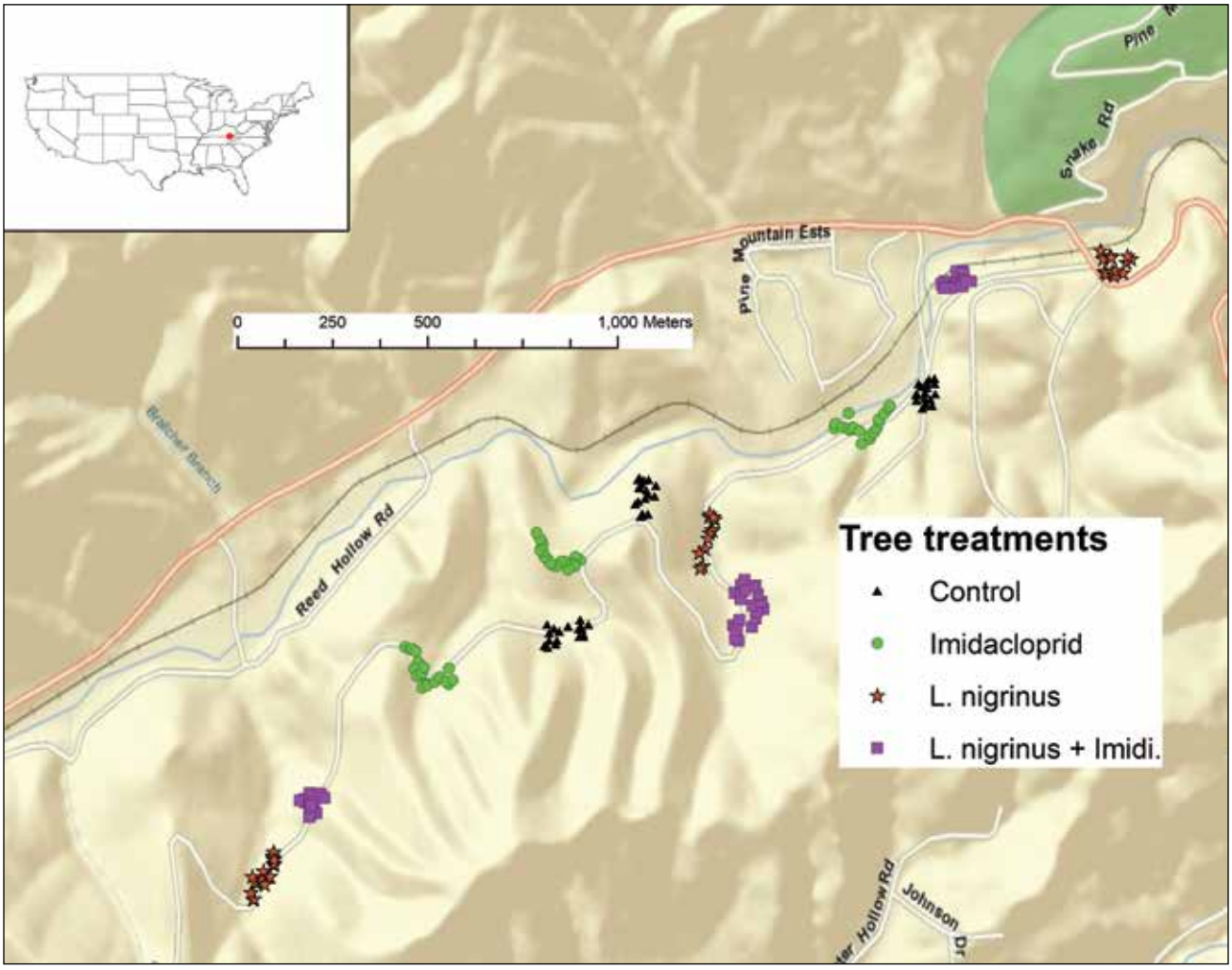

Figure 1. Chemical and biological control plots in Kentucky Ridge State Forest, KY.

in May 2010. In plots assigned beetle-only and beetle plus chemical treatments, 6 intermediate/suppressed trees were chosen as predator (*L. nigrinus*) release trees. A total of 125 lab reared *L. nigrinus* adults were released per tree (750 per plot) in October 2010. An additional 6 untreated trees within each plot were randomly selected for data collection to contrast with treated trees.

Tree health and HWA population measures were made for all chosen trees before treatment and will be assessed annually for 3 more years. Tree health measures include percent live crown ratio, foliage transparency, new growth, and tip dieback. HWA populations will be measured by randomly selecting 10 branches per tree. The terminal 30 cm will be examined and the number of HWA counted until ten HWA are found and the next branch will be examined. The total number of HWA found on the ten branches will be summed and this number will be recorded as an index of HWA density for that tree.

Sampling for adult predators every fall using beat-sheet methods and larvae every spring by clipping infested branches and rearing will be carried out to assess predator establishment. Predator exclusion sleeve-cage evaluations may also be carried out to determine if the impact from predation differs between predator only and predator plus chemical treatments.

Additional sites over a wide geographic range will be added to this study in an attempt to evaluate the proposed strategy along the active front of HWA movement. We will choose locations that normally would be chosen for HWA predator releases (i.e., stands with building HWA populations and none to minimal decline in tree health). In these situations, HWA populations are often sporadically present throughout the stands.

While this design will provide more answers over a longer time period, it is likely to yield measurable results within 3 years of application. We will be able to compare overall stand health for each treatment tested. We will also be able to compare predator establishment under the two predator treatment regimes to determine if presence of chemically-treated trees impacts predator success. Perhaps when this is done, we will be able to recommend a new coordinated integrated control treatment that can save a higher percentage of trees at any one location.

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Chapter 20: Integrating the Early Steps of Host Selection Behavior into Biological Control of HWA

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INTRODUCTION

When developing new programs, biological control practitioners have sometimes overlooked the role of behavior in the successful introduction of potential biological control agents. This is surprising since both host herbivore and natural enemy behavior can influence the overall efficacy of the biological control effort. To increase the efficiency and efficacy of using biological control agents to manage populations of non-native invasive organisms, biological control practitioners need to better understand the direct and indirect effects of the control agent. One way of predicting the potential direct and indirect effects of the control agent is to understand host specificity and behavioral processes involved in the multiple steps of host selection that end in host use.

Host selection behavior is a series of direct and indirect behavioral responses to hosts. The steps in host selection behavior include habitat location, host location within the habitat, host acceptance, and host use (Kennedy 1965). This sequence of behavioral steps in host selection raises a number of questions that have consequences for host specificity testing in biological control programs. Perhaps the most important consequences are those that originate from lack of understanding of the early steps in the host selection sequence. For example, if habitat location and host location are absent in testing host specificity then the lack of that information may result in false positives and therefore, lead to an overestimation of host use in the field.

The same steps that dictate the acceptance of plants as hosts for herbivores also apply to natural enemies of the herbivores, such as predators (Vinson 1998). Natural enemies also use visual and chemical cues associated with the food plant to locate their hosts or prey. Known chemical attractants that are common in plants and attractive to insects include green leaf volatiles and terpenoids (Bukovinszky et al. 2005). Once at the plant, natural enemies rely on chemical, tactile, gustatory, and visual cues to locate their prey. In the case of specialized natural enemies, chemical cues used to locate suitable plants may differ between exotic and native plant species. Predators may not initially respond to novel odors associated with the exotic plant, and these behaviors should be evaluated and incorporated into control efforts.

Testing to assess host use of potential biological control agents (arthropods) needs to incorporate as many steps of the host selection behavior sequence as possible. Options include using large assay arenas, outdoor arenas, or open field-testing. Unfortunately, false positives and false negatives can occur in cage experiments due to the lack of behavior filters that occur early in the sequence. For example, Turanli and Schaffner (2004) reported that the sessiid moth *Tinthia myrmosaeformis* (Herrich-Schaffer) showed little host specificity in contact bioassays with test plants, great specificity in multiple-choice cage tests, and the highest level of specificity in open field tests. Habitat location and pre-aligning cues often rely heavily on olfaction (Bernays and Chapman 1994) and still air or cage assays do not allow

testing insect responses to cues experienced during these early steps in the host selection sequence. Therefore, laboratory tests need to include wind tunnels, olfactometers, and/or airflow though cages.

Olfactometers are well-known tools used to identify host finding cues in parasitoids (Mills and Wajnberg 2008), phytophagous insects (Bernays 2001), and mosquitoes (Gillies 1990) but are less used in the development of biological control with predatory natural enemies. Nonetheless, there is no evidence that suggests that the behavioral responses of predators to host cues would be any different to that of parasitoids or phytophagous insects (Nakashima et al. 2002, Vanas et al. 2006). Therefore, including olfactometer assays to complement methods currently used in biological control program could lead to greater success due to increased establishment in the novel environment.

In addition to using multiple behavioral biological assays, it is important to incorporate external and internal factors that interact to influence the behavioral responses to host selection and use (Vinson 1984, Vet and Dicke 1992, Turlings et al. 1993, Powell et al. 1998). External stimuli are often prey or plant derived (Misell et al. 1984, Rhoades 1985, Price 1991), and may relate directly to host nutritional suitability or defensive capacity of the preys host plant (Hugentobler and Renwick 1995; Chambers et al. 1997; Frankfater and Scriber 1999, 2003). Insects use visual (Prokopy and Owens 1983), auditory (Drosopoulos and Claridge 2006), olfactory (Wood 1982), gustatory (Glendinning et al. 2009), and tactile stimuli (Rehman and Powell 2010) to identify and locate potential hosts. Once received by the insect, they are filtered, integrated, and interpreted as an attractant or deterrent at each step in the host selection sequence. Individual insect condition such as lipids (Mayhew 1997, Wallin and Raffa 2000), age (Stienberg et al. 1992), prior experience (Cunningham et al. 2001, deBoer and Dicke 2006), and hosts associated with pre-adult development (Tamo et al. 2006) influence an insect's response(s) to the external stimuli encountered during each step of the host selection sequence.

Several natural enemies discriminate between volatiles emitted by infested or uninfested trees (Vet et al. 1990, Harmel et al. 2007). The source of these chemical stimuli can be from the herbivore, the plant, or from the interaction of the two. In the early steps of host selection sequence stimuli from the host plant of the prey may be more reliable for the insect predator. Understanding the role of habitat or hosts infested with the prey in the host selection process is an important but often difficult step in evaluating the overall efficacy of a biological control agent. Stimuli generated by the herbivore prey are the most reliable source of information to a predator because they can inform the predator of the presence, identity, availability, and suitability of the prey (Whitman 1988), but they may not be apparent or available. Herbivore-derived information has two inherent constraints that limit its detectability and, therefore, its use as stimuli for prey location. Generally, herbivore prey are a small component of a complex environment and any information they provide will be in small amounts. Additionally, prey tend to be inconspicuous to avoid predation. Stimuli from the host plant of the herbivore prey are usually more readily available because of the plants comparatively large biomass, but are less reliable predictors of herbivore prey presence and suitability (Lima and Dill 1990, Dicke 1999, Cortesero et al. 2000). Understanding interactions among host plants, herbivore prey, and predator behavior may uncover important aspects of the biology of the predator that would otherwise be unnoticed, such as the influence of pre-release handling (e.g., age, mating status, level of satiation), the response of predators to plant odors, and herbivore host-induced plant odors.

Natural enemies are often held without exposure to their prey before release with unknown consequences to their host selection behavior. Physiological changes within an individual insect have been shown to affect orientation to hosts and responses to external cues (Kennedy 1977, Miller and Strickler 1984, Wallin and Raffa 2000). Physiological changes predict that factors linked to time limitation (e.g. age, time

since last meal) should increase the selection of suboptimal resources. However, in *Papilio glaucus* (Lepidoptera: Papilionida) the apparent direction of age effects are towards a greater specificity (Scriber 1993). A reversal from time limitation to egg limitation appears counterintuitive as age increases, unless there is an increased cost of egg production associated with aging.

The influence of food deprivation on host acceptance behavior suggests that satiated predators introduced into choice tests are not as likely to attack and feed on less preferred but otherwise acceptable test species than insects that are previously deprived of food (Withers et al. 2000, Barton and Whithers 2002). Predators that are satiated might reject test species in no-choice tests if the period without food or feeding on a non-target prey was short. Consequently, such tests would fail to reveal the fundamental host range (sensu Nechols and Kikuchi 1985, van Klinken 2000) therefore producing a false negative result. It is lesser known how recent food deprivation and lipid content of individual insects might influence the early steps of host selection sequence. Hence determining an optimal period of food deprivation prior to initiating testing host location and specificity is likely a good practice. Understanding consequences of food deprivation on host acceptance behavior may guide the release of potential biological control agents.

Change in preference by ovipositing or feeding in adults due to previous experience with a host has been documented in various insects (e.g., Vinson et al 1977, Prokopy et al. 1982, Jaenike 1983, Rausher 1983, Stanton 1984, Szentesi and Jermy 1990, Bjorksten and Hoffmann 1998; see also Agrawal et al. 2002). The phenomena of host selection being altered by previous experience with a host has been well reviewed for hymenopteran parasitoids by Turlings et al. (1995) and Vet et al. (1995). The general prediction is that there is a positive relationship between time of the last feeding and responsiveness to a lower ranked host. However, this is not always supported in laboratory tests. For example, Scriber (1993) exposed *P. glaucus* adults from the same population for two days to four differently preferred hosts. Oviposition specificity was influenced only on the first day after being placed in the arena with the four hosts present and not after.

There are fewer studies that have investigated the effects of experience on host selection behavior of predators. However, this is a very important step in developing a biological control program because one challenging effect to avoid is any enhanced responsiveness to the rearing host or environment. For example, increased attraction following experience with a host plant volatiles was demonstrated in the introduced predator *Anthocoris nemoralis* (F.) (Heteroptera: Anthocoridae) (Drukker et al. 2000). Specifically, wild-caught predators preferred odors from the host plant when offered either clean air, whereas laboratory reared first generation predators did not. However, after the laboratory reared predators were exposed to the host plant or host plant volitiles they showed a strong preference for the volatiles. The predatory mite *Phytoseiulus persimilis* Athias-Heriot is more attracted to the host that the prey was reared on when previously exposed to volatiles emanating from the prey's host (Dicke et al. 1990, de Boer et al. 2005). These results are similar to those seen in some parasitoids and suggest that experience-induced changes in host selection behavior could be expected to occur with predators. Any bias for the previously consumed species and/or its substrate can be avoided or reduced by parallel experimental approaches used for parasitoids.

A particular difficulty in identifying larval conditioning is separating early adult experience from larval experience. This difficulty led to a period in which the presence of Hopkins' Host Selection Principle was largely discounted (e.g. Courtney and Kibota 1990, van Emden et al. 1996, Barron 2001). However, several studies in which larvae have been exposed to specific host plant chemicals have indicated that induction of adult ovipositional preference may occur during the larval period (e.g., Del Campo et al. 2001, Ikkei at al. 2010; see also Jaenike 1983, Tully 1994). The capacity to alter behavior with experience is now well documented in parasitoid insects, with associative learning in

adults and learning through contact or exposure to host-derived chemicals during development (Vet et al. 1990). Parasitoids use of a variety of host and host-plant cues in host selection behavior sequences was thought to be fixed and innate. However, it is now clear that behavior is often plastic and varies between individuals with different exposure histories as well as their genetic composition. Identifying how past exposure to host herbivore and host-plant derived chemicals as a source of intraspecific behavioral variation is of interest for the utilization of parasitoids as biological control agents (Vet and Groenewold 1990). The host selection behavior of the generalist predatory paper wasp, *Polistes dominula* (Hymenoptera: Vespidae), is influenced by larval experience (Rayor and Munson 2002). They tested the role that larval experience with unpalatable prey played in subsequent foraging choices by adult predators. The results of the study demonstrate that previous experience with deterrent chemicals during larval development altered patterns of prey acceptability to the adult insects. It is not clear if these trends will also be seen with additional predatory insects but they are observed in herbivores and may also apply to natural enemies of the herbivores, such as predators.

Understanding these cues, interactions between host plant and herbivore prey, and behaviors associated with predation are especially important with the increased attention on biological control of invasive organisms. We have conducted and are in the process of conducting host selection behavior studies of several potential predators of hemlock woolly adelgid, *Adelges tsugae* Annad (Homoptera: Adelgidae) (HWA) and provided information to increase overall efficacy in the efforts to control HWA in hemlock forests in eastern United States.

OVERVIEW OF METHODOLOGY

Behavioral bioassays were conducted to test the responses of adult beetles to HWA and several tree species (Table 1). Bioassays are ongoing. Methods are fully described in Wallin et al. (in press). Briefly, beetles were brought to the laboratory and held at 16 °C; 12:12 on HWA infested hemlock branches for 24-48 hours. All beetles were randomly chosen from one laboratory population, tested once, and moved to a separate laboratory population. The four-arm olfactometer (Analytical Research Systems, #OLFM-4-C-2440PE. Gainesville, FL USA) was used in all bioassays. The olfactometer consisted of three parts: the base with the air output, the intermediate part, which delimited the walking chamber with a 9-cm circular central opening to introduce insects, with the four air inputs, and the transparent lid. Air was drawn in at a flow rate of -0.1Mpa. Air flow was controlled at 1.2Mpa in all four arms into the glass chambers containing the test material and carrying volatiles into the olfactometer. Arms with chambers receiving air but without test material were regarded as "blank" or control chambers.

All ambulatory responses were tested at 20 °C with artificial overhead lighting. For each experimental run an individual beetle was introduced at the center of the olfactometer. Each test lasted 15 min. Two criteria quantified behavior: (1) time spent in each field and (2) if the insects chose an odor field at the beginning of the test and remained in that field (final position). When air passed through the chambers into the olfactometer stage each of the airfields were considered to be a separate field and an additional field, central field (CF), at the center of the arena. We considered that an insect entered a given field when its entire thorax crossed the field boundary. A test was not retained when an insect remained motionless in the CF for more than 5 minutes.

SUMMARY OF FINDINGS

Laricobius nigrinus, *S. coniferarum*, and *S. tsugae* responded to odors from HWA's host trees, but not to odors associated with HWA alone. HWA being inconspicuous to all three predators suggests that HWA is extremely difficult to detect. This suggests these predators rely on volatiles produced by HWA's host trees to locate potential prey. This is true for many parasitoid species (Lima and Dill 1990, Vet et al. 1990, Tumlinson et al. 1993, Dicke 1999, Cortesero et al. 2000).

Table 1: Series of laboratory experiments quantifying the interactions of internal physiology and external stimuli on an early step in host selection sequence. The behaviors of *Laricobius nigrinus* (Ln), *Scymnus coniferarum* (Sc), *Sasajiscymnus tsugae* (St), *Laricobius rubidus* (Lr), and *Laricobius* hybrids (Ln x Lr) were tested using four-armed olfactory behavioral bioassays.

	Internal physiology		
External stimuli	**Starvation/lipids**	**Sex**	**Host during pre-adult development**
HWA	Ln[1], Sc[2], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], Sc[2], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], St[3,5]
Eastern Hemlock	Ln[1], Sc[2], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], Sc[2], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], St[3,5]
Western Hemlock	Ln[1], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], St[3,5]
Eastern White Pine	Ln[1], Sc[2], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], Sc[2], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], St[3,5]
Western White Pine	Ln[1], Lr[4], LnxLr[4], LrxLn[4]	Lr[4], LnxLr[4], LrxLn[4]	Ln[1], Lr[4], LnxLr[4], LrxLn[4]
Douglas-Fir	Ln[1]		Ln[1]
Spruce	Ln[1]		Ln[1]
Ponderosa Pine	Ln[1]		Ln[1]
Eastern Hemlock with HWA	Ln[1], Sc[2], St[3], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], Sc[2], St[3], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], St[3,5]
Western Hemlock with HWA	Ln[1], Sc[2], St[3], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], Sc[2], St[3], Lr[4], LnxLr[4], LrxLn[4]	Ln[1], St[3,5]

[1]Collected by Glenn Kohler from 16 sites in WA and OR (see Kohler et al. 2008, Wallin et al. in press).
[2]Collected by Dr. Richard McDonald from 1 site in WA (see Wallin et al. in prep).
[3]Reared and supplied by Virginia Poly Technical Institute and State University Biological Rearing facility.
[4]Collected by Dr. Richard McDonald from release sites in NC (assays to begin in 2011).
[5]Collected by Dr. Ashley Lamb from sites in Japan.

Host odors are less reliable than odors generated by the prey. This challenge may be overcome in systems where the predator, prey, and host have co-evolved with plants (Vet et al. 1990, Harmel et al. 2007). Volatiles induced by feeding of the prey may provide specific information to the predator and greatly increase its reliability (Harmel et al. 2007). However, the presence of feeding HWA did not increase the attractiveness of hemlock branches to *L. nigrinus* (Wallin et al. in press), *S. coniferarum* (Wallin et al. in prep) or *S. tsugae* (Wallin et al. in prep) suggesting that these predators are responding to hemlock volatiles produced by the cut branch, but not specific odors produced in response to adelgid feeding.

The condition of individual beetles influenced the response to host plant odors in the olfactometer. All three beetle species were more likely to respond to host plant odors if they were starved. The effects of starvation on response to host plant odors manifested within 24 hr without access to HWA for *L. nigrinus* and 4 hr without access to HWA for both *S. coniferarum* and *S. tsugae* (Wallin et al. in prep). However, the response to host plant odors decreased as the length of starvation increased for *L. nigrinus*, *S. coniferarum*, and *S. tsugae*. Total lipid content declined with the duration of starvation; however, there was not a linear relationship between lipid content and the mean time response time of *L. nigrinus*, *S. coniferarum*, and *S. tsugae* to host plant odors (Wallin et al. in prep).

Another way predators can overcome the low reliability of host odors is through conditioning during pre-adult development to respond to volatiles from the host plant they developed on (Tamo et al. 2006) or conditions in which they were reared. We found that *L. nigrinus* collected from western hemlock walked toward the chamber with western hemlock, whereas those reared in the laboratory on eastern hemlock showed no preference. However, *S. tsugae* collected from hemlock in Japan responded to both eastern and western hemlock, whereas those reared in the laboratory on eastern hemlock did not respond in the assay arena (Wallin et al. in prep). It is not clear why adults raised on eastern hemlock did not walk toward their natal hemlock species. Multi-generational studies on the effect of rearing on predator-prey location behavior are needed as are studies on associative learning. However, it should be noted that our results apply to an enclosed environment where chemosensory cues are highly concentrated and easily discernible. Whether or not the difference in volatiles would be detectable by insects in a field situation is unknown and requires further study.

Overall, our study suggests that these three potential predators of HWA that have been tested to date use chemosensory cues from HWA host trees and that these responses are plastic and depend on both season, external, and internal cues. These factors should be taken into consideration when planning releases of predators as biological control agents.

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Chapter 21: The Introduction of Laricobius nigrinus as a Biological Control Agent for the Hemlock Woolly Adelgid: Is There a Threat to the Native Congener, L. rubidus?

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BACKGROUND

Laricobius Rosenhauer is one of four genera in the family Derondontidae (Coleoptera) that occupies the temperate regions of the Northern Hemisphere (Lawrence 1989). Members of this genus are only known to prey on adelgids (Hemiptera: Adelgidae) (Lawrence and Hlavac 1979, Lawrence 1989). There are three species native to North America: *L. nigrinus* and *L. laticollis* are native to western North America, and *L. rubidus* is native to eastern North America. *Laricobius nigrinus* is being used in the eastern United States as a biological control of the hemlock woolly adelgid. Previously, *L. erichsonii* was introduced to both coasts of North America from Europe for control of the balsam woolly adelgid (summarized in Montgomery et al. 2011). This species was reported to have established, but its most recent recorded recovery was in 1978 (Schooley et al. 1984). A molecular study of the relationships among the four *Laricobius* species reported in North America, plus *L. kangdingensis* and *L. osakensis* from Asia, showed that, surprisingly, the two species from western North America were not the most closely related (Montgomery et al. 2011). Instead, it was found that *L. nigrinus* is very closely related to the eastern species, *L. rubidus*. In fact, the genetic distance between these species (using a portion of the mitochondrial COI gene) was only slightly higher than within each species (Davis et al. 2011). This suggests that they diverged very recently and may not be reproductively isolated. Recent work (described in more detail below) has shown that *L. nigrinus* and *L. rubidus* are in fact interbreeding at sites in the eastern U.S. where *L. nigrinus* was released. It is not yet known if this will enhance or hinder hemlock woolly adelgid (HWA) biological control.

ACKNOWLEDGING RISK

It is important to weigh the benefits and risks when making decisions for natural resource management (Loomans and van Lenteren 2005). Benefits of hemlock woolly adelgid are nil, while the risks and costs are great. Loss of hemlock timber and pulpwood (Burns and Honkala 1990, Ward et al. 2004) and residential property values (Holmes et al. 2006) can be tabulated, while calculating costs associated with intangible environmental and aesthetic benefits are much more difficult (Anders 1977, McConnachie et al. 2003). Left uncontrolled, HWA has the potential to cause hemlock mortality within all 25 forest cover types of which it is a component (Burns and Honkala 1990, Orwig et al. 2002). Loss of hemlock alters eco-hydrological systems (Ford and Vose 2007) and accelerates growth of invasive plants (Eschtruth and Battles 2008). It can also negatively impact temperature-sensitive streams (Snyder et al. 2002, Ross et al. 2003) and habitat

for numerous wildlife species (Yamasaki et al. 1999, Onken and Souto 2000, Lishawa et al. 2007).

Some of the risk associated with a biological control agent can be evaluated by laboratory experiments prior to its introduction, yet it is recognized that environmental variability and other sources of uncertainty are cause for continued post-release assessment (Louda et al. 2003, Hopper et al. 2006). An unexpected risk that was recently discovered in association with the release of *L. nigrinus* is its ability to hybridize with a native species, *L. rubidus*. In this report, we summarize what is currently known about interbreeding between *L. nigrinus* and *L. rubidus*, and we discuss research directions to evaluate the implications for biological control of HWA.

Laricobius nigrinus Fender

Laricobius nigrinus Fender is a small (2-3 mm), black beetle native to western North America (Fender 1945, Zilahi-Balogh et al. 2006) where it has been found to be a widespread and abundant natural enemy of HWA, at both low and high densities of the pest (Kohler et al. 2008).

Both adults and larvae feed on *A. tsugae* eggs, nymphs, and adults. Eggs of *L. nigrinus* are laid in late winter and early spring. Larvae develop through four instars, feeding on HWA progrediens eggs, and drop to the forest floor to pupate. Adults diapause during summer in the soil and emerge in fall to feed on HWA sistens nymphs in the fall and winter (Zilahi-Balogh et al. 2003). The life cycles of *L. nigrinus* and HWA are highly synchronized (Zilahi-Balogh et al. 2003).

Laricobius nigrinus was imported into the eastern United States from Victoria, British Columbia for further evaluation and was determined to be host-specific in the laboratory (Zilahi-Balogh et al. 2002). Federal and State approval for environmental release of *L. nigrinus* was granted in 2000. Laboratory mass-rearing methods were developed for *L. nigrinus* and adults are currently being reared in a number of laboratories (Lamb et al. 2005). Free releases of *L. nigrinus* began in 2003. As of 2009, *L. nigrinus* adults were released in 15 eastern states, spanning USDA plant hardiness zones 5a to 7a (Roberts et al. 2010). It was found to establish in 13/22 (59%) of initial release sites (Mausel et al. 2010). The probability of establishment was greater at sites with higher minimum annual temperatures and where more beetles were released. Additional *L. nigrinus* from Idaho has been released in several New England states in an attempt to establish a more cold-hardy strain in the north.

Laricobius rubidus

Laricobius rubidus is the only species of *Laricobius* native to eastern North America (Clark and Brown 1960; Lawrence 1989). Its known distribution extends from the District of Columbia, north to New Brunswick, west to Minnesota, and south to North Carolina (Brown 1944, Raske and Hodson 1964, Lawrence 1989, Wallace and Hain 2000). Its primary host is the pine bark adelgid (PBA), *Pineus strobi* Hartig (Clark and Brown 1960). *Laricobius rubidus* has also been found to occasionally feed on the balsam woolly adelgid, *Adelges piceae* Ratz. (Lawrence and Hlavac 1979) and has been collected from eastern hemlock infested with HWA throughout its introduced range (Montgomery and Lyon 1996, Wallace and Hain 2000, Mausel et al. 2008). Laboratory studies have shown that it can reproduce and complete development on HWA, but has an ovipositional preference for pine bark adelgid (PBA) (Zilahi-Balogh et al. 2005).

The life cycle of *L. rubidus* is well synchronized with that of PBA (Clark & Brown 1960). Adults are active between late March and early June with peak activity between mid-April to mid-May (Clark and Brown 1960, Zilahi-Balogh et al. 2005). Four instars are present late April through early June (Clark and Brown 1960), migrating to the soil to pupate by late June (Zilahi-Balogh et al. 2005). Emerging adults undergo an aestival diapause, becoming active in October through early November (Zilahi-Balogh et al. 2005). In Virginia, *L. rubidus* adults have been observed migrating from the branches to the duff where they are thought to undergo a hibernal diapause, but adults associated with HWA have also been found to be active in the winter (Zilahi-Balogh et al. 2005, Mausel et al. 2008).

POTENTIAL FOR INTERBREEDING

Adult *L. nigrinus* can be distinguished morphologically from *L. rubidus*. *Laricobius nigrinus* (Fig. 1) has unicolorous (black) elytra, the distance across the posterior of the pronotum is greater than across the anterior, and the apices of the lateral parameres of the male genitalia are narrowly acute. In contrast, *L. rubidus* (Fig. 2) has bicolor (red and black) elytra, the distances across the posterior and anterior of the pronotum are subequal, and the apices of the lateral parameres are truncate (Montgomery et al. 2011, Leschen 2011). The immature life stages are morphologically indistinguishable.

Several observations prompted questions about the potential of *L. nigrinus* and *L. rubidus* to interbreed: 1) both species are routinely recovered from HWA-infested hemlock trees in the eastern U.S. at sites where *L. nigrinus* was released; 2) molecular analysis of the genus *Laricobius* found, surprisingly, that *L. nigrinus* and *L. rubidus* are very closely related suggesting that they are recently diverged species that may have the ability to produce viable offspring (Klein et al. 2010, Montgomery et al. 2011); 3) members of the two species were observed copulating with each other on HWA infested hemlock at the Virginia Tech field insectary, which neighbors a white pine stand infested with pine bark adelgid (Mausel et al. 2008); and 4) morphological and molecular species identification were found to be in conflict for two beetles collected from a *L. nigrinus* release site in Maryland, suggesting that these individuals could be of hybrid origin.

This prompted the development of microsatellite markers that could be used to distinguish *L. nigrinus* and *L. rubidus* from their hybrids (Klein et al. 2010). This method exposed a trend of an increasing proportion of hybrids recovered at *L. nigrinus* release sites in Pennsylvania, North Carolina, and Tennessee between 2007 and 2009 (Havill et al. 2010). It was also used to identify *Laricobius* adults collected from HWA-infested hemlock the Virginia Tech field insectary where PBA-infested white pine grows in close proximity (N. Havill, unpublished data). Data from six microsatellite loci analyzed with the software NEWHYBRIDS (Anderson & Thompson 2002) were used to classify beetles. In 2008 we collected 27 *L. nigrinus*, 15 *L. rubidus*, and 13 hybrids. In 2010 we collected 87 *L. nigrinus*, 4 *L. rubidus*, and 8 hybrids, and in 2011 we collected 87 *L. nigrinus*, 7 *L. rubidus*, and 9 hybrids. Further confirmation that these species can interbreed was shown in a 2009 laboratory study where three interspecific pairs produced viable offspring (T. Dellinger, unpublished data).

Figure 1. Laricobius nigrinus (photo by Gina Davis).

Figure 2. Laricobius rubidus (photo by Gina Davis).

Field collected beetles that were identified as having mixed parentage had morphological characters that resembled either parent species or were intermediate—i.e., they had black or bicolored elytra, and the parameres of the male genitalia were either accute, truncate, or intermediate (Fig. 3). It is therefore not possible to use morphology to distinguish beetles of mixed parentage from the parent species.

The ecological niches occupied by *Laricobius* species and their offspring may affect the geographic distribution and extent of interbreeding. Laboratory host range studies show that *L. nigrinus* prefers HWA on hemlock (Zilahi-Balogh et al. 2002), and *L. rubidus* prefers PBA on white pine (Zilahi-Balogh et al. 2005). It may therefore be more likely for the two species to encounter each other in areas where hemlock and white pine co-occur than in areas with only one host species is present. The extent to which *Laricobius* adults migrate between stands would also affect the rate of interbreeding. *Laricobius nigrinus* was found to be common within 300 m of the original release trees by the fourth generation (G. Davis, unpublished data). Other observations suggest that *L. nigrinus* can disperse greater distances. For example, McDonald (2010) recovered *L. nigrinus* from at least 1.6 km from the release area, five years post-release. Preliminary data suggest that the geographic overlap of hemlock and white pine may indeed affect the rate and incidence of interbreeding between the species. Recovery of *L. rubidus* on hemlock was lower where eastern white pine was sparse or absent from stands in which *L. nigrinus* was released (G. Davis, unpublished data). In addition, we collected *Laricobius* from white pine at the Virginia Tech field insectary in 2011, and all 47 were classified as pure *L. rubidus* (Havill, unpublished data). Additional samples from hemlock and white pine in *L. nigrinus* release sites, as well as laboratory choice tests with hybrid beetles will help to further predict the importance of ecological factors in determining the outcome of interbreeding.

POSSIBLE HYBRIDIZATION SCENARIOS

Introductions of nonnative species can have large impacts on the genetics of native species through hybridization and introgression (i.e. gene flow) (Mooney and Cleland 2001, Mallet 2007). Hybridization between *L. rubidus* and *L. nigrinus* could have several outcomes, including:

- Hybrid Incompatibility
  - Sterility of hybrids
  - Outbreeding depression
  - Reinforcement of premating isolation
- Hybrid vigor
  - Speciation
  - Genetic assimilation

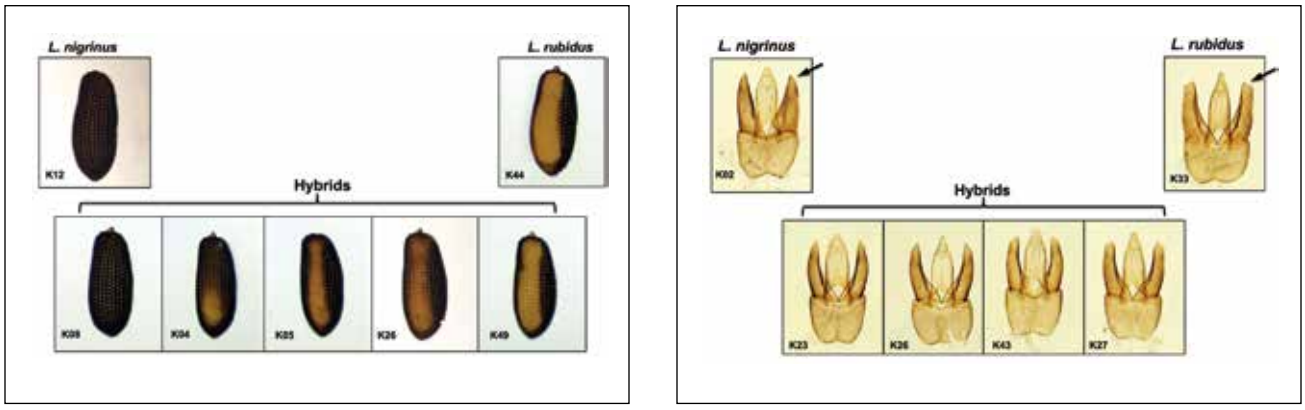

Figure 3. Examples of elytra (left) and slide-mounted male genitalia (right) of L. nigrinus, L. rubidus and their hybrids. Arrows point to the lateral parameres that are acute in L. nigrinus and truncate in L. rubidus. Hybrids can resemble either parent species or can be intermediate.

Hybrid Incompatibility

Reproductive isolation between populations can result in the accumulation of genetic incompatibilities over time. This could make reproductive isolation permanent, even if the cause of isolation were removed (Palmer and Feldman 2009). Hybridization produces recombinant genotypes that have not previously been subjected to selection. These genotypes will typically be less well adapted than those of their parents, resulting in selection against hybrids (Burke and Arnold 2001).

Sterility

Selection against hybrids is often exhibited as sterility or inviability (Haldane 1922, Mallet 2007). The production of sterile or inviable offspring would result in a decrease in fitness of the parental species due to an overall decrease in reproductive output. Although *Laricobius* hybrid sterility is a possibility, there is evidence of F_2 hybrid individuals and backcrosses in the field (N. Havill, unpublished data), suggesting that at least some of the F_1 hybrids are fertile.

Outbreeding depression

Outbreeding depression is a reduction in hybrid fitness, possibly due to the hybrid offspring being less well adapted to environmental conditions than the parental species (Klug and Cummings 2003). Outbreeding could result in lower reproductive potential (Arnold 1997). The reduction in reproductive output may occur as a result of a decrease in the number of offspring produced or as a result of lower levels of fertility or vigor among the hybrid progeny (Arnold 1997). We do not know whether *Laricobius* hybrids are less fit than their parents. Ongoing laboratory and field studies are explicitly testing this.

Reinforcement of pre-mating isolation

Hybridization can lead to an increase in reproductive isolation between parent species when mating barriers evolve due to selection against unfit hybrids (Mallet 2007). If reinforcement is occurring as *L. nigrinus* is released into sites where *L. rubidus* is present, over time we will see a decrease in hybridization and eventually a termination of hybridization as reinforcement becomes more powerful. This would also allow the parent species to remain genetically intact. Assessment of whether this is occurring will require long-term monitoring of the frequency of interbreeding.

Hybrid Vigor/Heterosis

Hybrids are often assumed to be less fit than their parents, but this is not always the case (Arnold 1997). A review by Arnold and Hodges (1995) found that hybrids were not uniformly less fit than parental genotypes.

Speciation

Unique adaptations might arise from combining divergent genomes (Arnold 1997, Mooney and Cleland 2001, Mallet 2007). The increased genetic variability that results from crossing divergent genotypes can result in offspring that are better adapted to changed and changing environments, allowing them to occupy a habitat that was not available to the parents (Arnold 1997, Mooney and Cleland 2001). If hybrids tend to assemble in habitats different than the parents by means of seasonality, drift in small populations, or change in host preference, then gene flow between hybrids and parents will be reduced, and hybrid speciation (the origin of a new species) can occur (Mooney and Cleland 2001, Mallet 2007). Adelgids on hemlocks in the eastern U.S. is a new niche that was created when HWA was introduced from Japan. *Laricobius* hybrids could be better adapted to this niche than their parents if they receive a preference for hemlock woolly adelgid from their *L. nigrinus* parents, and hardiness in eastern climates from *L. rubidus*. This possibility is being evaluated in laboratory and field studies.

Genetic assimilation

Open niches are not the only possible habitats for hybrids to invade (Arnold and Hodges 1995). If hybrids have an equivalent or higher fitness than the parents in their own habitat, the hybrids may replace the "pure" parental species due to competition (Arnold 1997, Mallet 2007). For

example, if hybrids were to show greater feeding efficiencies than those of the parental species, this could result in a greater reproductive capacity of hybrids and the displacement of the parental species locally (Grant and Grant 1996).

HYBRIDIZATION IN OTHER CLASSICAL BIOLOGICAL CONTROL PROGRAMS

There are very few examples in the literature of introduced biocontrol agents interbreeding with native species. We are aware of just three systems in which this was investigated in the laboratory, one of which also tracked hybridization in the field. Naka et al. (2005, 2006) found that a *Chrysoperla carnea* (Chrysopidae) introduced from Germany was able to produce fertile F_1, F_2, and backcrossed offspring with native Japanese *C. nipponensis* in the laboratory, but concluded that they were unlikely to hybridize extensively in the field because hybrid fertility was low, and the parent species have different courtship songs. Davies et al. (2009) used DNA sequence data to confirm that introduced *Diadegma semiclausum* (Ichneumonidae) can hybridize with native Japanese *D. fenestrale* in the lab, and encouraged field studies to follow up. Finally, Moriya et al. (1992) showed that an introduced parasitoid of chestnut gall wasps, *Torymus sinensis* (Torymidae), from China can hybridize with a native Japanese species, *T. beneficus*. The native species has an early-spring and a late-spring strain. Using field-collected wasps from a single chestnut orchard, Yara et al. (2010) found that the early-spring strain was displaced by the introduced species without evidence of hybridization, while the late-spring strain showed increasing frequency of hybrids over time. The effects on pest control were not evaluated.

CONCLUSIONS

Hybridization between *L. nigrinus* and *L. rubidus* has been confirmed in several *L. nigrinus* release sites where eastern white pine and eastern hemlock co-occur. We know hybrids are feeding on HWA and are capable of reproducing but there is no indication as yet whether hybridization will negatively or positively affect the HWA biocontrol program. Laboratory tests are underway to assess the feeding preferences and fitness of hybrids relative to the parent species. Both species readily feed and reproduce on hemlock woolly adelgid although laboratory studies indicate *L. nigrinus* is not able to successfully reproduce on pine bark adelgid. Laboratory studies have also shown each predator species to have a preference for one or the other adelgids when presented with a choice. Based on preliminary results of genotyping more than 1700 specimens collected from across the landscape where *L. nigrinus* has been released, the rate of hybridization has thus far been shown to be approximately 7 percent. These results and the known differences in host preference suggest that species separation is likely to be maintained with infrequent gene flow between the two species. We will continue to monitor this unusual hybridization event as it plays out over time.

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Chapter 22: An Overview and Outlook for Biological Control of Hemlock Woolly Adelgid

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Surveys in the eastern United States indicated that the native natural enemy complex associated with hemlock woolly adelgid (HWA) consisted primarily of generalist predators which did not effectively control HWA populations. Since no known parasitoids are associated with Adelgidae, the classical biological control program for HWA has focused on prey-specific predator species and entomopathogens. Although the earliest investigations into HWA biological control dates back to the early 1990s, the program has been going strong for the past decade and has involved 28 federal and state agencies, 24 universities, seven institutions in China and Japan, and numerous private industries. The integration and focus of biological control into an integrated pest management program remains the option of primary interest for suppression of HWA.

OVERVIEW

In the early 1990s, the lady beetle *Pseudoscymnus* n. sp (now *Sasajiscymnus tsugae*) and the oribatid mite *Diapterobates humeralis* were discovered in association with HWA in Japan by Dr. Mark McClure (retired, CT Agricultural Experiment Station). The mite fed on the woolly material surrounding the eggs, thereby dislodging the eggs from the trees to the ground. However, the mite is an opportunistic predator that occurs in many areas where the adelgid is absent. Even though it was already widely distributed in coniferous forests throughout the northern hemisphere, it was released in 1993 at two sites in CT but proved difficult to mass culture and ineffective for HWA. *Sasajiscymnus tsugae* was first reared and released in Connecticut in 1995 and, beginning in 1999 through 2011, over 2.5 million adults have been reared by five laboratories and released in 15 states in the East. *S. tsugae* has overwintered, established, reproduced, and spread at a limited number of release sites, but generally beetle recovery in most areas has been disappointing. Although the reason for sporadic establishment is unknown, several explanations have been put forward including: 1) poor sampling methodology involving the use of beat sheets to sample for adults in the lower crown while adult beetles tend to be phototrophic; 2) the tendency for adult beetles to widely disperse following release; and 3) the original rearing colony was small and a genetic bottleneck may have occurred over the 30+ generations of beetles that have been lab reared since 1995. *S. tsugae* was recently rediscovered in association with HWA in Japan, and mass rearing colonies have been established at North Carolina Department of Agriculture and University of Tennessee (Lindsay Young Beneficial Insect Laboratory) using only this new genetic stock. Studies are underway to determine if there are any apparent behavioral differences between the new and original genetic stock.

In 1995, Dr. Mike Montgomery (retired, U.S. Forest Service, Northern Research Station) began exploration in China with efforts focused on the diversity of lady beetles present in the forests with hemlock. Over 50 species were collected from

adelgid-infested hemlock in China, 21 of which were new to science. Three species of *Scymnus* (*camptodromus*, *sinuanodulus*, and *ningshanensis*) were the most abundant lady beetles feeding on HWA. Over 30,000 *S. sinuanodulus* beetles were released in 8 states from 2005-2009, with most located in the southeastern United States. As of 2011, establishment of *S. sinuanodulus* has not been confirmed at any of the release sites and rearing colonies of this beetle have been greatly reduced. Two small experimental releases of *S. ningshanensis* occurred in 2010 in North Carolina but establishment has not yet been confirmed. Rearing this species has proven difficult to maintain and the small colony has since been abandoned. The biology and rearing of *S. camptodromus* is currently being evaluated at Penn State University in an effort to better understand this species, its obligate diapause, and its host range. Based on the distribution of *S. camptodromus* in China, it is likely this beetle will be more cold tolerant than the other *Scymnus* species investigated and better suited for the northern range and higher elevations in the south. Field releases of *S. camptodromus* are anticipated in 2013.

In the late 1990s, the Pacific Northwest became the focus for predators of HWA with Dr. Lee Humble (Canadian Forest Service) observing *Laricobius nigrinus* feeding on HWA in seed orchards in British Columbia. The predator was imported by Drs. Lok Kok and Scott Salom to the quarantine facility at Virginia Tech. *Laricobius nigrinus* was approved for release in 2000 and the first releases were made in 2003. Additional surveys and collections of predators of HWA in Oregon and Washington have resulted in the shipment of thousands of *L. nigrinus* (Seattle biotype) for establishing lab colonies at New Jersey Department of Agriculture (Phillip Alampi Beneficial Insect Laboratory), Virginia Tech, University of Tennessee, Clemson University, North Georgia College and State University, and field releases in the East. Over 150,000 *L. nigrinus* (Seattle biotype) have been released and are now becoming widely established in plant hardiness zones 6a and 6b. These establishments span 11 states from the southern Appalachians to New England and have become the first potential biological control agent of HWA to establish in such numbers that beetles can be field-collected and redistributed elsewhere in the East. An inland biotype of *L. nigrinus*, which is more cold hardy than the Seattle biotype, has been collected in northern Idaho and northwest Montana since 2007 and released at a limited number of sites in New England. Small numbers of this biotype have since been recovered, indicating its adaptability to the more northerly climate and colder plant hardiness zones.

Two species of *Leucopis*, *L. piniperda* and *L. argenticollis*, were recovered from HWA infested western hemlocks. One of the species, *L. argenticollis*, was recovered feeding on both progredientes and sistentes eggs and nymphs in fairly large numbers from several collection sites in Washington and Oregon. *L. piniperda* and *L. argenticollis* also occur in eastern North America but are rarely recovered from HWA infested hemlock. Additional study and monitoring of these species in the West is warranted as *Leucopis* spp. may be adaptable to a range of climates because of their wide geographic distribution and host specificity toward HWA. Also, several species are among the biocontrol agents that have been responsible for measurable control of Adelgidae, specifically *Pineus* species in Chile and Hawaii. The current focus is to continue studies of biology and to develop rearing methods for *L. argenticollis*.

In 2005, an accelerated three year effort was initiated to explore for natural enemies of HWA in Asia, with a focus on China and Japan, followed by evaluation and quarantine studies. It was felt that the approach to foreign exploration before this time was "restrictive and inefficient." A full-time person, Dr. Wenhua Lu, was hired to be in China 3-6 months each year for two years. During this period she worked with Chinese scientists at intensive study sites in Sichuan and Yunnan Provinces as well as expanded the search for predators in additional provinces. This effort provided a more detailed assessment of HWA phenology and its associated natural enemies and the opportunity to study the full range of potential predators from the pests' native range. Unfortunately, this

effort did not result in the discovery of additional promising predator species in China. One species, *Tetraphleps galchanoides*, was investigated but soon abandoned as the larvae of *T. galchanoides* are also effective predators of *L. nigrinus* larvae.

The continued mass rearing and release of the old genetic stock of *S. tsugae* in the southern range has not produced many recoveries and only recently is being phased out of the overall program with initiation of field releases of the new genetic stock of *S. tsugae*. Also, the mass rearing of *L. nigrinus* has often been difficult due to high mortality during the pre-pupae and pupae life stages in the soil. Now that populations are becoming established in the East, field insectaries supplemented by wild collections in the Pacific Northwest will become the primary source of beetles which will provide the opportunity for laboratories to rear other HWA predators.

In 2005, the discovery by Drs. Montgomery and Shiyake (Museum of Natural History, Osaka, Japan) of a new species of *Laricobius*, now *osakensis*, in Japan created a renewed interest in the classical biological control program. In 2008-09, Dr. Ashley Lamb (University of Tennessee) conducted extended studies at several HWA locations in Japan to assess the phenology of HWA and its predators, and the impact of natural enemies on HWA in its native habitat. It was determined that *Laricobius osakensis* was the most abundant and frequently encountered predator of HWA in Japan, sharing many similarities to *L. nigrinus* in western North America.

In laboratory and field cage studies, *L. osakensis* has shown to be more robust in consumption of HWA and in producing a greater number of progeny than *L. nigrinus*. *L. osakensis* comes from the same location as the source population of HWA in the eastern U.S.; therefore, this species has evolved with the HWA affecting *T. canadensis*. Because of the geographically broad range this predator is found in Japan (elevations of 80-1850m), it is likely it will adapt well across the varied climate range in the United States. In 2010, this species was approved for release from quarantine.

The exploration for additional HWA predators in western North America continued into the early 2010s, but at a much reduced level. Only the previously mentioned *Leucopis* species and the coccinellid predator *S. coniferarum* warrant further investigations. *S. coniferarum* is another adelgid specialist often found throughout the year associated with HWA but typically in lower numbers than *L. nigrinus*. It readily feeds on both generations of HWA but is believed to be a primary predator of the spring progredientes generation. Host range testing and rearing methodologies for this predator are currently under investigation. Field releases of *S. coniferarum* are anticipated in 2012.

As exploratory efforts wind down, more emphasis will go toward the predators currently under review by improving the efficiency of collecting, rearing, and releasing the two biotypes of *L. nigrinus*, *L. osakensis* and *S. tsugae*. Maximizing the production of quality HWA predators requires highly skilled laboratory managers, adequate facilities and staffing, and an abundance of healthy host material (HWA) for food. The mass rearing of HWA predators by numerous laboratories with the primary goal of maximizing production over the shortest time has caused concern about the quality of the predators being reared and released. Drs. Allen Cohen (Insect Diet and Rearing Research LLC, North Carolina) and Carole Cheah (Connecticut Agricultural Experiment Station) are developing a fitness-based system of quality control standards for rearing predators (using *L. nigrinus* and *S. tsugae*) among the rearing laboratories.

Efforts to maximize mass rearing of predators based on a supply of sufficient quantities of quality host material ((HWA) has been problematic for all of the rearing laboratories. The impact of declining tree health on the nutritional quality of HWA as a food source has forced personnel at the labs to greatly expand their search to feed the laboratory reared beetles. The development and testing of artificial diet supplements by Drs. Cohen and Cheah has helped to mitigate the issue but more work in this area is needed. The most

successful diets and diet-presentation systems allowed adults of both *S. tsugae* and *L. nigrinus* to survive for several months, but no oviposition occurs in the absence of live host material. When provided HWA for a few days following feeding experiments on the artificial diet, egg production returns. *L. nigrinus* larvae will feed readily on the chicken egg-based diets but fail to develop.

Maintaining healthy laboratory-reared predators is a tremendous challenge that can be easily compromised if pathogens are inadvertently introduced to the colonies. The discovery by Dr. Lee Solter (Illinois Natural History Survey, Champaign, IL) of microsporidia infections in several laboratory and field collected populations of predators has resulted in heightened monitoring and periodic screening of predators. Microsporidia are single-cell organisms related to fungi and are obligate pathogens, typically chronic in nature, causing slow larval development, increased larval mortality, decreased adult lifespan, and reduced fecundity. Entire rearing colonies can be easily lost if adequate screening for microsporidia infections is not addressed. As of 2011, there are 8 laboratories mass rearing HWA predators including: Clemson University, New Jersey Department of Agriculture, North Carolina Department of Agriculture and Consumer Services, North Georgia College and State University, University of Georgia, University of Tennessee, Virginia Tech, and Young Harris College in North Georgia. The four predators currently being reared for mass release include: *L. nigrinus* (Seattle and Idaho biotypes), *L. osakensis*, *S. camptodromus*, and *S. tsugae*.

Investigations into potential entomopathogens have been ongoing since the mid-2000s. Dr. Bruce Parker (University of Vermont) conducted one survey in China and several surveys in the eastern U.S. for entomopathogens of HWA. Even though some non-host specific, native fungi have been found to infect HWA, naturally occurring epizootics of primary pathogens have seldom been observed. One particular fungus, *Verticillium lecanii* (now *Lecanicillium muscarium*), was recovered from HWA populations in the East and is currently being investigated by Dr. Scott Costa (University of Vermont). *L. muscarium* strain Ve6 is registered and commercially available in Europe to control whitefly pests in greenhouses. Laboratory, ground, and aerial application trials to document its efficacy on HWA have thus far been promising but the rapid degradation of the fungus once applied remains a challenge. Entomopathogenic fungi in general tend to be highly sensitive to photo-degradation and low humidity conditions. The commercially available formulation of the fungus Mycotal® (Koppert Biological Systems, a Netherlands-based company) along with MycoMax, a whey bi-product, has been added to the tank mix in an effort to stimulate fungal growth and spore production in the field. This formulation has been aerially applied to small replicated plots in Tennessee. Preliminary efficacy results are promising, although larger scale pilot tests are still needed (planned for 2012). Meanwhile, registration of Mycotal® in the United States is being pursued by Koppert.

The classical biological control program has encountered several "bumps-in-the-road" along with the many successes. A major deterrent from the onset was our inability to import predators from China into U.S. quarantine facilities in a timely manner to prevent large numbers of predators from dying in transit or while waiting to be shipped. In spite of attempts to resolve some of the specifically identified issues, this appears to be a major problem common to many U.S.-supported biological control foreign exploration efforts in China.

The recent utilization of molecular genetics by Dr. Nathan Havill (U.S. Forest Service, Northern Research Station) revealed that HWA in the eastern U.S. originated from southern Japan, and that HWA found in western North America is genetically unique from other HWA found worldwide, indicating that this biotype is likely native to this region. DNA sequencing technology and the use of molecular markers such as DNA barcodes and microsatellites helped characterize genetic variation within species of HWA predators as well as identify potential issues involving closely related predator species that would have otherwise been difficult to

realize. The first example of this involved the release of *L. nigrinus* (native to western North America) in the East, which was later found by Dr. Gina Davis (Virginia Tech) to have hybridized in some areas with a closely related species, *L. rubidus*. *L. rubidus* is a native predator of the pine bark adelgid *Pineus strobi*, an adelgid that attacks eastern white pine, *Pinus strobus*. *L. rubidus* had been reported in association with HWA, but were typically few in numbers and only in areas where white pine and hemlock were in close proximity. The outcome of this hybridization event is currently being studied in the laboratory and at *L. nigrinus* release sites. A second example of how this technology is being used is that it can distinguish the immature stages of *L. nigrinus* from that of *L. rubidus*, which are otherwise indistinguishable morphologically. Prior to implementing this technology, larvae needed to be reared to adults for identification. This is labor intensive, space consuming, and less reliable than molecular diagnostics. Finally, the third example of incorporating the science of molecular genetics into the HWA biological control program involves *L. osakensis*. In this case, a previously undescribed species of *Laricobius* (now *L. naganoensis*) was found contaminating the rearing colony of *L. osakensis* prior to it being released. *L. naganoensis* is separated morphologically as a different species primarily by differences in the male genitalia, but it is genetically distinct. It was DNA sequencing of the parent population of *L. osakensis* used to establish the rearing colony that led to this discovery, and this technology will be used to help "purify" the rearing colony in the future.

Assessment of the establishment, spread, and effectiveness of the predators released in the East is a long-term process with a constant need for refinement that begins by locating "ideal" sites and optimizing the timing of releases for specific predators. Unfortunately, the persistent challenge in predator field sampling efforts continues to be the use of the beat sampling technique, which only samples accessible lower canopy foliage of hemlock. We suspect the use of this technique might result in misinterpretation of the abundance of several species of predators, and other means of sampling for establishment should continue to be explored. For example, shifting from sampling for adult *L. nigrinus* predators to sampling for immature predator life stages is generally more labor intensive as it also requires laboratory processing, but it has been shown to be a more sensitive measure for detection of smaller predator populations. Assessment of predator impact on HWA and protecting tree health at the stand level, however, is even more of a challenge. In plant hardiness zones 6a and warmer, tree decline often occurs within a few years, and the small number of predators released in these areas has little chance to populate sufficiently to prevent this decline. At best, we will only be able to assess tree health recovery in areas where the ratio of predator to prey populations begin to balance out.

There have been numerous field evaluations of predator effectiveness as well as competition among predators using mesh cages over portions of hemlock branches containing various densities of HWA and predators. Whole tree canopy enclosures have been used by Dr. Jerome Grant et al. (University of Tennessee) to enhance understanding of the survival, colonization, and establishment of predators and to assess the impact of these agents on population-densities of HWA and on tree health.

Prior to 2007, data pertaining to the release, monitoring, and recovery of predators were maintained on paper data forms or in small local databases and, as a result, were inaccessible to HWA scientists and managers at regional and national levels. In 2007, the HWA Predator Release and Recovery Database (PDB) was initiated and is to include all historic release and monitoring information, as well as to provide a mechanism for field personnel to enter and update current and future records. The PDB uses standardized field protocols and data forms. It will facilitate improved access to the data, provide project-wide reports and maps, and is a tool for analysis and improved decision-making for future actions. View-only public access to the PDB is available at: http://hwa.ento.vt.edu/hwa/hwa.cgi.

In 2010, Dr. Richard McDonald (Symbiont Biological Pest Management) began a 2-year effort to monitor predators released and assess their ability to establish, disperse, and impact HWA across the pest's geographic range at a selected number of sites. Only 3+ year old release sites are being sampled to confirm establishment and the data will be entered into the HWA Predator Database. Thus far, at least 5 *L. nigrinus* release sites in NC, NJ, and PA have been identified as having sufficient predator densities that allow for collection and redistribution of beetles to other HWA infested areas. These are also some of the oldest release sites that were established between 2004 and 2006.

The guiding principles for developing a classical biological control program have been followed for HWA, especially with the release of climatically matched species or biotypes, including those adapted to local temperature conditions. Collections overseas were made not only in forests but also in ornamental sites and on susceptible exotic hosts, where factors that affect herbivore population dynamics more closely resemble those that characterize introduced habitats (e.g. eastern United States). Even though we are still collecting data on the ecological requirements and performance following introduction, this complex of predators has not yet provided a detectable regulation of HWA populations and corresponding protection of tree health. We suspect that, as our sampling techniques improve and the predators have additional time to increase in density and spread, their role in suppressing HWA population will become more apparent.

OUTLOOK

High mortality of hemlocks in the eastern U.S. has been attributed to a combination of host tree susceptibility and lack of effective natural enemies. Therefore, the dynamics of adelgid populations in the eastern U.S. is believed to be driven mainly by weather (cold winter temperatures) and the negative density-dependent consequences of host deterioration on adelgid survival. Significant effort has focused on classical biological control in an effort to alleviate the lack of natural enemies. The establishment of these additional predators in the northern part of the HWA range, along with periodic HWA density reductions due to cold temperatures, may provide the additional mortality necessary to effectively suppress populations of HWA below damaging levels. In the absence of these regulating cold winter temperatures in the southern part of the HWA range, this is more problematic. The intolerance of eastern hemlock species to attack by HWA may seriously constrain our biocontrol efforts, because exceptionally high mortality from natural enemies may be needed to maintain HWA at innocuous levels.

We have realized that natural enemies that are effective control agents in native natural habitats (where their hosts typically occur at low, innocuous densities) may have limited ability for pest populations that outbreak in introduced habitats. Natural enemies collected and evaluated for HWA biocontrol originated from trees growing on as well as off their preferred growing sites, which often renders them less resistant to insect herbivores, presumably due to stress from less adequate growing conditions.

Successful biological control of HWA in the eastern U.S. will require a suite of predators that attack all of the life stages of HWA. The rearing of predators in laboratories should continue, along with the release of predators into numerous geographical areas to establish and promote their natural spread. The number of field insectaries should be expanded in natural or planted settings in an effort to build up predator populations for harvesting and redistribution. These "wild" individuals are more adapted to climate and other local variables and would supplement the release of lab-reared individuals. Field insectaries resulting from several of our oldest release sites are already providing thousands of *L. nigrinus* beetles for release in other infested areas.

The classical biological control program needs to continue, but, as we understand more about the ecological requirements of these predators, we need to shift toward manipulation of these predators and integration into a management program. Even though biocontrol needs to be the focal tactic in an integrated program, no single control method will enable managers to meet their HWA management objectives in all environments. Early detection surveys conducted for HWA well in advance of symptoms of tree decline is critical if damage is to be prevented. Likewise, continued monitoring of pest densities throughout the management effort is recommended. A combination of control methods consistently applied through time will be necessary to obtain management objectives for HWA, especially as HWA expands its range.

The integration of chemical and biological control is being conducted on a landscape scale and evaluated on smaller stands of hemlock. The general strategy is to maintain the health of a select number of large hemlocks with insecticide applications, and at the same time release and allow the predators to become established on understory and other non-treated trees. The idea is that by protecting the health of and reducing HWA population densities on the larger higher value hemlock trees, predators will have more time to populate sufficiently to offer long-term suppression of HWA. These integrated efforts are urgent for management of HWA in the southern United States as hemlocks are dying at a rapid rate and an integrated management program is essential.

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