

**Release and monitoring of *Laricobius nigrinus* (Coleoptera:  
Derodontidae) for biological control of the hemlock woolly adelgid  
in the eastern US**

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# **Release and monitoring of *Laricobius nigrinus* (Coleoptera: Derodontidae) for biological control of the hemlock woolly adelgid in the eastern US**

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(ABSTRACT)

Different *Laricobius nigrinus* Fender release locations, numbers of predators, and timing of release were evaluated for biological control of the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). It established at 59% of the sites and location was the most important factor related with establishment and abundance, HWA density, and hemlock vigor index. Cold locations had poor establishment or low abundance, declines in HWA density, and increases in hemlock vigor over time. Paired release and control sites detected a predator impact on HWA density, but densities remained high and tree vigor declined. The phenology of *L. nigrinus*, *L. rubidus* LeConte, and HWA were studied at a field insectary and the species were highly synchronized. A cage exclusion study showed that HWA survival and density were lower and ovisac disturbance was higher when exposed to predation. To improve *L. nigrinus* monitoring, we compared beat sheets for adults or branch clipping for immatures, and the host searching behavior of *L. nigrinus* was studied to understand how it locates a tree and HWA. In the Appalachians, beat sheet sampling resulted in false negatives as larvae were collected by branch clipping. Adults orientated to a tree visually, fed when prey were present and flew when absent, and showed different search patterns on infested versus uninfested trees. In Seattle, both sampling methods detected *L. nigrinus* because the predator was common. Predator : prey ratios were high at heavily infested sites in Seattle and low in the eastern US, where it has been released recently. Partial life tables were constructed for HWA sistentes at four sites for 2 yr in Seattle. Unspecified causes of nymph and adult mortality were high and *L. nigrinus* was the dominant predator of ovisacs. Adult *L. nigrinus* abundance was positively related to HWA density and immature abundance was related to ovisac density, indicating an aggregation and numerical response to its prey. *Laricobius nigrinus* has not demonstrated complete biological control of HWA to date, but it may do so in the future and continued release is justified.

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# Chapter 1 Introduction

## 1.1 Biology and damage of the hemlock woolly adelgid

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is invasive in eastern North America on eastern hemlock [*Tsuga canadensis* (L.) Carrière] and Carolina hemlock (*Tsuga caroliniana* Engelmann). The Virginia Department of Agriculture and Consumer Services (VDACS) first reported HWA at Maymont Park, Richmond, VA in the early 1950's on planted eastern hemlock (Miller 1988, Souto et al. 1996). However, the introduction of HWA likely occurred in the early 20<sup>th</sup> century by James and Sallie Dooley, the original owners and plant collectors for Maymont Park (T. McAvoy, Virginia Tech, pers. comm.). Since then, HWA spread among ornamental hemlocks to the north and west of Richmond and VDACS reported HWA in native hemlock forests in the Blue Ridge Mountains near Rocky Mount, VA in the late 1960's or early 1970's. The rate of HWA spread increased at this time, as hemlock is common in Appalachian forests. HWA and a scale insect, *Fiorinia japonica* (Kuwana) (Hemiptera: Diaspididae), caused significant damage and mortality to ornamental and native hemlocks (Miller 1988). In 1985, HWA reached Connecticut and was recognized as a major ecological problem (McClure 1987b).

The current HWA infestation ranges from Maine to Georgia inland to Kentucky, West Virginia, and upstate New York (Anonymous 2007a). Isolated outbreaks exist in Michigan and western New York, most likely through the movement of infested nursery stock (Burns et al. 2005). HWA is established in 17 states, which represents approximately 26% of eastern hemlock's (Morin et al. 2005) and most likely 100% of Carolina hemlock's range in the US. Chemical insecticide applications (*i.e.* soaps, oils, imidacloprid) can control HWA on ornamental trees and a limited amount of forest trees (McClure 1991d, 1995b, Webb et al. 2003). However, forests will require less expensive control methods that can be applied efficiently over large areas and safely in riparian areas (McAvoy et al. 2005). Extinction of Carolina hemlock is possible and *ex situ* conservation of germplasm is in progress (Tighe et al. 2005). Development of HWA resistant eastern hemlock hybrids is also in progress (Lagalante and Montgomery 2003, Bentz et al. 2005, Montgomery et al. 2005, Playfoot and Ward 2005, Lagalante et al. 2006). HWA is

capable of adapting to cold climates (Butin et al. 2005), which indicates that it will continue to spread throughout eastern hemlock's range, especially to the north and west. In central Connecticut, few stand and landscape variables affect hemlock susceptibility and mortality, but trees succumb rapidly on xeric sites (Orwig et al. 2002). The duration of infestation is the primary factor that explains patterns of hemlock decline in this region. Currently, extreme winter cold limits HWA's northern spread into northern New England (Parker et al. 1999, Gouli et al. 2000, Skinner et al. 2003, Shields and Cheah 2005, Evans et al. 2007b).

HWA is native to Asia and western North America (Annand 1924, Cheah et al. 2004, Havill et al. 2006) but most of the basic and applied HWA research has been conducted in the eastern US. The primary host of HWA is tiger-tail spruce, *Picea polita* (Siebold & Zucc.) in Japan (Blackman and Eastop 1994) and other spruces in Asia (Anonymous 2007c). Secondary hosts are restricted to the genus *Tsuga* of which there are nine species worldwide (Farjon 1990). Evidence from HWA mitochondrial DNA suggests that the origin for the eastern North American population is Osaka, Japan (Havill et al. 2006) where its hosts are *Tsuga diversifolia* (Maxim.) Masters and *Tsuga sieboldii* Carrière. In China, the hosts are *Tsuga chinensis* (Franchet) E. Pritzel, *Tsuga forrestii* Downie, and *Tsuga dumosa* (D. Don) Eichler. In Taiwan, the host is *T. chinensis* and in northern India, Bhutan, and Nepal the host is *T. dumosa*. In western North America, the hosts are western hemlock [*Tsuga heterophylla* (Raf.) Sargent] and mountain hemlock [*Tsuga mertensiana* (Bong.) Carrière] (Cheah et al. 2004).

HWA has caused damage to hemlocks in eastern North America because of inadequate population regulation [possibly due to ineffective natural enemies (Wallace and Hain 2000)], inadequate host-plant resistance, an open niche, suitable environment, and a high reproductive capacity. Another exotic pest, the elongate hemlock scale, *Fiorinia externa* Ferris (Hemiptera: Diaspididae), has also been damaging to hemlocks, especially coupled with HWA (McClure 2002). The gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), can also defoliate and kill hemlocks during outbreaks (Lovett et al. 2006). Native pests like the eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Guenée) (Lepidoptera: Geometridae), the hemlock borer, *Melanophila fulvoguttata* (Harris) (Coleoptera: Buprestidae), spider mites, hemlock needle miner, spittlebugs, root rots, and Fabrella needle cast cause occasional outbreaks and damage

trees. In addition to pest and disease pressure, hemlocks are shallow rooted and very susceptible to drought and fire (Burns and Honkala. 1990).

The HWA nomenclature used throughout follows that described in Havill and Footit (2007). Anholocyclic HWA exules consist of two wingless parthenogenetic generations per year (McClure 1989). White cottony flocculence covers the apterous HWA generations, called the sistentes (summer-early spring) and progredientes (spring), except during the summer (s. sistens, progrediens). They are reddish black to dark purple (0.4 - 1.4 mm length) and have six female developmental stages: egg (amber colored), four nymphal instars, and the adult. Eggs are laid within the flocculence, which is called an ovisac. Only first instar nymphs of each generation are capable of moving and white flocculence is not present during this stage. Sistentes can lay up to 350 eggs and progredientes up to 75 eggs but fecundity varies greatly with host condition (McClure 1991c). The sistentes eggs hatch in late spring, nymphs settle on the needles pulvinus (woody “peg-like” part of the stem), aestivate in the summer, complete development in the fall/winter, and lay progredientes eggs in the late winter/early spring (McClure 1987b, McClure 1989, Gray and Salom 1996, Zilahi-Balogh et al. 2003c). Specific timing of HWA phenological events depends on the region of interest. Sistentes survive for approximately nine months and avoid the chemical defenses present in the new green shoots by entering a non-feeding aestivation stage (Lagalante et al. 2006). The progredientes hatch in the early spring, nymphs develop, and lay sistentes eggs in late spring. This generation exists for approximately three months. Sistentes also produce a sexual winged generation (sexuparae) in response to poor host quality with high densities of HWA (McClure 1991c). Ancestral holocyclic species of *Adelges* have spruce as a primary host and *Tsuga*, *Larix*, *Pseudotsuga*, or *Abies* as secondary hosts (Moran 1992, Blackman and Eastop 1994, Havill and Foottit 2007). The HWA life cycle in the eastern US is anholocyclic because sexuparae offspring apparently do not survive past the first instar on spruce or hemlock species in this region (McClure 1987a,b). Thus, the ability to migrate and sexually reproduce is lost. Alate sexuparae have not been observed in western North America (Annand 1924, Zilahi-Balogh et al. 2003c, Kohler 2007) indicating that this HWA population has completely lost the sexual part of their life-cycle (Blackman and Eastop 1994, Havill and Foottit 2007).

Mobile first instar HWA “crawlers” of both generations disperse by crawling and phoretically on birds and other forest animals during April - July for a short period after eclosion (McClure 1990). Wind also passively disperses crawlers over large distances and HWA appears to spread 7-16 km/yr into uninfested areas (Evans and Gregoire 2007b). HWA eggs are spread when an ovisac’s sticky flocculence become attached to animals. In the southern Appalachians, roads, hiking trails, and riparian areas appear to enable spread perhaps because the vectors mentioned previously are more prevalent in these corridors (Graham et al. 2005, Koch et al. 2006). Otherwise, the insect is sedentary on a needle’s pulvinus. HWA inserts its piercing-sucking mouthparts (*i.e.* stylet bundle) just below the needle abscission layer and feeds on ray parenchyma cell contents, inhibiting shoot growth, causing bud mortality, twig dieback, foliage discoloration and premature defoliation (Young et al. 1995). As living parenchyma cells in the sapwood primarily store and distribute carbohydrates that supply energy for the tree, HWA damages the tree’s energy reserves such that growth and survival declines, similar to the balsam woolly adelgid, *Adelges piceae* (Ratzeburg), (Hemiptera: Adelgidae) (Barbosa and Wagner 1989).

HWA inhibits new shoot growth when the percentage of infested shoots (*i.e.* presence/absence) reaches 30% (Fidgen et al. 2006), which represents a physiological damage level. Similarly, when HWA reaches 25-30 individuals per 100 needles the percentage of shoots with new growth declines rapidly, and as crown transparency reaches 60%, high stand-level tree mortality begins (Mayer et al. 2002). High HWA densities reduce shoot growth the following growing season after an outbreak, with a simultaneous reduction in HWA density because HWA survives poorly on old shoots and nodes compared with new growth (McClure 1991c). Hemlocks and HWA populations typically resume growth the next growing season but hemlocks never fully recover. As such, the trend in tree health is towards death with iterations of decline and recovery. HWA eventually kills all ages and sizes of hemlock trees including seedlings in as little as four years (especially in the southern Appalachians), although some trees may survive for decades in a moribund state (Orwig and Foster 1998, McClure et al. 2001). Other scenarios include HWA mortality and tree survival at high latitudes (Skinner et al. 2003) and elevations (Graham et al. 2005) due to extreme winter cold events. Some infested trees may survive for decades amongst

other dead trees and these potentially resistant eastern hemlocks have high phosphorus and low nitrogen content in the needles compared with declining trees (Pontius et al. 2006).

Annand (1924) first described HWA from the Pacific Northwest (PNW) on western hemlock. It is present throughout western North America, but at typically harmless densities (Furniss and Carolin 1977, Tait et al. 1985, McClure 1992). Havill et al. (2006) determined that this population is molecularly distinct from populations in Asia and the eastern US. It is likely native to the region. Some researchers indicate that the difference in HWA's pest status between the western and eastern US is perhaps due to host-plant resistance mechanisms (McClure 1992, McClure and Cheah 1999, Sántamour 1999, Lagalante and Montgomery 2003, Montgomery et al. 2005). McClure (1992) determined that *progredientes* in Connecticut oviposit fewer eggs and survive poorly on western and mountain hemlock compared with eastern and Carolina hemlock and concluded that they are resistant. This is debatable because HWA is genetically different in Connecticut, and the western and mountain hemlocks were young trees that were recently planted off-site, which may have reduced their nutritional quality. McClure (1992) also observed little predation on *sistentes* in the PNW during the summer, however HWA and a common predator *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) are both dormant in the summer.

In the PNW, HWA is rare on mountain hemlock. HWA populations are typically low on western hemlock, but local populations occasionally rise, and damage or kill trees (Annand 1924, Keen 1952, Collman 1972, Furniss and Carolin 1977, McClure 1992). It is possible that natural enemy communities suppress HWA populations below damaging levels and are as important as host-plant resistance mechanisms for western hemlock. Results of a small study conducted with *sistentes* on mature trees in Seattle, Washington showed results that are in contrast to the findings of McClure (1992). HWA fecundity was significantly higher on western hemlock than on eastern hemlock and predators were frequently observed, thus calling into question established reasons for HWA's non-pest status in this region (Mausel 2005). Eastern hemlock was planted off-site in this case in the 1950's, which also may have reduced its nutritional quality and HWA fecundity. In Asia, natural enemies and host resistance likely regulate HWA populations (McClure 1995c, Sasaji and McClure 1997, McClure et al. 1999, Montgomery et al. 1999,

Montgomery et al. 2002). For example, ornamental eastern hemlock trees have survived in Japan and numerous natural enemy species exist. In Seattle, ornamental eastern hemlocks and Carolina hemlocks have been alive for up to 50 years, while infested with non-damaging HWA population sizes (D. Mausel, unpub. data) and are host to many HWA natural enemies (Kohler 2007). This suggests that natural enemies regulate HWA populations or that the western North American HWA may be a biotype that is less damaging than the eastern North American HWA. A combination of both explanations is also possible.

## 1.2 Classical biological control of the hemlock woolly adelgid

Classical biological control is a pest management strategy that attempts to maintain pest populations below damaging levels by the strategic release of effective self-sustaining populations of natural enemies from the pest's native range (Debach and Rosen 1991). Since the early 1990's, foreign exploration for HWA-specific predators began in Asia for release in the eastern US, where there are currently ineffective predators (McClure 1987b, Montgomery and Lyon 1996, Wallace and Hain 2000). A survey of entomopathogenic fungi in the eastern US indicates that several generalist species exist and have potential for use as microbial pesticides against HWA (Gouli et al. 1997, Reid et al. 2002).

The Adelgidae family lacks parasitoids (Clausen 1978a) and the search for an effective HWA biological control agent focuses on predators (Montgomery and Lyon 1996), entomopathogenic fungi (Costa et al. 2005), and killing bacterial endosymbionts (Shields and Hirth 2005). HWA is suitable for biological control because they are sessile and apparent on trees for a long period of time, which renders them exposed to predation and pathogen infection. The only defense adelgids have is their cottony flocculence and HWA is active in the cooler parts of the year when many predators are inactive. Even so, biological control successes against the Adelgidae have only been completely successful for the *Pineus* genus (Mills 1990).

Biological control of the balsam woolly adelgid has not been successful to date despite introduction of 29 predators from eight families and establishment of at least nine (Mitchell and Wright 1967, Humble 1994, Zilahi-Balogh 2001). Early in that program Franz (1958b)

recognized that one predator, *Laricobius erichsonii* Rosenhauer, was not likely to be a good agent. It feeds on many adelgid species and is not synchronized with BWA in Europe, implying that BWA is not its primary host. In contrast, *Leucopis* spp. (Diptera: Chamaemyiidae) were effective against *Pineus* spp. (Hemiptera: Adelgidae) in Chile, New Zealand, and Hawaii (Culliney et al. 1988, Zondag and Nuttall 1989, Mills 1990). *Leucopis obscura* Haliday was one of the dominant predators of *Pineus pini* (Macquart) in Europe and had many positive traits in Hawaii: it was host-specific, bivoltine, adaptable, established easily, synchronized, density dependent, and it oviposited on the host. The primary difference between the *P. pini* biological control program in Hawaii and the BWA program in North America was that *P. pini* has a high damage threshold (50 adelgids/2.5 cm twig) and rarely kills trees. In contrast, BWA kills trees and has a low damage threshold, which the established natural enemies failed to keep BWA populations below.

### Japanese predators

A mite, *Diapterobates humeralis* Hermann (Oribatida: Ceratozetidae), which eats the cottony HWA flocculence and causes HWA eggs to be exposed to the environment and predation, was initially selected for study because it is common and an effective HWA natural enemy in Japan (McClure 1995a). However, it is not a suitable biological control agent because it already exists in North America, has low fecundity, is difficult to rear, and feeds exclusively on HWA flocculence (Cheah and McClure 1996).

A multivoltine coccinellid predator, *Sasajiscymnus* (=*Pseudoscymnus*) *tsugae* (Sasaji and McClure), was found to be a common HWA natural enemy in Japan (Sasaji and McClure 1997). Ecological traits suggested it had potential to be successful in the eastern US (Cheah and McClure 1996, Cheah and McClure 1998). To date infested hemlock forests throughout the HWA distribution in the eastern US have received over three million *S. tsugae* that were produced in mass rearing laboratories (B. Onken, USDA Forest Service, pers. comm.). Release methods varied widely from eggs (Grant et al. 2005) to releases of approximately 2,000 - 20,000 adult beetles per site on one or several occasions in the spring or early summer (Salom et al. 2001). There are reports of establishment and impact of *S. tsugae* (Salom et al. 2001, Blumenthal 2002, Palmer and Sheppard 2002, Blumenthal and Werner 2005, Cheah et al. 2005,

Conway and Culin 2005). Overall, *S. tsugae* has been difficult to establish and has not shown significant population growth, nor impacted HWA (Casagrande et al. 2002, Asaro et al. 2005, Montgomery et al. 2007). The outcomes of a majority of the releases have not been reported. Complete success by this beetle may be limited due to immediate dispersal after release, lack of successful oviposition (Asaro et al. 2005), and impact on HWA (Butin et al. 2003). In addition, the summer active *S. tsugae* may suffer when HWA is dormant and alternate food sources are not available (Cheah and McClure 2000). Three Japanese coccinellids *Scymnus posticalus* Sicard, *Adalia conglomerata* (L.), and *Scymnus giganteus* Kamiya have been identified (Yu 2001) but are not currently in quarantine. A newly discovered species of *Laricobius* collected in Osaka, Japan from hemlock infested with HWA is currently undergoing host-range evaluation and study in the Virginia Tech quarantine facility.

### **Chinese predators**

China has an abundance of HWA natural enemies, especially in the Coccinellidae (Yu et al. 2000, Yu 2001). Three coccinellids *Scymnus camptodromus* Yu and Liu, *Scymnus sinuanodus* Yu and Yao, and *Scymnus ningshanensis* Yu and Yao are common and avid predators, and appear to be host-specific in China. They have different univoltine life cycles and acceptable host ranges (Butin et al. 2004). *Scymnus sinuanodus* was released in the southern Appalachian mountains of northeast Georgia in 2004 (Asaro et al. 2005) and *S. ningshanensis* was released in Massachusetts in 2007 (Montgomery et al. 2007). Both species significantly reduced HWA populations in sleeve cage experiments (Butin et al. 2003) and post-release monitoring is in progress. Releases of *S. camptodromus* will begin as soon as rearing is productive (Montgomery et al. 2007). Host-range testing has begun for *Tetraphleps galchanoides* Ghauri (Hemiptera: Anthocoridae) in the US, and more testing is required before its release (McAvoy et al. 2007). This predator exists in many Chinese localities, is an avid HWA predator, and is able to complete nymphal development on a diet of HWA.

Recent foreign exploration yielded new *Laricobius* species from China, *Laricobius boaxingensis* Zilahi-Balogh and Jelínek, and *Laricobius kandingensis* Zilahi-Balogh and Jelínek (Zilahi-Balogh et al. 2007). Collections of both species came from hemlocks infested with HWA. *Laricobius kandingensis* fecundity and temperature developmental thresholds are lower than for

*L. nigrinus*. Adults and larvae appear host-specific, but no larvae completed development on HWA during an initial laboratory testing (Gatton 2005).

### Taiwanese predators

Numerous individuals of one *Laricobius* species were collected in 1994 but no work has continued in this location (Murphy 1995).

### North American predators

One of the few predators of HWA in eastern North America, *Laricobius rubidus* Leconte (Coleoptera: Derodontidae), feeds exclusively on adelgids, completes development, and survives well on a diet of HWA (Zilahi-Balogh et al. 2005). It is present in Connecticut (Montgomery and Lyon 1996), North Carolina, Virginia (Wallace and Hain 2000), Maine, New Hampshire, Massachusetts, District of Columbia, Pennsylvania, New York, Michigan, Quebec, Ontario, and New Brunswick (Lawrence 1989, Downie and Arnett 1996). The primary host of *L. rubidus* is the pine bark adelgid, *Pineus strobi* Hartig (Hemiptera: Adelgidae) on white pine, *Pinus strobus* L. (Clark and Brown 1960).

In the Pacific Northwest, Kohler (2007) identified 55 predator species from HWA-infested western hemlock. *Laricobius nigrinus*, *Leucopis argenticollis* Zetterstedt, and *Leucopis atrifacies* (Aldrich) (Diptera: Chamaemyiidae) comprise 59% of all the predators collected, with *L. nigrinus* being the most abundant. *Laricobius nigrinus* and *L. atrifacies* are good candidates for biological control of HWA. They are active at different times of the year, are common at low- and high-HWA density, and appear to have a density-dependent response to HWA. Since *L. argenticollis* exists in the eastern US (McAlpine and Tanasijtshunk 1972) it should not be introduced from the PNW unless this population is determined to be a different biotype.

### 1.3 Biology of *Laricobius nigrinus*

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) (Borror 1960, Downie and Arnett 1996). The beetles inhabit the temperate latitudes of the northern and southern

hemispheres but are cryptic and rarely observed. There are four genera in the family with three being mycophagous and one, *Laricobius* Rosenhauer, that is predaceous on adelgids, which are also holarctic (Lawrence and Hlavac 1979, Lawrence 1989, Leschen 2000). There are 14 known *Laricobius* spp. worldwide and three are native to North America (Zilahi-Balogh 2001, Zilahi-Balogh et al. 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* species only feed on the Adelgidae, and all 70 adelgid species in the family feed on Pinaceae hosts (Blackman and Eastop 1994, Havill and Foottit 2007).

*Laricobius nigrinus* was first collected and described from Bear Springs, Oregon (Fender 1945). The known distribution ranges from northern California to British Columbia, Alberta, and northern Idaho, indicating adaptability to different ecological and climatic conditions (Hatch 1962, Furniss and Carolin 1977, Tait et al. 1985, Lawrence 1989, Bright 1991). Some specimens of *L. nigrinus* in the US National Museum of Natural History, Washington D.C. have been collected from Wyoming and the southeastern Yukon, outside of the range of hemlock (M. Montgomery, USDA Forest Service, pers. comm.). *Laricobius nigrinus* adults are small (2.0 - 3.0 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 (Zilahi-Balogh et al. 2006).

Donald E. Bright (Agri-Food Canada, Systematic Entomology Section) confirmed the species identification when *L. nigrinus* was imported from Victoria, British Columbia for study in Virginia in May 1997. In the lab, *L. nigrinus* completed development on HWA but not on several other eastern adelgids and aphids tested therefore gaining release from quarantine by USDA-APHIS (Zilahi-Balogh et al. 2002, Zilahi-Balogh et al. 2003c, Zilahi-Balogh et al. 2003a, b). This set in motion written approvals for field release of *L. nigrinus* by the US Fish and Wildlife Service, USDA Forest Service, USDI National Park Service, and several eastern states.

*Laricobius nigrinus* is univoltine and has good phenological synchrony with HWA throughout the year in the PNW (Zilahi-Balogh et al. 2003c). Both the predator and prey are active in the fall, winter, spring, and diapause in summer. Adults emerge from the soil in the fall, disperse to

hemlock branches, and feed on HWA sistentes nymphs, adults, and progredientes eggs (available late winter/early spring) then die. The adults have pointed mandibles and pierce the HWA's body or eggs and suck out the contents as in *L. erichsonii* (Franz 1958b, Franz 1958a). Antennal morphology and preliminary behavioral studies suggest that *L. nigrinus* use host volatiles in host searching behavior (Franz 1958a, Broeckling and Salom 2002, 2003a, b). The multiporous basiconic sensilla on *L. nigrinus* terminal antennal annuli likely function for olfaction (Kiel 1999). It oviposits up to 396 eggs (mean = 101) in the lab, typically one per HWA sistentes ovisac, in early spring, but more than one is common (Zilahi-Balogh et al. 2003a) if predator density is high. *Laricobius nigrinus* females synchronize oviposition with HWA sistentes oviposition of progredientes eggs. The predaceous oligopod larvae consume these eggs and sistentes nymphs as described previously and have four instars. Predator eggs and early instars are obscured within HWA flocculence. Later instars become mobile on stems in search of food. Larvae are inefficient feeders of HWA eggs because they feed on numerous ovisacs and often leave uneaten eggs. This feeding style could enhance HWA mortality because the uneaten eggs are within loosened HWA wool, which wind and rain dislodge, as described in other natural enemies (McClure 1995a). By June, mature larvae drop to the soil. They enter a pharate prepupal stage for 10 days before an exarate pupal stage in an earthen cell, which lasts 10-14 days (Zilahi-Balogh et al. 2003b). Development from egg to adult takes 57 and 44 days at 15 and 18 °C, respectively. Adults are cold hardy and active at temperatures above 0.0 °C and immature stages have minimum developmental thresholds at or below 5.0 °C. The predator is freeze intolerant but adults are physiologically able to avoid freezing down to -16 to -19 °C (Humble and Mavin 2005). Eggs had supercooling points of -27.5 to -26.9 °C. Larval supercooling capability diminished from -22.1, -17.0, -15.0, and -13.0 °C for each consecutive instar. As such, the predator can feed on HWA from the fall to early spring when other predators are not active. Field cage studies conducted in 2001 and 2002 showed that the predator survived at high elevation in southwestern Virginia under caged conditions during winter and spring, and feeds and oviposits considerably on eastern HWA (Lamb et al. 2005b, Flowers et al. 2006). A caged egg release of *L. nigrinus* was conducted in spring 2003 and significant predation, oviposition, and recovery of F<sub>2</sub> adults occurred in fall 2004 (Lamb et al. 2006). No adults were recovered in fall 2005 or 2006 (Mausel unpub. data). Rearing methods have been developed and are being

used at several insectaries in the eastern US to produce adults for field release (Lamb et al. 2005a, 2007).

Few natural enemies are currently known to affect *L. nigrinus* in the eastern US. Generalists likely prey on *L. nigrinus* occasionally and other previously released HWA predators probably do not drastically compete or prey on *L. nigrinus* due to phenological differences (Flowers et al. 2005, 2006). In Europe, *Earobia* (=*Echthrolaricobius*) *paradoxus* Perkins, *Phrudus brachyscleroma* Cushman, *Phrudus icariomimus* Seyrig, *Phrudus erythrodolius* Seyrig, and *Phrudus melanodolius* Saussure (Hymenoptera: Ichneumonidae) are parasitoids of *L. erichsonii* (Franz 1958b, Anonymous 2006b). Also described by Franz (1958b) in Europe is a pathogen that caused a “black spot disease” epizootic.

#### **1.4 Research rationale and objectives**

Hemlocks are some of the longest-lived, most shade tolerant, and tallest trees in eastern North America (Burns and Honkala 1990). HWA is causing landscape-level mortality, and in some cases, pre-emptive salvage logging of stands resulting in a loss of cool and damp microclimates that many species require (Kizlinski et al. 2002, Orwig et al. 2002, Foster and Orwig 2006). The current hemlock decline is preceded by a mid-Holocene decline likely due to insects or disease (Davis 1981, Bhiry and Filion 1996). Landowners today have several options to monitor and manage HWA and tree mortality (Ward et al. 2004, Orwig and Kittredge 2005, Costa and Onken 2006), but they have no practical options to prevent hemlock forest decline and these stands are transitioning to hardwoods and invasive plants (Orwig and Foster 1998, Orwig et al. 2002, Eschtruth et al. 2006). The negative effects on biodiversity are extensive (Yamasaki et al. 1999; Brooks 2001; Evans 2002; Snyder et al. 2002; Tingley et al. 2002; Ross et al. 2003, 2004; Buck et al. 2005; Ellison et al. 2005; Lishawa et al. 2007). Hemlock mortality is also changing ecosystem processes, structure, and function (Jenkins et al. 1999; Stadler et al. 2005, 2006; Cobb et al. 2006), water quality (Yorks et al. 2003), and care of ornamental plantings. Effective control of HWA is urgently needed.

The USDA Forest Service has prioritized biological control in its HWA research and technology development, but biological control introductions against arthropod pests fail to establish in approximately three out of five attempts and therefore fail after considerable investment (Hall and Ehler 1979, Williamson 1996). Some introductions are bound to fail due to poor synchrony, adaptability, climate matching, dispersal, geographical race, or searching abilities of the agent (Lockett and Palmer 2003). Programs that have failed perhaps may have had a better chance with an optimal introduction or release strategy. Experiments on different release methods (*i.e.* release type, size, location) could aid in developing a strategy that maximizes successful establishment and impact, as done in other release programs (Campbell 1976, Memmott et al. 1998, Center et al. 2000, Clark et al. 2001b). Adequate and long-term sampling is also required since released organisms may be very rare after introduction and suddenly appear many years post-release (Soper et al. 1988, Humble 1994, Mo et al. 2000).

Since the introduction of a natural enemy is one aspect of a biological control program where the introducer has some control, experiments that test factors believed important for establishment and success are important in new release programs (Hopper and Roush 1993, Strong and Croft 1996, Daane and Yokota 1997, Dray et al. 2001). A limitation in the HWA system is that the biological control agents must establish quickly and reach population sizes that can control HWA before irreversible damage occurs to trees. In some cases, irreversible damage can occur very rapidly as in old growth trees and in the southern Appalachians, because the former are not resilient and the latter's HWA populations are not subjected to mortality from extreme cold. Release size is an important factor and knowing the relationship between release size and percentage frequency of establishment or effectiveness has resulted in an optimal release strategy (Shea and Possingham 2000, Clark et al. 2001b, Grundy and Maelzer 2002, Jung et al. 2004). Initial release sizes could be a few large (Hopper and Roush 1993, Memmott et al. 1998, Grevstad 1999b), numerous small (Center et al. 2000, Clark et al. 2001b) and mixed (Grevstad 1999b, Shea and Possingham 2000) depending on the supply and ecology of the organism. Other factors affecting establishment and success include release frequency, release season, release stage of the biological control agent, release site characteristics, pre-release acclimation, release equipment, pest density, weather, alternate hosts, competition, natural enemies, dispersal,

genetics, and detection and monitoring efforts (Center et al. 2000, Clark et al. 2001b, Damon and Valle 2002, Grundy and Maelzer 2002, Norris et al. 2002).

There are three general mechanisms for failure of an agent to establish or succeed. The demographic “Allee effect” is the correlation of per capita population size with population growth (Stephens and Sutherland 1999). For example, if a release size is small and the agent is free to disperse, it may have trouble locating mates, population growth is negative, and trends towards extinction. Caged releases or a release size above an establishment or effectiveness threshold for success is required and the best approach may be to use a few large releases (Freckleton 2000). Probable Allee effects for *L. nigrinus* would arise from small releases of unmated adult females, which results in a failure for the predator to find mates and reproduce. Additionally, because of small release sizes, hemlock health could decline due to inadequate pest suppression, which would lead to a decline in prey density and then starvation of the predator or an inability of the trees to recover. Environmental variability such as climate, weather, natural enemies, natural disturbance, host factors, and human interference could determine success or failure. The best approach may be to use numerous small releases to account for this unpredictability. Demographic variability such as sex ratio, fecundity, longevity, and health of the organism at the time of release are also important (Freckleton 2000). The relative importance of these factors early in a release program is unknown and should be tested with experimental releases.

The release phase of a natural enemy should continue to be the subject of comprehensive research. This is especially important in new releases, as in *L. nigrinus*. The primary objective of this study was to test different release approaches to permanently establish *L. nigrinus* in the eastern US and to evaluate different release factors for establishment, impact on HWA, and hemlock health for three generations post-release (Chapter 2). To support this objective, a *L. nigrinus* field insectary was established. Predator-prey synchrony and cage exclusion impact studies investigated *L. nigrinus* impact on HWA in detail (Chapter 3). Two sampling methods were studied to improve detection and monitoring (Chapter 4) and HWA mortality and *L. nigrinus* predation, prevalence, and numerical response were studied in the native range to see if a significant impact is probable where it is introduced (Chapter 5). Lastly, the host searching

behavior of *L. nigrinus* was studied in the laboratory to help sampling methods (Chapter 6). A practical goal of this research is to begin development of “best release practices” so that limited numbers of beetles may be released as effectively and efficiently as possible.

## **Chapter 2 Effect of release methodology on establishment and impact of *Laricobius nigrinus* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid, *Adelges tsugae***

### **Abstract**

Different release locations, numbers of predators, and timing of release were evaluated for establishment of *Laricobius nigrinus* Fender for potential biological control of the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). Adults were released at 22 sites between 2003 and 2005 in eight states. Release numbers were 75, 150, 300, 600, and 1,200 in fall, winter, spring, or sequentially (fall or winter and spring). Monitoring of establishment consisted of beat sheet sampling (adults) and branch clipping (immature stages) for three years post-release. Recoveries of  $F_3$  generation and increasing abundance over time indicate establishment at 59% of the sites. A logistic regression with the three release variables was significant. Release location (*i.e.* Plant hardiness zone or recorded minimum temperature) was the only significant coefficient and cold locations were related with establishment failure (*i.e.* plant hardiness zones 5a). Multiple regression and commonality analysis were used to relate the release variables with larval abundance. To detect impact, these analyses were conducted for changes in HWA sistentes density and eastern hemlock vigor index (HVI) between 2004 and 2007. Release location was most related with larval abundance, HWA density, and HVI. Cold locations were related with low predator abundance, decline in HWA density, and increases in HVI. Paired release and control sites were tested for goodness of fit to compare distributions of HWA density and HVI over time. Predator impact on HWA density was detected, but HWA densities remained high and HVI declined. Investment in releasing *L. nigrinus* appears worthwhile and guidelines for optimizing releases are described.

**Keywords:** Biological control, release strategy, location, number, timing, establishment success, impact assessment, commonality analysis

## 2.1 Introduction

The invasive hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) was first reported on eastern hemlock trees [*Tsuga canadensis* (L.) Carrière] at Maymont Park, Richmond, Virginia in the early 1950's (Miller 1988, Souto et al. 1996). In the following decades, HWA spread slowly among ornamental hemlocks around Richmond and was reported in forests of the Blue Ridge Mountains near Rocky Mount, VA in the late 1960's or early 1970's. HWA and a scale insect, *Fiorinia japonica* Kuwana (Hemiptera: Diaspididae), caused significant damage and mortality to hemlocks and the rate of HWA spread increased at this time (Miller 1988). HWA's occurrence with *F. japonica* and DNA evidence indicate that HWA was introduced from Japan (Havill et al. 2006). HWA is also native to China, Taiwan, northern India, Bhutan, Nepal and the Pacific Northwest of North America (Annand 1924, Cheah et al. 2004, Havill et al. 2006). In 1985, HWA was found in Connecticut and soon after widely reported as a major invasive pest (McClure 1987b).

HWA currently infests 17 eastern US states, which represents a large fraction of the eastern hemlock (Morin et al. 2005) and the entire Carolina hemlock [*Tsuga caroliniana* Engelmann] range in the US (Anonymous 2007a). It is capable of adapting to cold climates (Butin et al. 2005), which puts the rest of eastern hemlock's range, especially in the Northeast and Midwest, at risk. HWA is the primary cause of landscape-level hemlock mortality in many regions and in some cases, pre-emptive salvage logging of stands (Kizlinski et al. 2002, Orwig et al. 2002, Foster and Orwig 2006). The result is a loss of unique habitats that many species require (Brooks 2001, Snyder et al. 2002, Ross et al. 2003, Ross et al. 2004, Buck et al. 2005, Ellison et al. 2005, Lishawa et al. 2007). Forest managers have options to monitor and ameliorate hemlock forest decline (Ward et al. 2004, Orwig and Kittredge 2005, Costa and Onken 2006), but they have no practical solutions to prevent mortality and sites once occupied by hemlock are transitioning to hardwoods and invasive plants (Orwig and Foster 1998, Orwig et al. 2002, Eschtruth et al. 2006).

Chemical insecticide applications can control HWA on ornamental trees (McClure 1991d, 1995b, Webb et al. 2003) and forest trees (McAvoy et al. 2005). However, control at the

landscape-level will require less expensive methods that can be applied efficiently and safely in riparian areas. As such, the USDA Forest Service prioritized biological control in its HWA research and technology development program (Cheah et al. 2004). The Adelgidae family lacks parasitoids (Clausen 1978a) but numerous predators have been identified in Asia and western North America, (McClure 1995a, Murphy 1995, Sasaji and McClure 1997, Yu et al. 2000, Yu 2001, Kohler 2007, McAvoy et al. 2007, Zilahi-Balogh et al. 2007). Few predators exist in the eastern US (McClure 1987b, Montgomery and Lyon 1996, Wallace and Hain 2000) and several generalist entomopathogenic fungi have potential for use as microbial pesticides (Gouli et al. 1997, Reid et al. 2002, Costa et al. 2005). One of the few predators of HWA in the eastern US, *Laricobius rubidus* (Coleoptera: Derodontidae), completes development and survives on a diet of HWA (Zilahi-Balogh et al. 2005). It is native to eastern North America (Lawrence 1989, Downie and Arnett 1996) and its primary host is the pine bark adelgid, *Pineus strobi* Hartig (Hemiptera: Adelgidae) on white pine, *Pinus strobus* L. (Clark and Brown 1960).

HWA kills trees and can severely reduce growth and development with relatively low densities (McClure 1991c) compared with *Pineus strobi* Hartig and *Pineus pini* (Macquart) (Hemiptera: Adelgidae) (Culliney et al. 1988). To control HWA, its density needs to be below a physiological damage threshold and 30% shoots infested has been used (Fidgen et al. 2006). In northeastern US, cold temperatures are an important HWA mortality factor (Parker et al. 1998, Parker et al. 1999, Skinner et al. 2003, Shields and Cheah 2005) and predation could contribute to this mortality, if the predators can establish and are effective in these locations. In the South, where temperatures are less limiting, predation will be relied on to a greater extent for reducing HWA densities. Currently several coccinellids and *Laricobius nigrinus* Fender are being released (Cheah et al. 2004, Flowers et al. 2005, 2006, Montgomery et al. 2007).

Since 1997, tooth-necked fungus beetles (Coleoptera: Derodontidae) in the genus *Laricobius* Rosenhauer have been studied at Virginia Tech, Blacksburg for control of HWA. The Derodontidae is a small family known for their characteristic dentate or flattened lateral margins of the pronotum (Der = neck; odon = tooth) (Borror 1960, Downie and Arnett 1996). There are four genera in the family with three being mycophagous and one, *Laricobius*, that is predaceous on adelgids (Lawrence and Hlavac 1979, Lawrence 1989, Leschen 2000). 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. All 14 known *Laricobius* spp. feed solely on the Adelgidae, and all known adelgid species in the family feed on Pinaceae hosts (Blackman and Eastop 1994, Zilahi-Balogh 2001, Havill and Foottit 2007, Zilahi-Balogh et al. 2007).

*Laricobius nigrinus* was imported from Victoria, British Columbia because it is highly host-specific and synchronized with HWA temporally and in abundance (Zilahi-Balogh et al. 2002, Zilahi-Balogh et al. 2003c). The predator is univoltine (Zilahi-Balogh et al. 2003c) and HWA is bi-voltine, with *sistentes* (summer-spring) and *progredientes* (late-spring) generations (McClure 1989). Both predator and prey are active in the fall, winter, spring, and aestivate in the summer. *Laricobius nigrinus* is small (2-3 mm length), shiny black, and covered with fine gray hairs (Fender 1945, Hatch 1962, Zilahi-Balogh et al. 2006). The adults and larvae have pointed mandibles that pierce HWA eggs, nymphs, and adults and then suck out hemolymph as described for *Laricobius erichsonii* Rosenhauer (Franz 1958b, Franz 1958a). Research in the Pacific Northwest has shown that *L. nigrinus* is the most common HWA natural enemy (Kohler 2007) and has a numerical response to HWA (see Chapter 5). Field cage studies conducted in 2001 and 2002 showed that the predator survives in southwestern Virginia under caged conditions during winter and spring, and feeds and oviposits considerably on HWA (Lamb et al. 2005b, Flowers et al. 2006). An egg release of *L. nigrinus* was conducted in spring 2003 and significant predation, oviposition, and recovery of F<sub>2</sub> adults in fall 2004 occurred (Lamb et al. 2006). The predator, propagated at a field insectary near Blacksburg, VA was synchronized with and significantly reduced HWA populations (see Chapter 3). Rearing methods have been developed and are being used at several insectaries in the eastern US to produce adults for field release (Lamb et al. 2005a, 2007). Previously, *L. erichsonii* established successfully in North America on balsam woolly adelgid (BWA), *Adelges piceae* (Ratzeburg), but was ultimately unsuccessful in controlling it (Mitchell and Wright 1967).

The introduction of an agent is the sole aspect of biological control that the researcher has control over (Hopper and Roush 1993) and experiments that test important factors for establishment and impact are critical in new release programs (Strong and Croft 1996, Daane and Yokota 1997, Memmott et al. 1998, Center et al. 2000, Dray et al. 2001). Release number is a

crucial factor and could be a few large, numerous small, or mixed depending on the supply and ecology of the organism (Campbell 1976, Grevstad 1999b, Shea and Possingham 2000, Grundy and Maelzer 2002, Jung et al. 2004). Other factors include release frequency, timing, developmental stage of the biological control agent, site characteristics, pre-release acclimation, climate, pest density, alternate hosts, competition, natural enemies, dispersal, and genetics (Pschorr-Walcher 1977, Debach and Rosen 1991, Center et al. 2000, Clark et al. 2001b, Damon and Valle 2002, Grundy and Maelzer 2002, Norris et al. 2002). The relative importance of these factors for *L. nigrinus* is unclear. A lesson learned from the BWA program is that releases of predators must establish quickly and reach population sizes that can control HWA before irreversible damage occurs to trees.

The release phase of a natural enemy should continue to be the subject of comprehensive research to justify continued releases, optimize the releases, and to make the discipline more scientifically thorough (Stiling 1990). In this study, we evaluated the effect of release location, number, and timing on *L. nigrinus* establishment and abundance, as well as the impact on HWA density and hemlock vigor for three generations post-release. A practical goal of this research is to begin development of “best release practices” so that forest health specialists can release a limited supply of predators as effectively and efficiently as possible.

## 2.2 Materials and methods

### Predators released

The *L. nigrinus* used in releases were lab-reared adults from parents collected from HWA infested western hemlock, *Tsuga heterophylla* (Raf.) Sargent, seed orchards near Victoria, B.C., Canada (UTM Zone 10, 0473021E 5370328N) in late winter 2003. Donald E. Bright (Agri-Food Canada, Systematic Entomology Section) confirmed the species identification when *L. nigrinus* was in quarantine. Rearing occurred at the Virginia Tech Insectary using established methods (Lamb et al. 2005a, Lamb 2005). After aestival diapause, adults emerging from soil containers in fall 2003 (*i.e.* F<sub>1</sub> lab generation) were placed in 3.8 L containers with up to 50 conspecifics on HWA infested eastern hemlock. The predators were given fresh HWA every 2 wk until field-release. Depending on the season, adults were maintained at 2-10 °C, varying day length, and 70-90% relative humidity in environmental chambers before release. Predators were screened

for missing appendages, off-color, small size, abnormal behavior, and vigor. Three sub-samples of adult beetles were examined for entomopathogens before release and results were negative (Dr. L. Solter, Illinois Natural History Survey; Dr. A. Lawrence, Mississippi State University; and Dr. J. Becnel, USDA-ARS Gainesville, unpub. reports).

### **Study site selection and characteristics**

Federal, State, University, and private forest health specialists assisted in the location of release sites after one or two days of scouting (Table 2.1, Fig. 2.1). Guidelines for site selection included a preference for public land along the advancing edge of the HWA distribution, >10 ha of forest with a hemlock component, mesic sites, trees with high live crown ratios, early stage of HWA infestation, and <10% HWA associated hemlock crown decline. However, not all preferred factors were possible at each site. Trees receiving predators (release trees) were clustered at an infestation epicenter, if present. A preference was made for individual trees that had <10% crown decline, light-moderate HWA levels, and a crown accessible from the ground for beat sheet sampling. A GPS coordinate (UTM WGS84/NAD83) was recorded with a Garmin III (Garmin International Inc, Olathe, KS) unit in the center of the release area (Appendix 1). Each release tree's GPS location was also recorded, sketch mapped, and permanently marked by attaching a blue tree number with an aluminum nail below stump height, to aid re-measurement. Control sites were established to match as best as possible the attributes of each release site. Emphasis was placed on matching the HWA infestation level, tree vigor, and the number of control trees. Control sites averaged  $1.6 \pm 0.3$  km (mean  $\pm$  sem) from the release sites. The lowest minimum and highest maximum temperatures during the three years post release were recorded for each release site from the nearest weather station, typically within 20 km of the site. Weather data were acquired through the Southeast Regional Climate Center at North Carolina State University.

### **Release method**

Adults were released throughout the eastern US infestation in the southern Appalachians, Mid-Atlantic States, and Northeast in groups of 75, 150, 300, 600 (sequential releases of 300), and 1,200 per site (Fig. 2.1, Table 2.1). There were four replications of each release number except for 75 and 1,200 releases, which had three replicates. The 75 adult releases had two release trees

with each receiving 35 or 40 predators. The 150 adult releases had 30 predators released on five trees, 300 releases had 30 predators released on 10 trees, and 600 releases had 30 predators released on 10 trees in the fall and again on the same trees in the spring. At the PA-Rothrock and VA-North Fork sites, five additional release trees were added during the second release because HWA densities were low on some of the trees. The 1,200 adult releases had 80 predators released on 15 trees. Fall releases corresponded with early instar HWA sistentes, winter with late instars and adults, and spring with the beginning of sistentes oviposition of progredientes eggs until peak oviposition. A sequential release was made in the fall or winter and again in the spring.

At the Virginia Tech Insectary, a bouquet of HWA infested hemlock was made of 10-15 twigs (10 cm length) inserted into wetted Oasis® floral foam (Smithers-Oasis Co., Kent, OH), which was wrapped in parafilm. The bouquets were placed in ventilated escape proof polyethylene containers (950 mL, Rez Tech Corp., Kent, Ohio) with wetted filter paper. Based on release number, 30, 35, 40 or 80 predators were put in each container for release on individual trees. A larger container (3.8 L, polyethylene terephthalate, General Bottle Supply Co., Los Angeles, CA) and bouquets (20 cm long twigs) were used for the 80 predators/tree release. The predators were hand-carried to release sites in Styrofoam coolers with APHIS PPQ 526 permits for the interstate movement of HWA from Virginia, kept at ambient temperature, and shaded before release typically within 24 h. The hemlock bouquets containing predators were tied to the most heavily infested branch available with surveyors flagging on each release tree in the morning. Occasionally, one or two predators died in the containers during transit. Releases were timed to avoid inclement weather but this was not always successful. Site-specific release information was recorded on an AD-943 form “Biological shipment record non-quarantine” and submitted to the Biological Control Documentation Center, USDA-ARS, Beltsville, MD.

### **Establishment**

Standardized sampling for *L. nigrinus* establishment began the year following release at each site. Sampling initially targeted F<sub>1</sub> generation adults up to F<sub>3</sub> adults for the 2003/2004 releases (3 complete generations) or up to F<sub>3</sub> larvae for the 2004/2005 releases (partial 3<sup>rd</sup> generation). The monitoring period was chosen based on the theory that if a natural enemy does not establish

and show evidence of impact within three generations then the probability of complete biological control is low (Clausen 1951). Establishment was considered successful if F<sub>3</sub> adults or larvae were recovered and a failure if none or only F<sub>1</sub> or F<sub>2</sub> were recovered.

**Beat sheet sampling procedure.** In fall 2004 and 2005, beat sheet sampling was conducted six times per year from mid-September through November to collect *L. nigrinus* adults with canvas (71 cm<sup>2</sup>) beat sheets (Bio-quip, Rancho Dominguez, CA). Several Federal, State, University and volunteers assisted with this sampling. In fall 2006, the six sampling dates were from mid-October through December. Rarely, sites were sampled less than six times. Sampling was repeated every 2 wk from the lower canopy of the release trees and neighboring non-release trees (a minimum of 10 trees per sample date). Sampling was timed to avoid temperatures below 0° C when adults are not active (Zilahi-Balogh et al. 2003c), moderate-heavy wind, or rain and snow. Beat sheets were positioned under infested HWA branches around the circumference of each tree and tapped ~10 times with a stick. Predators were identified in the field using reference specimens of *L. nigrinus* in 4X magnifier boxes (Bio-quip, Rancho Dominguez, CA) and reference specimens were temporarily preserved in 70% ethanol for species confirmation. The date, number of *L. nigrinus* recovered, and amount of neighboring trees sampled were recorded on each sample date. Pinned reference specimens and species misidentified as *L. nigrinus* were placed in the Virginia Tech, Entomology Department Collection, Blacksburg, VA. The misidentified species were identified to the family level by the Virginia Tech Entomology Department, Identification Lab.

**Branch clipping sampling procedure.** Branch clipping was conducted once a year at each site in March, April, or May when HWA sistentes ovisacs reached a peak egg load and were 2-3 mm diameter. The specific sampling dates depended on a site's elevation and latitude (Appendix 2). Phenological indicator plants used to guide sampling were flowering eastern redbud, *Cercis canadensis* L. (Fabaceae), or *Forsythia x intermedia* (Oleaceae). Two 0.5-1.0 m long branches from the canopy of each release tree were cut with a 1.8 m fiberglass pole-pruner and up to three 1.8 m attachments (Fred Marvin Assoc., Akron, Ohio). For trees up to 10 m tall, two branches were cut from the upper crown of the tree. For taller trees, two branches were cut from the maximum height possible (~10 m). The branches were then cut down to 20-30 cm length twigs

with a hand-pruning shear. If a branch was not moderately to heavily infested or consisted of mainly dead HWA without ovisacs then another branch was cut from the upper crown, the lower crown, or a neighboring tree. If no HWA ovisacs could be recovered from these alternative sources clipping stopped and a poor sample was noted. The branches from a tree were combined, loosely placed in an individually labeled plastic bag (30x20x76 cm), and stored in a covered pick-up truck bed. Approximately 30x30x8 cm of foliage were collected from each tree. Samples were transported to the insectary and each sample was placed in a modified Berlese funnel used to rear out larvae. After larvae developed through four instars, they dropped into mason jars that were inspected daily in the AM and PM for pupation-site seeking larvae. The larvae were counted, pooled by site, and transferred to soil containers for pupation and aestivation. Each funnel represented a release tree and the percentage of release trees that larvae were reared from was recorded. *Laricobius nigrinus* and *L. rubidus* adults that emerged from the soil in the fall were counted by site and their ratio calculated to estimate the number of larvae collected of each species. A sub-sample of larvae was also preserved in 95% ethanol and frozen, and if emergence was poor or completely failed, the larval sub-samples were identified by a polymerase chain reaction, restriction fragment length polymorphism (PCR-RFLP) diagnostic assay using the COI gene [N. Havill, D. Mausel (Virginia Tech), and A. Caccone, Yale University, unpub. report].

### **Impact on HWA and tree vigor**

We monitored *L. nigrinus* impact on HWA, hemlock growth, and crown vigor at each release and control site by collecting baseline data and two (2004/2005 sites) or three (2003/2004 sites) years of post-release data. One branch was selected at each of the four cardinal points of a tree and a 30 cm long section of each branch, from 30 to 60 cm from the distal shoot tip toward the trunk of the tree, was demarcated with cable-ties. Occasionally, branches were missing at a cardinal point due to shading and the point was skipped. Within this demarcation, the number of new shoots (*i.e.* produced the previous growing season) infested with at least one HWA sistentes and the total number of new shoots were counted in the winter (Fidgen et al. 2006). The percentage of shoots infested was calculated as a proxy for sistentes population density. The number of new shoots was recorded as a measure of tree growth and the number of dead tips was recorded as a measure of HWA damage. Dead shoot tips were distinguished from shoots dying

from non-HWA causes by characteristic needle loss and desiccated xylem at the tips, which is symptomatic of previously high HWA infestations. The average percent shoots infested, number of new shoots, and dead tips per tree were calculated from the four branches. The average per site was calculated from the individual trees.

Crown vigor measures included the live crown ratio and transparency that were estimated for each tree by a single person to eliminate inter-observer variability. The live crown ratio (*i.e.* % of tree height with foliage) was recorded in 5% gradations up to 99% (*i.e.* foliage touches the forest floor) (Smith 1986, Anonymous 2005). Crown transparency was also recorded in 5% gradations and was an estimate of the amount of light visible through the foliated portions of the crown (Anonymous 2006a). A hemlock vigor index (HVI) was calculated by summing scalar indexes created for the four hemlock parameters (Appendix 3). The numbers of new shoots (McClure 1991c) and transparency (Mayer et al. 2002) have been shown to be affected by HWA. New shoots and dead tips were weighted more heavily because they are quantitative measures. Lastly, each tree was assigned a decline code to correlate with the HVI: 1). Healthy = <10% of the foliage with shoot tip dieback, off color foliage, or defoliation symptoms; 2). Light decline = 10-25% symptomatic; 3). Moderate decline = 25.1-50%; 4). Severe decline = >50.1%; 5). Dead = no green foliage on tree (B. Onken, USDA Forest Service, pers. comm.). The HVI was significantly correlated with the USFS decline code system during each year ( $r^2 = 27\text{-}62\%$ ). The average HVI per site was calculated from the individual trees. A summary of means for the HWA and each tree parameter over time were tabulated by site (Appendix 4).

### Statistical analyses

To analyze release location with the goal of facilitating management decisions in the field, each release location's 1990 USDA plant hardiness zone was determined. The map was created from average annual minimum temperatures (*i.e.* the coldest temperatures likely each year) between 1974 and 1986 at 8,000 weather stations (Cathey 1990). Releases were made in zones 5a (-26.2 to -28.8 °C), 5b (-23.4 to -26.1 °C), 6a (-20.6 to -23.3 °C), 6b (-17.8 to -20.5 °C), and 7a (-15.0 to -17.7 °C). Changes in climate, particularly warming, have likely made the locations of the zones shift. However, our release location's minimum temperatures relative to each other did not change even though the minimum temperatures may be higher than stated in 1990. The

predictive value and utility of the map has not changed for identifying general climatic relationships with *L. nigrinus*, HWA density, and HVI. We re-ran our analyses with the actual lowest minimum temperature recorded during the study to confirm the soundness of the plant hardiness zones. Highest maximum temperature was found to negate the significance of all the regressions and was removed from analyses. Release number was analyzed as the total number of predators released per site. The release date of each site was classified into fall (Oct+Nov), winter (Dec-Feb), spring (Mar+Apr), or sequential (fall or winter and spring) to analyze the timing of release. We calculated simple correlation coefficients between the predictor variables to check for intercorrelation but no significant problems were found.

A logistic regression was used to study the effect of three variables: plant hardiness zone (or lowest minimum temp.), release number, and timing on establishment success or failure (*i.e.*  $F_3$  adult or larval presence/absence) using SPSS<sup>®</sup> version 10.0. Three variables were entered in the model and the adequacy of the model was confirmed with the Hosmer and Lemeshow goodness-of-fit test. Multiple regression analyses were used to analyze the effects of the release variables on each of three dependent variables: *L. nigrinus* larval abundance, change in HWA density, and change in HVI from 2004 to 2007. Adult abundance was not analyzed due to the numerous zero values and false negatives that beat sheet sampling produced. Larval abundance was analyzed as the total number of  $F_2$  and  $F_3$  larvae collected divided by the number of release trees. Change in HWA density was analyzed as the mean percentage of shoots infested per site in 2006/2007 minus 2004/2005. A positive value indicates an increase in HWA density and negative value indicates a reduction. Similarly, the change in HVI was analyzed and a positive value indicates an improvement in tree vigor and a negative value indicates a reduction. After each multiple regression, commonality analysis was used to assign a percentage of the coefficient of determination ( $r^2$ ) to each predictor variable in isolation and in each possible combination of unique, first-order, and second-order common effects (Seibold and McPhee 1979). Simple correlation coefficients were calculated for each predictor with the dependent variable and squared. Then multiple correlations between the predictors were calculated and squared. Computational formulas were used to determine unique, first-order, and second-order common effects. These effects for each predictor variable were summed and compared to each other to

ascertain which was most important (*i.e.* accounted for the greatest amount of the explained variance in the dependent variable,  $r^2$ ).

Release and control sites were analyzed by comparing the distribution of mean HWA densities in 2003/2004, 2004/2005, 2005/2006, and 2006/2007 by  $\chi^2$  goodness of fit tests. Of the 12 sites with established *L. nigrinus* populations, only three sites with similar baseline HWA densities were appropriate for analyses. Control sites represented the expected distribution and release sites represented the observed distribution. The analyses were repeated for the HVI at the three sites. The 0.05 significance level was used in all analyses.

## 2.3 Results

### Establishment

Beat sheet sampling for adults recovered F<sub>1</sub> *L. nigrinus* at 23% (5 of 22), F<sub>2</sub> at 27% (6 of 22), and F<sub>3</sub> at 30% (3 of 10) of the sites (Table 2.1). Overall, 41% (9 of 22) of the sites have had adults recovered. Fifty-six percent (5 of 9) of these sites had their first adult collected during the F<sub>1</sub> generation, 33% (3 of 9) during the F<sub>2</sub> generation, and 11% (1 of 9) during the F<sub>3</sub> generation. The number of adults recovered has increased over time at all the sites except VA-Hurricane and VA-Lick Creek where no F<sub>2</sub> were recovered. Of these, only one site, NC-Hemlock Hill showed recoveries and increase in each of the three years. Misidentified species included *L. rubidus*, an unidentified silken fungus beetle (Coleoptera: Cryptophagidae), and a minute brown scavenger beetle (Coleoptera: Lathridiidae).

Recovery of larvae from branch clipping was 59% F<sub>2</sub> and F<sub>3</sub> (13 of 22) (Table 2.1). Overall, larvae were recovered at 64% (14 of 22) of the sites. Eighty-two percent (18 of 22) of the sites also had recoveries of *L. rubidus*. Total larvae collected and reared to adults in 2005, were 25% *L. rubidus* and 75% *L. nigrinus* (*i.e.* F<sub>2</sub> generation) and in 2006, 34% *L. rubidus* and 66% *L. nigrinus* (*i.e.* F<sub>3</sub> generation). At the three WV sites, VA-Big Stony, and VA-Dickey Ck., all larvae collected were *L. rubidus* while at NC-Hemlock Hill and NC-Ivy Ck., all larvae were *L. nigrinus*, but no larvae of either species were collected at MA-Mt. Tom, MD-Finzel, and GA-Overflow Ck. In general, both species coexisted and *L. nigrinus* was regularly more abundant on hemlock. The number of *L. nigrinus* larvae recovered from F<sub>2</sub> to the F<sub>3</sub> generation has increased

over time at 64% (9 of 14) of the sites. The average population growth rate at the 13 established sites was  $7.4 \pm 2.6$  with a maximum of 31.4 at NC-Hemlock Hill ( $F_3/F_2$  larvae). At the five sites where larval abundance decreased, the samples had a light HWA ovisac density due to a lack of new hemlock growth. The prevalence of larval recoveries (*i.e.* the percentage of trees with larvae) increased at 58% (11 of 19) of the sites from the  $F_2$  to the  $F_3$  generation, 21% (4 of 19) of the sites had no change, and 21% (4 of 19) showed a decline.

To date, 59% (13 of 22) of the sites have established *L. nigrinus* populations. Establishment at VA-Lick Ck. is not confirmed because no  $F_3$  were recovered, perhaps due to declining HWA. This site had poor HWA samples collections due to a near complete collapse of the population and failures to recover subsequent generations is to be expected. The logistic regression model with the three predictor variables (Plant hardiness zone, numbers of *L. nigrinus* released, and timing of release) correctly and significantly ( $\chi^2 = 18.7$ , df = 3,  $P < 0.0001$ ) classified sites as established or non-established 92.3% and 87.5% of the time, respectively. The model significantly explained 80.2% (Nagelkerke  $r^2$ ) of the variation in establishment. Only plant hardiness zone had a significant coefficient (Table 2.2). No *L. nigrinus* was recovered at the GA-Overflow Ck. location and was excluded from the release location variable because it was the lone site located in zone 7a, on the fringe of hemlock's distribution. (when included, the regression remained significant;  $P = 0.009$ , Nagelkerke  $r^2 = 55\%$ ). When the actual lowest minimum temperature was used in the model as a substitute for plant hardiness zones, the regression remained significant but explained a lesser amount of the variance ( $\chi^2 = 15.2$ , df = 3,  $P = 0.002$ ). The model classified sites as established or non-established 84.6% and 87.5% of the time, respectively, and explained 70.2% (Nagelkerke  $r^2$ ) of the variation in establishment.

There was a significant relationship between the three predictor variables and larval abundance ( $F = 5.96$ , df = 3, 17,  $P = 0.006$ ,  $r^2 = 51\%$ ). The only significant regression coefficient ( $\beta$ ) was plant hardiness zone (Table 2.3). Plant hardiness zone accounted for 34% of explained variance when unique and common effects were summed and confirmed that it was most related with larval abundance (Table 2.4). Release number or timing accounted for 8.7 or 11.7%, respectively, of the explained variance. The GA-Overflow Ck. site was removed from the release location variable in the larval abundance analysis (when included the regression remained

significant ( $F = 4.78$ ,  $df = 3,18$ ,  $P = 0.013$ ,  $r^2 = 44\%$ ). The regression remained very significant when actual lowest minimum temperature was used as a substitute for plant hardiness zones ( $F = 5.84$ ,  $df = 3,17$ ,  $P = 0.006$ ,  $r^2 = 51\%$ ).

### **Impact on HWA and tree vigor**

There was a significant relationship between the three predictor variables and change in HWA density from 2004 to 2007 ( $F = 5.63$ ,  $P=0.007$ ,  $df = 3,18$ ,  $r^2 = 48\%$ ). The only significant regression coefficient ( $\beta$ ) was plant hardiness zone (Table 2.5). Plant hardiness zone accounted for 29% of explained variance when unique and common effects were summed and confirmed that it was most related with larval abundance (Table 2.6). Release number or timing accounted for 19.8 or 18.9%, respectively, of the explained variance. When the actual lowest minimum temperature was used as a substitute for the plant hardiness zones, the regression explained less of the variance in HWA density, but remained significant ( $F = 3.26$ ,  $P=0.046$ ,  $df = 3,18$ ,  $r^2=35\%$ ).

There also was a significant relationship between the three predictor variables and the change in hemlock vigor index from 2004 to 2007 ( $F = 8.60$ ,  $P = 0.001$ ,  $df = 3,18$ ,  $r^2 = 59\%$ ). Again, the only significant regression coefficient ( $\beta$ ) was plant hardiness zone (Table 2.7). Plant hardiness zone accounted for 53% of the combined explained variance and was most related with the hemlock vigor index (2.9). Release number or timing accounted for 9.9 or 6.9%, respectively, of the explained variance. When the actual lowest minimum temperature was used as a substitute for the plant hardiness zones, the regression was significant and explained more of the variance in HWA density ( $F = 15.4$ ,  $P < 0.0001$ ,  $df = 3,18$ ,  $r^2 = 72\%$ ).

There was a significant difference between the observed distribution of HWA density at the release site and expected density at the control site at TN-Laurel Ck. ( $\chi^2 = 9.8$ ,  $df = 3$ ,  $P = 0.02$ , Fig. 2.2) and MD-Rocky Gap ( $\chi^2 = 47.2$ ,  $df = 2$ ,  $P < 0.0001$ , Fig. 2.3). At the TN release site, HWA density declined from 2005 to 2007 but increased at the control site. At MD, the release site's HWA density remains unchanged and increased at the control site. There was no difference at VA-North Fork ( $\chi^2 = 3.1$ ,  $df = 3$ ,  $P = 0.4$ , Fig. 2.4). No significant difference was observed in the vigor index between the release site and control site at TN-Laurel Ck. ( $\chi^2 = 3.8$ ,

$\text{df} = 3, P = 0.3$ , Fig. 2.2) and MD-Rocky Gap ( $\chi^2 = 5.8, \text{df} = 2, P = 0.06$ , Fig. 2.3). There was a difference at VA-North Fork ( $\chi^2 = 10.7, \text{df} = 3, P = 0.01$ , Fig. 2.4), however this was because of the different initial health of the trees.

## 2.4 Discussion

### Establishment

Recovery of the  $F_3$  generation and increasing abundance at most sites indicate that *L. nigrinus* has become established in the eastern US. Its source location in Victoria, B.C. was in plant hardiness zone 8b and it has established in the eastern US from Pennsylvania to Tennessee (Zones 5b-6a). This suggests its adaptability to different temperature conditions, as the maritime climate of the coastal Pacific Northwest is different in terms of seasonal temperature extremes and rainfall patterns from the continental eastern US climate. Equally important is that the predator has been able to adjust to HWA originating from Japan (Havill et al. 2006), eastern hemlock, and different soils in which it pupates and aestivates. Sites where larval abundance decreased does not necessarily represent a population in decline but small host collections from release trees in decline.

The establishment rate of 59% is encouraging considering the small release sizes and might be indicative of its future success for biological control of HWA. Clausen (1951) showed that successful biological agents established easily and showed at least some impact within three generations. Those that do not establish easily or show impact within this period have little chance of success, but there are exceptions (Debach and Rosen 1991). At sites where *L. nigrinus* was not collected, or establishment is unclear (*i.e.* VA-Lick Ck.), the predators may be recovered in the future even though we consider them to have not established (Humble 1994). Although possible, it is improbable for *L. nigrinus* because larval sampling appears to be a reliable sampling method. For example, VA-Highland was the only site where  $F_2$  larvae were not recovered but  $F_3$  larvae were found. Our studies suggest that larval sampling is preferred to adult sampling for detecting *L. nigrinus* presence and abundance regularly. Adult recovery was poor due to their small size and behavior, which is not well understood. Inconsistency of adult recoveries among established sites could also be due to differences in sampler experience with *L. nigrinus*.

The amount of variance in *L. nigrinus* establishment and abundance accounted for primarily by plant hardiness zone or lowest minimum temperature was high considering the various climatic variables not included in the model, the complex structure of forests, HWA population dynamics, and other factors such as soil characteristics. Climate has long been recognized as a major factor in establishment and impact of beneficial insects (Pschorn-Walcher 1977, Clausen 1978b). *Laricobius nigrinus* is freeze intolerant but adults are physiologically able to avoid freezing temperatures from -16 to -19 °C (Humble and Mavin 2005). Survival at lower temperatures is likely if behavioral mechanisms exist, such as the use of shelter in duff, bark crevices, hemlock cones, or moss. This has not been investigated in detail with *L. nigrinus*, but it was implied that they do seek shelter because they are not found on branches when temperatures are below 0 °C (Zilahi-Balogh et al. 2003c). Eggs have supercooling points of -27.5 to -26.9 °C and larval supercooling capability diminishes from -22.1, -17.0, -15.0, to -13.0 °C for each consecutive instar as they develop later in the spring (Humble and Mavin 2005). Death occurs if temperatures fall below their supercooling points and because eggs are laid in late winter/early spring, they are particularly susceptible. *Laricobius nigrinus* did not establish in zone 5a (-26.2 to -28.8 °C), which has minimum temperatures below the eggs supercooling points and may explain the lack of establishment in these locations. The actual lowest minimum temperature recorded at these sites was -22.8 °C in 2004/2005 and other factors like duration of cold temperatures could have reduced the predator's survival (Humble and Mavin 2005). Cold conditions likely explain failures in zone 5a and marginal establishment rates in 5b (-23.4 to -26.1 °C), which are not below the eggs supercooling point. The actual minimum temperatures recorded at the 5a sites ranged from -25.5 to -23.3 °C. The HWA rate of spread also slowed in zone 5b (Evans and Gregoire 2007b), probably because of their lower survival and densities in cold areas. HWA and *L. nigrinus* have similar lower developmental temperature thresholds and climatic tolerances (Salom et al. 2002, Zilahi-Balogh et al. 2003b). HWA has spread into zone 5a (Anonymous 2007a) but not *L. nigrinus*, and predators with better cold tolerance may be required in zones 5a and 5b. Zones 6a (-20.6 to -23.3 °C) and 6b (-17.8 to -20.5 °C) had high establishment rates due to the milder climate. Actual minimum temperatures ranged from -18.9 to -13.1. At the VA-Lick Creek site, the actual minimum temperature recorded was -21.1 and was colder than other sites in zone 6a, which may explain the lack of establishment. Zone 7a (-

15.0 to -17.7 °C) was the warmest release location and the lack of establishment in Georgia is unclear because there were no replications in this study. It may also suggest that there is a range of temperatures where *L. nigrinus* thrives. The actual minimum (-12.2 °C) or maximum temperature (35.5 °C) were not extreme. An analysis of releases in this zone made subsequent to this study would shed more light on the establishment rate and effectiveness of releases in this zone. Other factors besides climate could affect *L. nigrinus* establishment, but competition with *L. rubidus* and other predators does not seem likely (Flowers et al. 2005, 2006). Additional factors such as site characteristics, predator and prey synchrony, generalist soil predators, beetle vigor (*i.e.* lab versus field collected beetle releases), predator : prey ratios, and *L. nigrinus* genetics should be investigated.

Release number (Campbell 1976, Memmott et al. 1998, Grevstad 1999a) and timing (Strong and Croft 1996, Daane and Yokota 1997) have been shown to be important factors for establishment of biological control agents by controlled field experiments of small spatial scale. Grevstad (1999a) found that release number was the most significant factor for establishment of *Galerucella pusilla* Duftschmidt and *Galerucella calmariensis* L. (Coleoptera: Chrysomelidae), for purple loosestrife control, and location was secondary in central NY state. Release number and timing were minor factors relative to location for *L. nigrinus* establishment and abundance perhaps because of the large spatial scale in this study. Some large release numbers, spring, and sequential releases had the highest larval counts recorded in the study. The explanation for the former is obvious. The latter may be explained by the fact that female predators released in spring can oviposit immediately after release, while those released in the fall or winter must survive adverse winter weather conditions before oviposition. Furthermore, female predators released in the fall may not have mated and become subject to demographic Allee effects if they cannot locate males or vice versa. A sequential release may facilitate establishment if the first release failed due to adverse weather conditions and the second was successful (Norris et al. 2002).

### **Impact on HWA and tree vigor**

The amount of variance in HWA density and HVI change accounted for by plant hardiness zone or lowest minimum temperature was also high. Minimum temperature has been shown to be an

important cause of HWA mortality at northern locations (Parker et al. 1998, Parker et al. 1999, Skinner et al. 2003, Shields and Cheah 2005). A lack of extreme cold in the warmer plant hardiness zones (*i.e.* the south and low elevations) explains the greater increase in HWA density and decline in hemlock vigor compared with the colder zones in this study. A faster rate of HWA spread in the south compared with the north supports this relationship (Evans and Gregoire 2007b). Release size and timing were not significant but accounted for a considerable amount of explained variability in HWA density change. The larger release numbers, spring, and sequential releases tended to have small increases or decreases in HWA density. The larger release numbers tended to have smaller decreases in HVI, and release size may be a significant factor if larger releases >1,200 per site were made.

Individual trees have declined in vigor at most release and control sites (Appendix 4). Shoot growth declined, number of dead tips increased, transparency increased, live crown ratio declined, and USFS decline class increased. From year to year there were alternations of tree decline and meek recovery that was related to HWA outbreaks and collapses as described by McClure (1991c). No release trees died in the three or four years of monitoring but many trees were in a moribund state in the final re-measurement year, particularly in the south. There were exceptions to this pattern when hemlocks recovered slightly from their initial conditions at cold locations (*i.e.* VA-Big Stony and the WV sites). The MD-Finzel site was an atypical case because it was located in zone 6a, *L. nigrinus* did not establish, yet tree health has remained very healthy and HWA density has been below the threshold density. The actual minimum temperature recorded was -21.7 °C and was colder than other sites in this zone during this study. Dead HWA were observed in 2003/2004 and 2004/2005 and was likely due to winter mortality (Shields and Cheah 2005). Another explanation is that the trees may have effective chemical defenses against HWA and foliage samples have been sent to researchers for laboratory assays (R. Casagrande, University of Rhode Island, pers. comm.).

At TN-Laurel Ck. and MD-Rocky Gap, two sites where *L. nigrinus* was abundant, differences between release and control sites were likely due to the activity of the predators because the control sites had very similar initial conditions. *Laricobius rubidus* was common at these sites but we believe it was at similar densities at the release and control sites because the control sites

were nearby and well matched in terms of white pine and pine bark adelgid abundance. Even so, HWA density is above hemlocks physiological damage threshold and tree health has declined. For biological control to be successful HWA populations must be reduced below this threshold and further investigations are needed to determine if *L. nigrinus* can do this by itself. Long term monitoring of the 22 release sites is planned. At the VA-North Fork site, *L. nigrinus* established and *L. rubidus* was collected but both were rare. This site had HWA density dip below the threshold in 2005/2006 and an increase in HVI starting in 2005/2006 at the release and control site. This pattern is probably driven by its location in zone 5b and the low minimum temperatures reached in the winter of 2004/2005.

### **Best release practices recommendations**

Some guidelines can be suggested from the research described here in addition to the criteria for release site selection described in the “Study site selection and characteristics” section of methods. First, *L. nigrinus* from the coastal regions of the Pacific Northwest may prove most effective by focusing releases in plant hardiness zones 6b, 6a, which will increase the overall establishment rate and potential impact. Releases in zone 5b appear marginal for establishment but releases should continue in these locations. The predator does not appear to establish in zone 5a and operational releases in this zone are not recommended. Releases in 7a should be suspended until an analysis of releases in this zone becomes available. Predators may have established in the southern Appalachians, but with the rapidity of tree decline due to a lack of abiotic HWA mortality and drought conditions in this study, the chance of successful biological control is low.

Second, since there was no significant effect of release size, releases as small as 75 predators should be seriously considered by forest health specialists for stands at an early stage of infestation at widely dispersed sites. This will require an accurate knowledge of HWA’s expanding front. As such, the spread of HWA could be potentially checked before a prolonged outbreak occurs. Releases of 80 predators of the Eurasian pine adelgid brought about successful biological control in Hawaii (Culliney et al. 1988). The larger release sizes we tested may increase establishment and impact on HWA density, however this would be at the risk of reducing the total number of releases given a limited supply of predators. The primary objective

of releases is to obtain establishment and not immediate control, so large releases may be wasteful of insects (Campbell 1976). In addition, if dispersal is slow, as in *L. erichsonii* (Buffam 1962, Mitchell and Wright 1967), then numerous small releases will be necessary to cover the range of HWA.

Third, spring and sequential (*i.e.* fall or winter and spring) releases may increase establishment and impact on HWA. Releases in the fall are ideal because this is the earliest that suitable prey stages are present and increases the time for predators to feed on HWA in the field. Releases in the fall ease the mass-rearing effort and reduce mortality in the laboratory. One possible approach is to make the sequential or spring releases in zones 5b to compensate or avoid adverse winter conditions. In zone 6a and 6b, any release timing should be successful.

**Table 2.1.** Location, number, and timing of *Laricobius nigrinus* adult releases in 22 hemlock forests in the eastern US between November 2003 and March 2005 and summary of post-release recoveries (right of vertical line).

Site no.	Location (State-site name)	Release						No. adults			No. larvae				% of trees with larvae		<i>L. nigrinus</i> establishment <sup>2</sup>
		Zone <sup>*</sup>	No.	Date(s)	Fall	Winter	Spring	<i>F</i> <sub>1</sub>	<i>F</i> <sub>2</sub>	<i>F</i> <sub>3</sub> <sup>1</sup>	<i>F</i> <sub>2</sub>	<i>F</i> <sub>3</sub>	<i>F</i> <sub>2</sub>	<i>F</i> <sub>3</sub>	<i>F</i> <sub>2</sub>	<i>F</i> <sub>3</sub>	
1	MA-Mt. Tom	5b	150	19 Nov 04	150	--	--	0	0	-- <sup>1</sup>	0	0	0	0	0	0	-
2	PA-Rothrock	6a	600	4 Dec 03	--	300	--	0	0	8	30	343	7	61	60	100	+ <sup>3</sup>
				20 Apr 04	--	--	300	0	0	0	0	0	0	0	0	0	
3	PA-Bear Run	6a	300	24 Mar 05	--	--	300	0	1	--	125	311	9	23	80	90	+ <sup>3</sup>
4	MD-Finzel	6a	300	18 Dec 03	--	300	--	0	0	0	0	0	0	0	0	0	-
5	MD-Rocky Gap	6b	1,200	23 Nov 04	1,200	--	--	31	49	--	517	440	64	54	93	93	+ <sup>3</sup>
6	MD-Frederick	6b	75	23 Nov 04	75	--	--	0	1	--	1	14	1	5	50	100	+ <sup>4</sup>
7	WV-Seneca	5a	600	28 Nov 03	300	--	--	0	0	0	0	0	10	158	50	80	-
				10 Mar 04	--	--	300	0	0	0	0	0	10	158	50	80	
8	WV-Watoga	5a	300	28 Nov 03	300	--	--	0	0	0	0	0	16	114	40	80	-
9	WV-Monongahela	5a	300	10 Mar 04	--	--	300	0	0	0	0	0	0	7	0	40	-
10	VA-North Fork	5b	600	8 Dec 03	--	300	--	0	0	0	23	32	5	7	60	50	+ <sup>3</sup>
				28 Mar 04	--	--	300	0	0	0	0	0	5	7	60	50	+ <sup>3</sup>
11	VA-Big Stony	5b	300	8 Dec 03	--	300	--	0	0	0	0	0	2	44	20	70	-
12	VA-Hurricane	6a	300	30 Mar 03	--	--	300	1	0	1	19	1	14	0	30	10	+ <sup>3</sup>
13	VA-Highland	6a	1,200	4 Nov 04	1200	--	--	0	0	--	0	62	6	0	7	27	+ <sup>3</sup>
14	VA-Lick Ck.	6a	150	4 Nov 04	150	--	--	1	0	--	5	0	5	0	60	0	-
15	VA-Dickey Ck.	6a	75	8 Feb 05	--	75	--	0	0	--	0	0	52	27	100	50	-
16	NC-Hemlock Hill	6a	300	31 Dec 03	--	300	--	3	13	93	10	314	0	0	40	40	+ <sup>3</sup>
17	NC-Holloway	6a	150	27 Oct 04	150	--	--	1	19	--	2	2	24	20	40	60	+ <sup>4</sup>
18	NC-Ivy Ck.	6b	1,200	14 Mar 05	--	--	1,200	0	1	--	42	868	0	0	27	100	+ <sup>3</sup>
19	NC-Locust Ck.	6b	75	12 Jan 05	--	75	--	0	0	--	42	109	42	109	100	100	+ <sup>3</sup>
				12 Jan 05	--	300	--	0	0	--	180	1,163	12	74	90	100	+ <sup>3</sup>
21	TN-Laurel Ck.	6b	300	17 Feb 04	--	--	300	0	0	0	118	31	31	67	80	80	+ <sup>3</sup>
				13 Mar 05	--	--	300	0	0	--	0	0	0	0	0	0	-
22	GA-Overflow Ck.	7a	150	26 Oct 04	150	--	--	0	0	--	0	0	0	0	0	0	-

\* Hardiness zones based on avg. ann. min. temp. (°C): 5a (-26.2 to -28.8), 5b (-23.4 to -26.1), 6a (-20.6 to -23.3), 6b (-17.8 to -20.5), and 7a (-15.0 to -17.7)

<sup>1</sup> (--) indicates that *F*<sub>3</sub> data were not available at the time of this publication

<sup>2</sup> (+) indicates establishment by confirmed *F*<sub>3</sub> *L. nigrinus* adults or larval presence

<sup>3</sup> Indicates confirmation by adult recovery or rearing of eggs and larvae to adults

<sup>4</sup> Indicates confirmation by PCR-RFLP of larvae

**Table 2.2.** Results (coefficients and  $P$ -values) from a logistic regression analysis predicting *Laricobius nigrinus* establishment or non-establishment.

Model variable	Established versus non-established	
	$\beta$	$P$ -value
Constant	-18.8	0.037
P.h. zone	4.6	0.041
Release number	1.5	0.202
Release timing	1.2	0.314

**Table 2.3.** Correlations and standardized regression weights of average annual minimum temperature, release number, and timing on *Laricobius nigrinus* larval abundance.

Simple correlations	$X_1$	$X_2$	$X_3$	$Y$	$\beta$	$P$
$X_1$ = P.h. zone	1.00*	0.001	0.008	0.343	0.620	0.002
$X_2$ = Release number	-0.034	1.00	0.162	0.087	0.181	0.335
$X_3$ = Release timing	-0.092	0.402	1.00	0.117	0.310	0.110
$Y$ = $\ln$ larvae	0.586	0.295	0.342	1.00		

Multiple correlations	$r^2_{y,123} = 0.513$	$r^2_{y,12} = 0.431$	$r^2_{y,13} = 0.484$	$r^2_{y,23} = 0.146$
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\*Note: Below the diagonal is the Pearson simple correlation coefficient ( $r$ ) and above the diagonal is the coefficient of determination ( $r^2$ ).

**Table 2.4.** Variance in *Laricobius nigrinus* larval abundance associated with the independent variables unique, first-order, and second-order common effects.

Effects	$R^2_{v,123} = 0.513$	Explained variance
Unique to:		
P.h. zone	0.367	36.7%
Release number	0.029	2.9%
Release timing	0.082	8.2%
Common to:		
P.h. zone+release number	0.0	0.0%
P.h. zone +release timing	-0.023	-2.3%
Release number+timing	0.059	5.9%
P.h. zone+release number+timing	-0.001	-0.1%
<b>Totals:</b>	<b>0.513</b>	<b>51.3%</b>

**Table 2.5.** Correlations and standardized regression weights of average annual minimum temperature, release number, and release timing on the change of HWA density from 2004 to 2007.

Simple correlations	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Y	β	P
X <sub>1</sub> = P.h. zone	1.00*	0.011	0.033	0.289	0.464	0.015
X <sub>2</sub> = Release number	-0.106	1.00	0.162	0.198	-0.304	0.118
X <sub>3</sub> = Release timing	-0.181	0.402	1.00	0.189	-0.229	0.238
Y = HWA density	0.538	-0.445	-0.435	1.00		

Multiple correlations	r <sup>2</sup> <sub>y,123</sub> = 0.484	r <sup>2</sup> <sub>y,12</sub> = 0.441	r <sup>2</sup> <sub>y,13</sub> = 0.407	r <sup>2</sup> <sub>y,23</sub> = 0.276
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\*Note: Below the diagonal is the Pearson simple correlation coefficient ( $r$ ) and above the diagonal is the coefficient of determination ( $r^2$ ).

**Table 2.6.** Variance in hemlock woolly adelgid density associated with the independent variables unique, first-order, and second-order common effects.

Effects	R <sup>2</sup> <sub>v,123</sub> = 0.484	Explained variance
Unique to:		
P.h. zone	0.208	20.8%
Release number	0.077	7.7%
Release timing	0.043	4.3%
Common to:		
P.h. zone+release number	0.01	1.0%
P.h. zone+release timing	0.035	3.5%
Release number+timing	0.075	7.5%
P.h. zone+release number+timing	0.036	3.6%
<b>Totals:</b>	<b>0.484</b>	<b>48.4%</b>

**Table 2.7.** Correlations and standardized regression weights of average annual minimum temperature, release number, and release timing on change in the eastern hemlock vigor index from 2004 to 2007.

Simple correlations	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Y	β	P
X <sub>1</sub> = P.h. zone	1.00*	0.011	0.033	0.530	-0.696	<0.0001
X <sub>2</sub> = Release number	-0.106	1.00	0.162	0.099	0.222	0.196
X <sub>3</sub> = Release timing	-0.181	0.402	1.00	0.069	0.048	0.778
Y = Vigor index	-0.728	0.315	0.263	1.00		

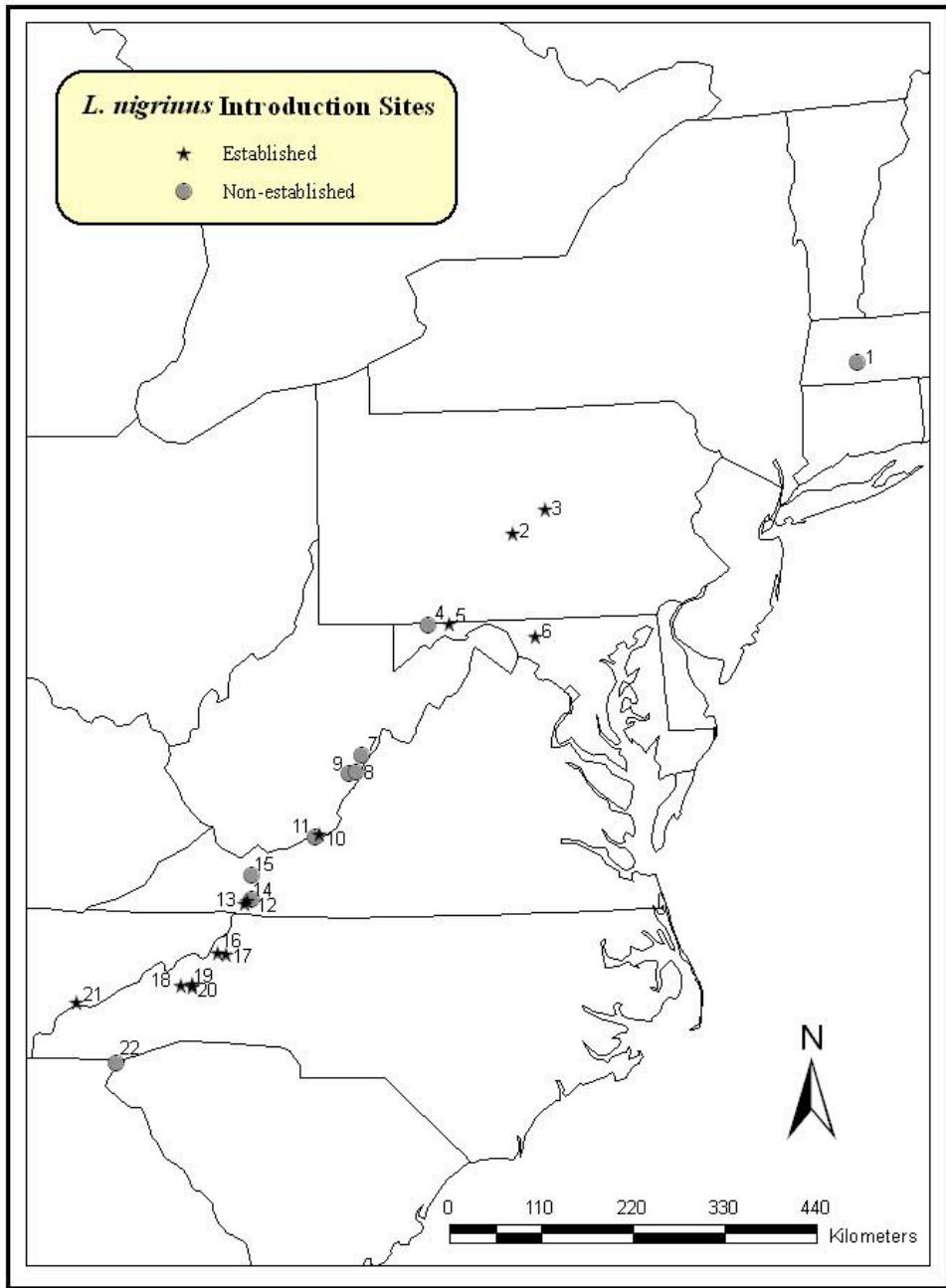
  

Multiple correlations	r <sup>2</sup> <sub>y,123</sub> = 0.589	r <sup>2</sup> <sub>y,12</sub> = 0.587	r <sup>2</sup> <sub>y,13</sub> = 0.548	r <sup>2</sup> <sub>y,23</sub> = 0.121
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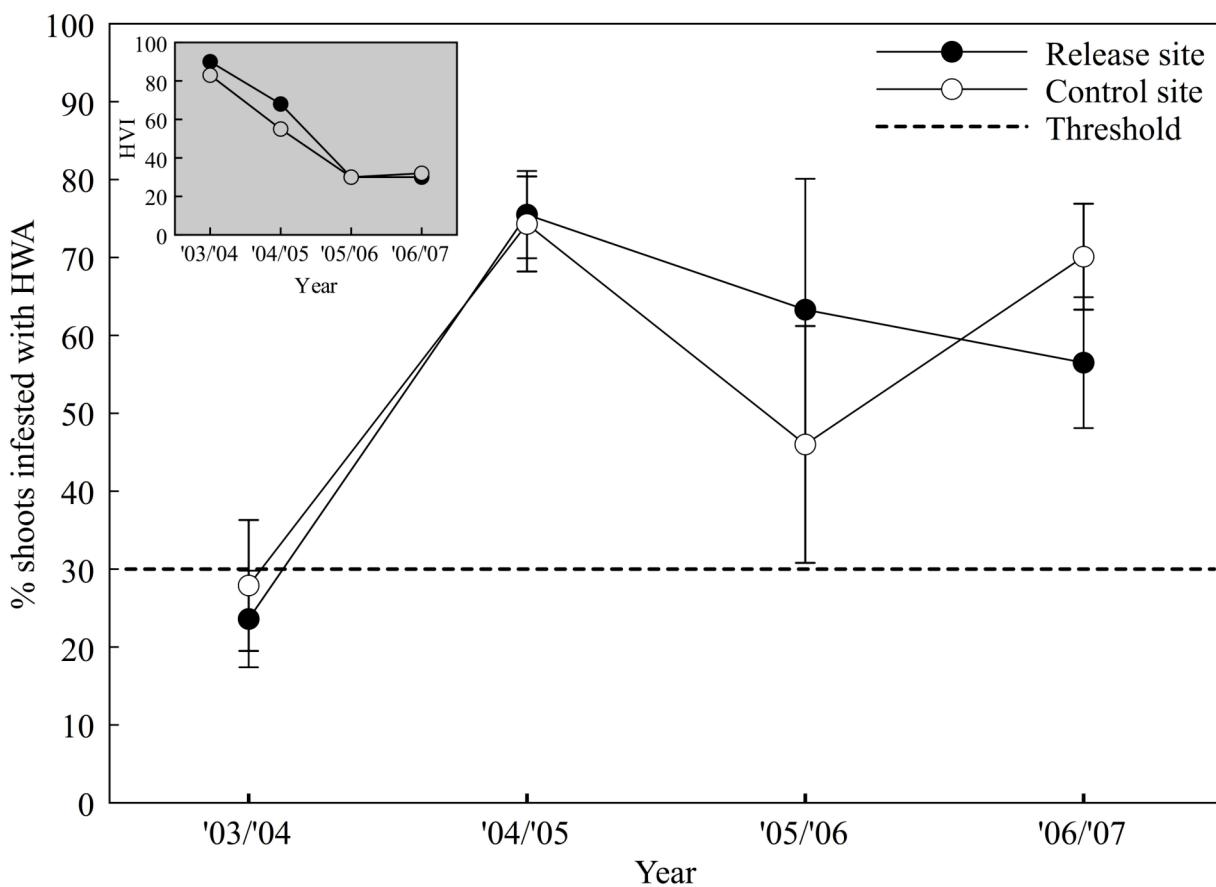
\*Note: Below the diagonal is the Pearson simple correlation coefficient ( $r$ ) and above the diagonal is the coefficient of determination ( $r^2$ ).

**Table 2.8.** Variance in eastern hemlock vigor index associated with the independent variables unique, first-order, and second-order common effects.

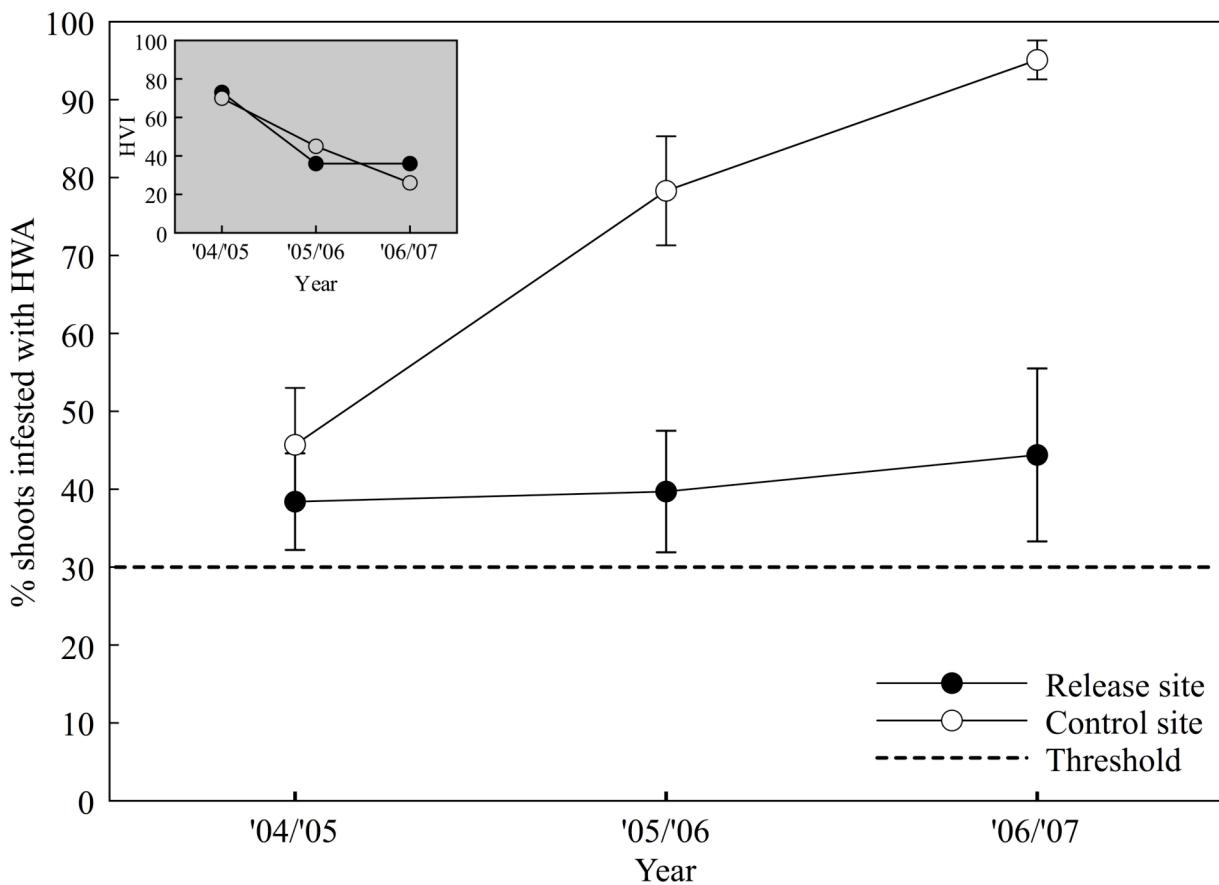
Effects	$R^2_{v,123} = 0.589$	Explained variance
Unique to:		
P.h. zone	0.468	46.8%
Release number	0.041	4.1%
Release timing	0.002	0.2%
Common to:		
P.h. zone+release number	0.011	1.1%
P.h. zone+release timing	0.02	2.0%
Release number+timing	0.016	1.6%
P.h. zone+release number+timing	0.031	3.1%
<b>Totals:</b>	<b>0.589</b>	<b>58.9%</b>



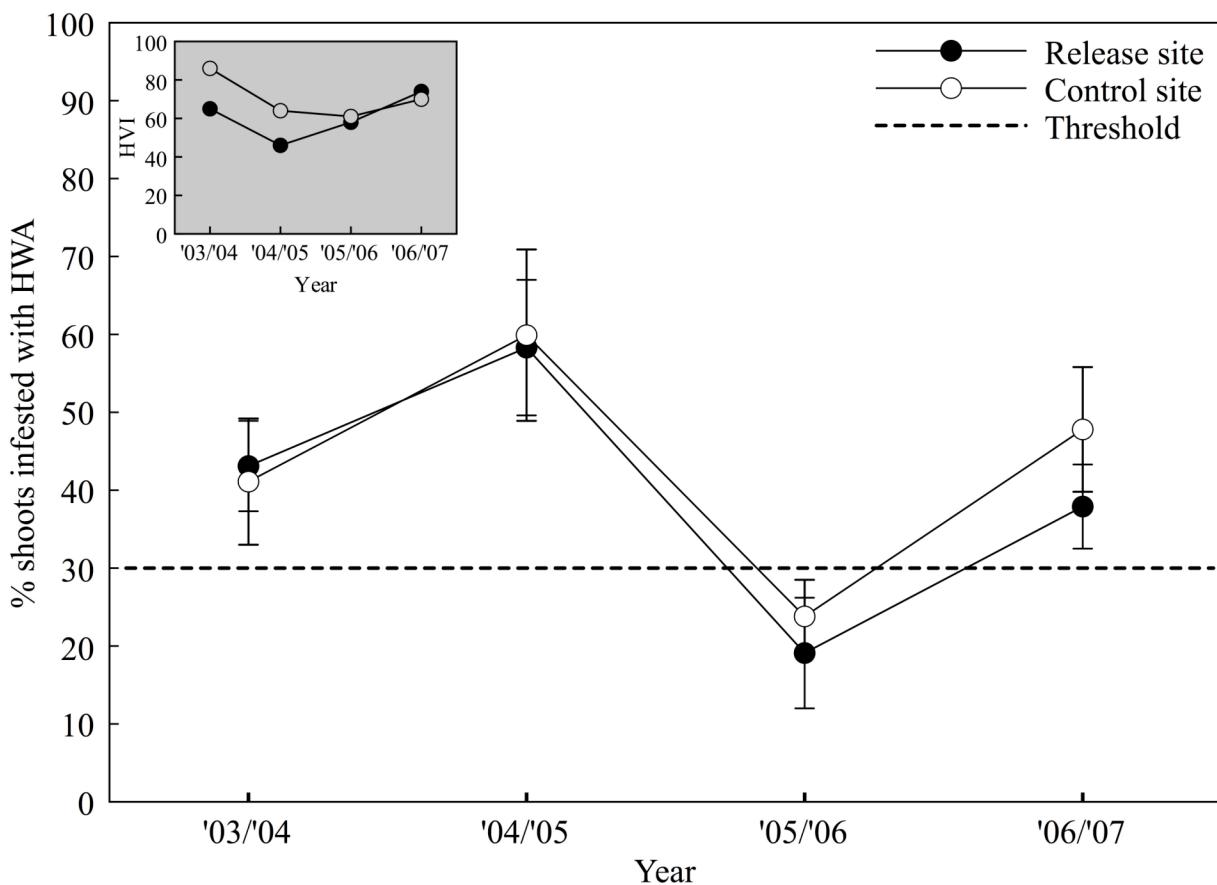
**Fig. 2.1.** Locations of 22 *Laricobius nigrinus* introduction sites in the Appalachian mountains of the eastern US between fall 2003 and spring 2004.



**Fig. 2.2.** Change in *sistentes* hemlock woolly adelgid density and hemlock vigor index (HVI, inset) over time at the *Laricobius nigrinus* release and control sites at Tennessee-Laurel Creek, dashed line signifies hemlocks physiological damage threshold.



**Fig. 2.3.** Change in *sistenes* hemlock woolly adelgid density and hemlock vigor index (HVI, inset) over time at the *Laricobius nigrinus* release and control sites at Maryland-Rocky Gap, dashed line signifies hemlocks physiological damage threshold.



**Fig. 2.4.** Change in *sistentes* hemlock woolly adelgid density and hemlock vigor index (HVI, inset) over time at the *Laricobius nigrinus* release and control sites at Virginia-North Fork, dashed line signifies hemlocks physiological damage threshold.

## **Chapter 3 Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera: Derodontidae) on the hemlock woolly adelgid (Hemiptera: Adelgidae) at a field insectary**

### **Abstract**

Phenological synchrony and impact of *Laricobius nigrinus* Fender, *Laricobius rubidus* LeConte, and the hemlock woolly adelgid (HWA) *Adelges tsugae* Annand, were studied in an eastern hemlock field insectary in Blacksburg, Virginia. A field insectary for propagating *L. nigrinus* was established by planting hemlocks in 2001, infesting them with HWA in 2002/2003, and introducing 258 *L. nigrinus* onto the trees in 2004. Annual monitoring from 2004 to 2007 was conducted to estimate HWA and predator populations. Beginning in 2004, HWA and *Laricobius* spp. populations increased annually from which 305 adult *L. nigrinus* were collected and re-distributed to forests in 2007. From Sep 2005-Aug 2006, adult *Laricobius* spp. were sampled with beat sheets every 2 wk and their numbers recorded by species. Hemlock clippings were sampled from the trees every 1 to 2 wk and the numbers of HWA and immature *Laricobius* spp. stages were recorded. Adult *L. nigrinus* were collected from 3 Nov 2005 to 10 Apr 2006 and *L. rubidus* from 3 Nov 2005 to 8 May 2006. The presence of *Laricobius* spp. adults overlapped with sistentes development and continued through oviposition. Immature *Laricobius* spp. were collected from 24 Feb to 8 May 2006 and overlapped the presence of progredientes eggs. Predator impact on HWA was studied with a cage exclusion experiment with three treatments: closed cage, open cage, and no cage. HWA survival was higher in the closed cage (71%) than open (59%) and no cage (56%). HWA density was higher in the closed cage (1.7/cm) than open (0.8/cm) and no cage (0.6/cm). The percentages of HWA ovisacs that were disturbed by predation were less in the closed cage (1%) than the open cage (12%) and no cage (35%). The ease of establishment, impact, and adaptability of *L. nigrinus* to a new environment and co-existence with *L. rubidus* indicate that these predators have potential for controlling HWA.

**Keywords:** *Adelges tsugae*, classical biological control, field nursery, post-release monitoring, predation, invasive species, synchronization, exclusion cage

### 3.1 Introduction

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is invasive in eastern North America on eastern [*Tsuga canadensis* (L.) Carrière] and Carolina hemlock (*Tsuga caroliniana* Engelmann), and the subject of biological control attempts since the mid-1990's. Several HWA-specific predators from its native range in Asia and the North American Pacific Northwest (PNW) have been released into eastern North America (Cheah et al. 2004). One promising species is *Laricobius nigrinus* Fender (Coleoptera: Derodontidae). In the PNW, Kohler (2007) identified numerous predators from HWA-infested western hemlock [*Tsuga heterophylla* (Raf.) Sargent], and *L. nigrinus* was the most widespread and consistently collected of all the predators at low- and high-infestation levels.

In eastern North America, the current HWA-infestation ranges from Maine to Georgia and inland to Kentucky, West Virginia, and upstate New York (Anonymous 2007a) with considerable mortality in 13 states. HWA uses its piercing-sucking mouthparts to feed on a tree's energy reserves in ray parenchyma cells, which results in no shoot growth, high bud mortality, branch dieback, foliage discoloration (graying) and premature defoliation (Young et al. 1995). HWA is mostly sedentary and has two wingless asexual generations per year called the sistentes (summer-early spring generation) and progredientes (spring generation) (McClure 1987b, McClure 1989).

*Laricobius nigrinus* is small (2-3 mm), black, and covered with fine gray hairs (Fender 1945, Zilahi-Balogh et al. 2006). It is univoltine and maintains good phenological and numerical synchrony with HWA throughout the year in the PNW (Zilahi-Balogh et al. 2003c). Both the predator and HWA are active in the fall, winter, spring, and aestivate in the summer. Adults emerge from the soil in the fall, disperse to hemlock branches, and feed on HWA sistentes nymphs, adults, and progredientes eggs before they die. *Laricobius nigrinus* females synchronize oviposition with the presence of progredientes eggs in sistentes ovisacs. The predaceous larvae preferentially consume eggs and if necessary, nymphs. By June, larvae develop through four instars and migrate to the soil and pupate (Zilahi-Balogh et al. 2003b). Adults are cold hardy (Humble and Mavin 2005) and active at temperatures above 0 °C and

immature stages have minimum developmental thresholds at or below 5 °C. Thus, *L. nigrinus* feeds on HWA in the fall through spring when other predators are inactive.

*Laricobius nigrinus* was imported from Victoria, British Columbia, Canada into Virginia in 1997 under quarantine (Zilahi-Balogh et al. 2002, Zilahi-Balogh et al. 2003c, Zilahi-Balogh et al. 2003a, b) and subsequently became unrestricted. Species in the genus *Laricobius* feed exclusively on the Adelgidae, which feed exclusively on the Pineaceae (Lawrence and Hlavac 1979, Havill and Foottit 2007, Zilahi-Balogh et al. 2007). Field cage studies showed that the adults survived in southwestern Virginia under caged conditions during winter (min. temp. endured was -16 °C) and fed on 3 to 6 sistentes nymphs per day (Lamb et al. 2005b). Another cage study and egg release of *L. nigrinus* demonstrated significant predation, oviposition, and recovery of F<sub>2</sub> adults post-release (Lamb et al. 2006). Currently, *L. nigrinus* is being mass-reared and introduced in the eastern US (Lamb et al. 2005a).

*Laricobius rubidus* is native to eastern North America and has been collected from HWA infested hemlock trees (Clark and Brown 1960, Lawrence 1989, Montgomery and Lyon 1996, Wallace and Hain 2000). The primary host of *L. rubidus* is the pine bark adelgid (PBA), *Pineus strobi* Hartig (Hemiptera: Adelgidae), to which it is phenologically synchronized (Clark and Brown 1960). However, it can survive as well on a diet of HWA as on PBA (Zilahi-Balogh et al. 2005). The natural history of *L. rubidus* is similar to *L. nigrinus* except that it is reported to have a hibernal diapause in addition to an aestival diapause in New Brunswick (Clark and Brown 1960), Massachusetts (Montgomery and Lyon 1996), and possibly in Virginia (Zilahi-Balogh et al. 2005). Adults are active in the fall and spring and larvae develop in the spring.

Field insectaries were recently advocated for rearing *L. nigrinus* (Kok and Salom 2002). In this study, the effectiveness of a field insectary for propagating *L. nigrinus* was evaluated. The phenological synchrony of HWA and *Laricobius* spp. were studied by monitoring their life cycles. The impact of the predators on HWA was evaluated by a cage exclusion experiment. Field insectaries can produce field-quality beetles for re-distribution to native forests and cage exclusion experiments realistically gauge the impact of predators on their prey (Luck et al. 1988). An understanding of *L. nigrinus* synchrony and impact on HWA can help determine why

the species may succeed or fail, aid in the development of an optimal release strategy, and provide assurance that *L. nigrinus* has potential for significant long-term impact on HWA.

### **3.2 Materials and methods**

#### **Founding the field insectary**

An eastern hemlock plantation was established at Virginia Tech's Kentland Farm, Blacksburg, Virginia in Oct 2001 (UTM Zone 17, 0536369E 4118129N) (Kok and Salom 2002). An old vegetable crop field that was left fallow with naturally occurring wild grass was selected for the location of a 0.4 ha field insectary. Adjacent to the field insectary was a 15 year-old white pine, *Pinus strobus* L., plantation that was naturally infested with pine bark adelgid, *Pineus strobi* Hartig (Hemiptera: Adelgidae). Soil depth exceeded 0.6 m on a northeast-facing slope ranging 10-15%. The area was mowed on 3 Oct 2001 to reduce competing vegetation. Twelve 12.2 x 19.5 m blocks were laid out in a 4 x 3 block rectangle and spaced 4.9 m apart. Trees were spaced 2.4 m within and 3.7 m between rows that were orientated northeast and southwest. There were six rows with five trees in each (30 trees per block).

Three hundred 1.2-2.4 m tall hemlock trees with 0.6 m diameter root balls wrapped in burlap and secured in a wire basket were purchased from a local nursery in Floyd, VA. The total cost of the trees including transportation was discounted to \$7,800 because some of these trees were lightly infested with HWA. Ten of the 12 blocks were planted with the large hemlocks. The other two blocks were planted at the same spacing but with potted 0.6 m tall hemlocks, for a total of 360 trees. A 0.6 m and 15 cm diameter augers were used to make holes for the root balls of the large hemlocks and the smaller potted hemlocks, respectively. Five of the 10 blocks with large hemlocks had 2-year-old bare root tulip poplars, *Liriodendron tulipifera* L. (0.9 to 1.2 m tall) planted on the south-facing side of each hemlock. The fast growing tulip poplar was expected to provide shade for the hemlocks until they established. The 15 cm diameter auger was used to make 21 cm deep holes to plant the poplars. All trees received 4 to 6 inches of bark mulch and watered with 3.8 - 7.6 liters immediately after planting and again at 3 and 6 wk after planting. Approximately 600 h were required for this work.

Surveys in 2002 revealed that 85 large hemlocks had a majority of their needles drop prematurely, 91 hemlocks had dead tops, and approximately 95% of the tulip poplars had died due to prolonged drought conditions. Unfortunately, irrigation was not available at this location. In 2003, another survey revealed that 75 hemlocks had died with a majority of those being the potted hemlocks in two blocks. The remaining dead trees were scattered throughout most blocks. Between 2002-2005, dead tops were pruned out, all the dead trees were removed, and replaced with potted hemlock trees, as described previously. During the growing seasons, starting in 2002, weeds in the plot were managed by mowing, clipping, and hand pulling on a monthly schedule. In summer 2002, 0.6 kg of pelletized lime was placed around the drip line of the large hemlocks and 0.2 kg of lime placed around the small trees. In addition, 0.5 kg of 5-10-10 (N-P-K) fertilizer was placed around the drip line of the large hemlocks and 0.2 kg around the small hemlocks. In Nov 2004, the trees were re-limed and fertilized, and the tallest hemlocks had their leaders pruned to favor a low and spreading crown form for accessibility. The trees were inoculated with HWA in March 2002 and April 2003 to increase the small amount already present. Two hemlock clippings (~30 cm long) infested with *progradientes* eggs from native forests were placed among the mid-crown of all living trees. Approximately 6 h were required to collect HWA and inoculate the trees.

For 193 trees greater than 1.0 m tall, the number of new shoots infested with at least one *sistentes* HWA and the total number of new shoots were counted on 30 cm segments at the middle third of a branch's length on 12 Nov 2003. One branch was measured at each cardinal point and the percentage of infested shoots was calculated and averaged for each tree. The number of predators released on each tree was based on the estimated density of HWA as follows: six adult *L. nigrinus* were released on each of four heavily infested trees ( $\geq 76\%$  of new shoots with at least one HWA), four were released on 42 moderately infested trees ( $25 \leq x \leq 75\%$ ) and one was released on 66 lightly infested trees ( $\leq 25\%$ ). In summary, 258 *L. nigrinus* adults of an unknown sex ratio were open-released on 112 hemlocks that ranged in height from 1.8 - 3.0 m.

The *L. nigrinus* adults we released were lab-reared from beetles collected in Victoria, B.C. in late winter 2003 (Lamb et al. 2005a). Adults emerging from the soil containers in the lab in fall 2003 (*i.e.* F<sub>1</sub> lab generation) were placed in plastic containers with 50 conspecifics on HWA infested

eastern hemlock. Containers were kept at 4 and 2 °C (day and night, respectively) and given fresh HWA every 2 wk until field-release. One, four, or six beetles were packaged in 50 mm x 9 mm Falcon® Petri dishes with lids (Becton Dickenson, Lincoln Park, NJ) for release on individual trees. Beetles were released on 18 Nov 2003 by placing them on an infested part of a tree with a paintbrush from 11:00 - 13:30 under light drizzle, ambient 11.2 °C, and 11.3 km/h wind conditions. Hourly weather data were collected at the Kentland Farm weather station 1.4 km from the field insectary. The weather during the 48 hours after release consisted of considerable wind with a maximum at 30 km/h, 20+ km/h winds for over 15 h, and rainfall that totaled 3.2 cm.

#### **Annual field insectary survey and *Laricobius nigrinus* collection**

In January of 2004, 2005, 2006, and 2007, live hemlock trees greater than 1.5 m in height were inspected for HWA-infestation level. These included the 112 trees that beetles were released on plus an additional 25 trees. HWA-infestation was visually estimated and recorded in these categories: heavily infested, moderately infested, and lightly infested trees, and no HWA detected (no HWA found within ~1 min. of searching). Infestation estimates were qualitative, but consistent with the quantitative estimates taken in 2003 to determine how many beetles to release per tree. Data were summarized as the total number of trees in each infestation category and presented graphically. In 2007, tree crown health was subjectively classified into five categories that represented: healthy (<10% HWA damage symptoms), light decline (10 - 25%), moderate decline (26 - 50%), severe decline (51 - 100%), and dead (no green foliage). Decline symptoms included no new shoot growth, bud mortality, twig dieback, foliage discoloration and premature defoliation (Young et al. 1995).

Population estimates of *L. nigrinus* and *L. rubidus* were conducted in January of 2005, 2006, and 2007 on 215 live hemlock trees greater than 1.0 m in height. Beat sheet sampling was first done on the most heavily HWA-infested branch of a tree, which was typically at the top of each tree's crown, followed by lower branches. Approximately 1 min. total sampling time was spent per tree. A canvas (71 cm<sup>2</sup>) beat sheet (Bio-quip, Rancho Dominguez, CA) was used to collect beetles dislodged by hitting branches with a 1.0 m long bamboo stick. Counts of each species were recorded by tree and the predators were 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* was hand-carried or mailed overnight to HWA infested release sites in Pennsylvania and Maryland. One hundred *L. rubidus* were shipped to Dr. Leellen Solter (Illinois Natural History Survey) for entomopathogen screening. This was done to serve as a surrogate for *L. nigrinus* health.

### **Phenological synchrony**

The Kentland Farm field insectary provided a unique setting for evaluating the comparative synchrony of HWA, *L. nigrinus*, and *L. rubidus*. The predators and HWA populations were common and could be consistently collected. Few other easily accessible *L. nigrinus* release sites had these qualities at this early stage of the release program. In addition, the site is comparable to the western hemlock seed orchard used to study *L. nigrinus* and HWA phenology in Victoria, B.C. (Zilahi-Balogh et al. 2003c).

To determine synchrony among *L. nigrinus*, *L. rubidus*, and HWA, 42 trees where *L. nigrinus* was collected in Jan 2005 were numbered with aluminum tags. At 2 wk intervals, beat sheets were used to monitor the numbers of predator adults on the trees in the afternoons between 1 Sep 2005 and 15 Aug 2006. The most heavily infested branches were tapped with a bamboo stick for a total of 1 min and the numbers of each species were counted before returning them to the tree. In the spring, third and fourth instars were found on the beat sheets but not identified to species, but 80 larvae collected on 28 Apr were placed in a soil container and reared out in the lab to determine the ratio of *L. nigrinus* to *L. rubidus*. Soil from the A-horizon at the field insectary was used, covered with hemlock organic matter, and kept at a constant 15 °C temperature and 12:12 (light : dark). Notes on the relative abundance of other predator adults and immatures were also recorded. Hourly weather observations on the sample dates were summarized as the mean daily soil temperature (at 10 cm depth) and ambient temperature from 24 measurements. Wind speed (km/h) during sampling was also summarized. Specimens were confirmed by the use of keys and descriptions and were deposited in the Virginia Tech Entomology Collection (Lawrence and Hlavac 1979, Downie and Arnett 1996, Zilahi-Balogh et al. 2006).

An HWA-infested shoot from the 2005 growing season was clipped at 2 wk intervals from 1 Sep 2005 to 15 Aug 2006 from the north- and south-side of each tree's mid-crown to monitor development of HWA and immature *Laricobius* spp. life stages. Age of shoots were identified by their unweathered bud scales at the base of the shoot and color of the shoots. For the 19 May to 15 Aug 2006 sample dates, current season growth was expanding and these shoots were cut in addition to the 2005 season's growth. From Mar - May 2006, shoots were clipped weekly to more precisely monitor the phenology of oviposition by HWA, *L. nigrinus*, and *L. rubidus*. The 84 shoots (*i.e.* 42 trees x 2 clippings) were pooled and placed into one 16.5 x 14.9 cm plastic bag. Each sample was labeled, frozen (-15 to -18 °C), and inspected with a dissecting microscope (12 - 20X) in fall 2006. On thawing, each shoot was placed into a brown paper sandwich bag, and randomly sub-sampled until a minimum of 200 HWA and a minimum of 40 cm of shoots were examined for each sample date. The HWA and shoots were inspected by probing with a dissecting tool. The lengths of the examined shoots were measured to the nearest 0.1 cm and summed for each sample. The percentage of HWA in each life stage was recorded for the sistentes and progredientes. No sexuparae were observed. N1 through N4 instars (N=nymphs), adults, and adults with eggs (ovisacs) were tallied. All ovisacs were counted even if the adelgid inside was dead or alive (at the time of freezing). Probing of HWA that was frozen live produced purple colored hemolymph if punctured, rebounding to the touch of a probe, or appeared normal shaped. Dead HWA had either desiccated bodies that did not rebound to touch (*i.e.* hardened), was cloudy brown or black, or had no hemolymph (McClure 1991c, Palmer and Sheppard 2002). The HWA instar was determined by adding one to the number of HWA molts (by counting exuvia within the flocculence). In cases of missing or disturbed flocculence, the body or antennae length were measured with an eyepiece micrometer to determine the instar (McClure 1989).

During microscopic examinations of HWA developmental stages, predators within the cottony flocculence or on the shoots were recorded. All the shoots in the sample that were not sub-sampled were probed for predators. *Laricobius nigrinus* and *L. rubidus* egg and larval identifications were confirmed with keys and descriptions (Lawrence and Hlavac 1979, Downie and Arnett 1996, Zilahi-Balogh et al. 2006). *Laricobius erichsonii* Rosenhauer has been considered established in high elevation fir (*Abies* spp.) forests of the southern Appalachians but

was not recovered in this study. There are no known morphological differences between the eggs and larvae of *L. nigrinus* and *L. rubidus* and therefore they were pooled. Synchrony of immature predators with HWA was not determined at the species level. The larval instars (L1 - L4) were determined by the use of head capsule measurements for *L. nigrinus* (Zilahi-Balogh et al. 2003a) and all specimens fell within the published ranges. Although no head capsule data exist for *L. rubidus*, head capsule sizes for *L. erichsonii* and *L. nigrinus* are non-overlapping and we assume that *L. rubidus* is similar (Franz 1958b, Zilahi-Balogh et al. 2006). Other predator larvae and eggs were identified to the family level (Chu and Cutkomp 1992). All the immature specimens have had reference specimens deposited in the Virginia Tech Entomology Collection.

The total numbers of adults and late instar larvae collected with beat sheets over time, percentages of *Laricobius* spp. and HWA populations in each immature life-stage from the shoot clippings, and total number of immature *Laricobius* spp. and HWA ovisac densities are summarized in Fig. 3.1 - 3.5. Overlap values ( $C_{ih}$ ) were calculated for pairwise comparisons of the predator and prey stages and values range from 0 to 1, with 1 being perfect overlap (Colwell and Futuyma 1971):

$$C_{ih} = 1 - \frac{1}{2} \sum |p_{ij} - p_{hj}|$$

Where  $p_{ij}$  equals the proportion of a predators' stage on a sample date out of all the sample dates and  $p_{hj}$  equals the proportion a HWA stage on a sample date out of all the samples. The overlap value incorporates both the presence and abundance of the two species' stages in question.

### **Cage exclusion impact study**

The Kentland Farm field insectary provided a unique opportunity to study the field impact of *L. nigrinus* on sessile stages of HWA. Scouting on 1 Nov 2005 identified 14 HWA infested trees that F<sub>2</sub> *L. nigrinus* adults were present upon and were not being used in the synchrony study. These trees had moderate to high HWA densities. Exclusion cages were used to estimate *Laricobius* spp. and other predators' impact on HWA survival, ovisac density, and disturbance of ovisacs (e.g. larval feeding). Three treatments were set up on 8 Nov 2005 and included a closed cage (*i.e.* closed at both ends), open cage (*i.e.* opened at both ends), and no cage. Cages were made of sewn white chiffon fabric (polyester) 45 cm wide and 60 cm long and open on one or

both ends. Identical cages had no significant effect on interior temperatures compared with exterior temperatures in other studies (Lamb et al. 2005b, Lamb et al. 2006).

Three branches with similar HWA densities were marked with an aluminum tag and each treatment was randomly assigned within a block (tree) ( $n = 14$ ). To set up the closed cage treatment, the branch was tapped 10 times along the length of the branch with a stick to dislodge predators. The open end of the cage was pulled over the branch until the branch tip reached the sewn end of the cage. The open end of the cage towards the tree stem was cinched with a cable-tie to the branch stem tight enough to prevent predators from entering. To set up the open cage treatment, a cage with both ends open was pulled over the branch until the apical tip of the branch reached the open end, and fastened along the top of the branch stem with thin wire poked through the screen. Three to four attachments were used to prevent wind from disturbing the cage and branch. Other than tagging, nothing was done to the no-cage branches.

After five months, on 11 and 14 Apr 2006, the treatments were cut down from the trees, clipped in the field, and placed in labeled 3.8 liter plastic bags. The timing of cage removal coincided with the tail end of *sistentes* oviposition and immature *Laricobius* spp. presence. Cage removal was conducted in mid-April because other generalist predators were becoming common and the objective was to ascertain the impact of *L. nigrinus*. The bags were placed in a freezer and the foliage was inspected with a dissecting microscope (12 - 20X) in fall 2006. Shoots from the 2005 growing season were cut off, placed into a brown paper bag, and sub-sampled until a minimum of 200 HWA and 100 cm of shoots were inspected by probing with a dissecting tool. The total length of examined shoots were measured to the nearest 0.1 cm and recorded. HWA N2 - N4 and adults were recorded as live or dead, as described previously. Each ovisac was counted and recorded as disturbed or undisturbed. Disturbed ovisacs had lost their round form, the flocculence was tattered, and loose on the branch due to larvae undermining the ovisac's attachment to the twig and feeding on the eggs within them. Feeding damage of this type is typical of the genus (Brown and Clark 1962). The ovisacs recorded as disturbed were probed and the cause was attributed to (1) *Laricobius* spp., by egg, larvae, or exuvia presence; (2) Syrphidae; by egg or larvae presence; (3) or unknown causes if there was no direct evidence.

The initial sistentes HWA density per centimeter was calculated as the total numbers of live and dead N2 - N4, adults, and ovisacs divided by the total length of shoots. Percent sistentes survival was calculated as the total numbers of live N2 - N4, adults, and undisturbed ovisacs divided by the total numbers of HWA (*i.e.* live + dead N2 - N4, adults, and total ovisacs). Sistentes that produced ovisacs were considered survivors even if they were dead inside the ovisac. Aestivating neosistentes were not included because the cages were not set up when they were present and it is likely that mortality at this stage is due to abiotic or hemlock defenses (Lagalante et al. 2006) and not predation. Aestivating neosistentes are not considered suitable prey for *L. nigrinus* (Zilahi-Balogh et al. 2003a). Sistentes ovisac density per centimeter was calculated as the total numbers of ovisacs divided by the total length of shoots examined. *Laricobius* spp. and Syrphidae immature stage density were calculated separately as the total numbers of predators divided by the total length of shoots examined. Percent sistentes ovisac disturbance was calculated as the total numbers of disturbed ovisacs divided by the total number of ovisacs. The percentage of disturbed ovisacs caused by *L. nigrinus* was calculated as the total number of ovisacs disturbed by *L. nigrinus* divided by the total number of disturbed ovisacs. This was repeated for syrphids and unknown causes.

Two-way ANOVA was used in the analysis and Tukey's HSD to differentiate between treatment differences in mean sistentes initial density, percent sistentes survival, ovisac density, and percent ovisac disturbance using SPSS® version 10.0. Proportion data were arcsine square root transformed before analyses (Zar 1999). The ovisac density was  $\sqrt{x+3/8}$  transformed to stabilize variance. In Table 3.2, data are presented untransformed. A paired t-test was used to compare mean *Laricobius* spp. and Syrphidae density between the open and no cage treatment because no predators were present in the closed cage treatment. The percentage of disturbed ovisacs due to *Laricobius* spp., Syrphidae, and unknown causes were compared between the open and no cage treatments by paired t-tests. All averages are presented as means  $\pm$  standard error and the 0.05 significance level was used. The relationship between immature predator density (dependent variable) and HWA ovisac density (independent variable) was determined for the no cage and open cage treatments (pooled).

### 3.3 Results

#### Annual field insectary survey and *Laricobius nigrinus* collection

The total number of uninfested and lightly infested trees decreased over four years after HWA inoculation and the number of moderate and heavily infested trees increased (Fig. 3.1). The amount of highly infested trees increased rapidly after 2004 and have fluctuated between 30 and 40 trees since 2005. The abundance of *L. nigrinus* and *L. rubidus* has been increasing sharply since introduction and F<sub>3</sub> adults were recovered (Fig. 3.1). In 2007, when 305 *L. nigrinus* were removed from the field insectary and re-distributed to HWA infested forests in Pennsylvania (121 adults) and Maryland (184 adults), 89% of the trees were healthy, 6% had light, 3% had moderate, and 2% had severe decline symptoms or were dead. The tree that died succumbed too rapidly to be killed by HWA and the cause is unknown. The field-reared beetles were large (*e.g.* 3 mm) and approaching the maximum size described for the species (Zilahi-Balogh et al. 2006). On two occasions, *L. nigrinus* and *L. rubidus* were observed mating on a beat sheet after being dislodged from a tree (21 Feb 2007). No entomopathogen infections were observed from the 100 *L. rubidus* sampled (Dr. L. Solter, Illinois Natural History Survey, pers. comm.).

#### Phenological synchrony

Beat sheet sampling from 3 Nov 2005 to 10 Apr 2006 collected 193 *L. nigrinus* adults (Fig. 3.2). From 3 Nov 2005 to 8 May 2006, 383 *L. rubidus* were collected. The soil temperature had dropped from 19.1 to 11.2 °C from 13 Oct to 3 Nov when the first predators were collected. No beetles were collected on 15 Dec when the mean ambient temperature was -2.1 °C and snow and sleet were accumulating. On 29 Dec, gusty winds (18 km/h) and light sleet were associated with reduced collections of *L. nigrinus* but not *L. rubidus*. Similarly, *L. nigrinus* collections were low on 10 Feb due to wind (19 km/h) and cold (mean daily temperature was -1.1 °C).

HWA sistentes commenced development after aestival diapause on 3 Nov, developed through four overlapping instars, and became adults starting on 28 Jan (Fig. 3.3). They began ovipositing progredientes eggs on 24 Feb, which peaked on 24 Mar and stopped on 8 May. The progredientes developed rapidly and the first adult was observed on 5 Jun. Progredientes ovisacs with sistentes eggs were recorded on 5 June and 20 June, respectively. Aestivating neosistentes were first observed on 20 Jun. *Laricobius* spp. eggs and larvae were collected from 24 Feb to 8

May ( $\Sigma$  329) (Fig 3.4) and overlapped sistentes oviposition of progredientes eggs (Fig. 3.5). *Laricobius* spp. L3 and L4 were not collected on 8 May from ovisac dissections (Fig. 3.4) but were collected with beat sheets (Fig. 3.2). Overlap values were very high for *Laricobius* spp. and the sistentes generation but less with the progredientes (Table 3.1). Overlap values for adult predators were highest with prey nymphs and adults, and overlap values for the predator immatures were highest with prey ovisacs. In fall 2006, 47 adult *Laricobius* emerged from the 80 mixed *Laricobius* spp. larvae collected with beat sheets in spring and reared in the lab. Of these 48% were *L. nigrinus* and 52% were *L. rubidus*.

Syrphidae eggs and larvae were first observed in ovisacs on 11 Mar and Coccinellidae larvae were observed on 28 Apr and 8 May. Other adult predators besides *Laricobius* spp. were found during beat sheet sampling in the spring only. Adult *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) were first observed on 11 Mar and were abundant on 10 Apr. Coccinellidae and lacewing larvae were first observed on 28 Apr and Coccinellidae larvae were abundant on 8 May and 19 May. Lacewing and Syrphidae larvae observed on 20 Jun were not abundant. After 20 Jun, observations on these predators were discontinued.

### Cage exclusion impact study

The initial HWA densities at the beginning of the experiment were noticeably different among the three treatments but not significantly different ( $F = 2.72$ ,  $df = 2, 26$ ,  $P = 0.08$ ; Table 3.2). Absolute densities in descending order were closed cage > open cage > no cage treatment. The mean percent HWA survival was different among the three cage treatments ( $F = 7.23$ ,  $df = 2, 26$ ,  $P = 0.003$ ; Table 3.2). The closed cage (predators excluded) had significantly higher survival than the open or no cage treatments (predator exposed), and mean ovisac densities were different among the three cage treatments ( $F = 3.29$ ,  $df = 2, 26$ ,  $P = 0.05$ ; Fig 3.6). The closed cage had a higher ovisac density than the no cage treatment, and the open cage was not different from either. There was no difference in the density of *Laricobius* spp. or Syrphidae eggs or larvae between the open cage and no cage treatments (*Laricobius* spp.,  $t = 0.29$ ,  $df= 13$ ,  $P = 0.77$ ; Syrphidae,  $t = 0.46$ ,  $df = 13$ ,  $P = 0.65$ ; Table 3.2). No predators were found in the closed cages.

The mean percentage of ovisacs that were disturbed was different among the three cage treatments ( $F = 36.2$ ,  $df = 2,26$ ,  $P < 0.001$ ; Fig 3.6). Disturbance was greatest in the no cage treatment followed by the open cage, which was higher than the closed cage treatment. The difference in disturbed ovisacs could not be attributed to any specific cause between the open cage or no cage treatments (*Laricobius* spp,  $t = 0.04$ ,  $df = 13$ ,  $P = 0.9$ ; paired t-test Syrphidae:  $t = 0.3$ ,  $df = 13$ ,  $P = 0.7$ ; Unidentified:  $t = 0.8$ ,  $df = 13$ ,  $P = 0.5$ ; Table 3.2). As no predators were found in the closed cage treatment, all disturbed ovisacs were from unknown causes. There was a significant relationship between immature predator and HWA ovisac density ( $F = 55.03$ ,  $df = 1,25$ ,  $P < 0.0001$ , Fig. 3.7)

### 3.4 Discussion

#### Annual field insectary survey and *Laricobius nigrinus* collection

Invasive weeds and insects of pastures, rangelands, agricultural, and aquatic habitats are often controlled with biological control agents produced in field insectaries or release sites that aid collection (Stoyer and Kok 1986, Debach and Rosen 1991, Kok and Salom 2002), but we are aware of only one case where a field insectary was used to rear a biological control agent of a forest pest: *Didea fasciata* (Diptera: Syrphidae) a predator of the fir bark aphid, *Cinara piceae* (Hemiptera: Lachnidae) in Europe (Stary 1976). *Laricobius nigrinus* is now being propagated at a field insectary and re-distributed to HWA-infested forests. The predator easily established in its new environment and several factors incorporated into the insectary design favored multiplication, collection, and limited dispersal. These were: an abundance of HWA; very fecund HWA due to nitrogen fertilization (McClure 1991b); hundreds of open grown hemlock trees with little vegetative competition; isolation from natural hemlock forests; easy access (low tree height and vehicle accessible); and healthy trees with full crowns and abundant new growth. After modest initial costs and labor, only weeding, fertilizing, and pruning are now required. Populations of *L. nigrinus* should be self-sustaining and provide a steady supply of predators. Field-reared predators are also likely more fecund and healthy than lab-reared beetles because of their large size. Zilahi-Balogh (2001) showed that there is a significant positive relationship between female size and fecundity. Beetles reared under field conditions are better acclimated to the environment than lab-beetles.

*Laricobius* spp. and other predators like *H. axyridis* at the site may play a role in limiting the number of heavily HWA-infested trees. Predators are easily observed with the naked eye on these trees and evidence of larval feeding is widespread in the spring. The abundance of *L. rubidus* was unexpected and they most likely migrated from the white pines infested with PBA. HWA is a suitable host for *L. rubidus* (Zilahi-Balogh et al. 2005) and its rapid increase in numbers from year to year highlights its numerical response to increasing HWA abundance. Similarly, *L. nigrinus* is increasing its population size rapidly and could surpass *L. rubidus* in the next few years, even though only 258 beetles were introduced. Attempts at mating between *L. nigrinus* and *L. rubidus* was observed and if they can produce fertile hybrids, it would be important to determine any positive or negative impacts for biological control of HWA or PBA. Further investigation in this area is needed. In spite of increasing HWA populations, the majority of the trees appear healthy, which may be due to their young age and good growing conditions.

Successful field insectaries could substantially enhance biological control of HWA by increasing the supply of vigorous beetles for release. Based on experience we recommend that every state have at least one field insectary near the expanding HWA front to service their needs. The low cost makes this feasible. Alternatives to buying trees and planting them include utilization of heavily infested hedges, nurseries, and open grown trees on campuses, cemeteries, and city parks that have not been nor are likely to be treated with insecticides.

### **Phenological synchrony**

Nearly twice as many *L. rubidus* adults were collected than *L. nigrinus* and both species were active in the fall, winter, and spring on hemlock. It is possible that *L. rubidus* dislodges more readily than *L. nigrinus* during beat sheet sampling and the difference in relative population size may be less. Clark and Brown (1960) observed that *L. rubidus* readily drops from trees when disturbed, but no comparisons have been made with *L. nigrinus*. On two sampling dates with windy conditions (29 Dec and 10 Feb) *L. nigrinus* collections were greatly reduced and *L. rubidus* were not, which suggests that *L. rubidus* willingly drops to escape adversaries or does not secure itself as firmly to a shoot, HWA, needle, or other structure.

The phenology of adult *L. nigrinus* was slightly different at Kentland Farm compared with Victoria, B.C. (Zilahi-Balogh et al. 2003c). The first adult *L. nigrinus* captured in the fall was 2 wk later than in Victoria and adult presence ended earlier. *Laricobius nigrinus* and *L. rubidus* were collected on a day when the mean daily temperature was below 0 °C (10 Feb). However, at the sampling time (14:30 - 16:10) the temperature was 7.5 °C. On the only day when predators were not collected (15 Dec), the temperature was -0.4 °C at the time of sampling (14:15 - 15:19) and the weather was adverse. These data agree with the observation that *L. nigrinus* is active on branches when ambient temperature is above 0 °C (Zilahi-Balogh et al. 2003c). January 2006 was unusually warm and predator activity increased accordingly. *Laricobius* spp. oviposition began one month later than in Victoria, and ended at approximately the same time. Larvae were first observed at the same time but were present for one month longer in Victoria. Synchrony with the phenology of HWA is important because *Laricobius* spp. adults need developing sistentes and larvae need eggs to develop (Zilahi-Balogh et al. 2003a). *Laricobius nigrinus* density was lower at the field insectary than in Victoria and compared with *L. rubidus*, since it was newly established. It is not unusual for the low levels of a newly established species to miss detection until they reach a threshold, which may explain some of the phenological differences.

In spite of the higher adult *L. rubidus* than *L. nigrinus* populations on the trees, the amounts of larvae of each species that we reared out in the lab were nearly equal. A possible explanation for this discrepancy is that *L. rubidus* oviposition began later than *L. nigrinus* and was not as well synchronized with HWA. In Fig. 3.5, it appears that larval abundance was bimodal with one mode from 11 Mar to 10 Apr and the second from 10 Apr to 8 May, perhaps corresponding to *L. nigrinus* and *L. rubidus*, respectively. As such, on 28 Apr when the larvae were collected for rearing, *L. nigrinus* larval abundance was declining as *L. rubidus* was increasing. It is likely that the second oviposition period was *L. rubidus*, which is in line with the oviposition period of the predator on PBA (Clark and Brown 1960). If this is the case, the more synchronized oviposition of *L. nigrinus* produces a greater larval impact on HWA in spite of the lower adult densities than *L. rubidus*.

The presence of active *L. rubidus* adults feeding on HWA in the winter was unexpected because it was described as having a hibernal diapause in addition to an aestival diapause (Montgomery and Lyon 1996, Zilahi-Balogh et al. 2005). The reported primary host of the predator is PBA and it has a hibernal diapause as well (Raske and Hodson 1964). However, Clark and Brown (1960) reported a *L. rubidus* specimen from Toronto, Ontario that was collected in December. Several possible explanations exist for it to remain active on HWA and not enter a hibernal diapause in Virginia. The species enters a quiescent dormancy in direct response to adverse conditions and not diapause dormancy. As winter in the southern Appalachians is relatively mild, it may not adversely affect *L. rubidus* activity most of the time. HWA is a viable, abundant, and apparent, food source in the winter. PBA typically is found in concealed and protected places at low density. Competition between *L. rubidus* and the diverse natural enemy complex that feeds on PBA in the spring - fall has made a switch to HWA a new and advantageous strategy to reduce niche overlap. What we identified as *L. rubidus* could in fact be a *L. nigrinus* and *L. rubidus* hybrid. Although this is highly speculative, the low level of sequence divergence between the two species (N. Havill, Yale University, unpub. data) suggests they may be subspecies.

The phenology of *A. tsugae* at Kentland Farm differed slightly compared with previous descriptions due to regional climate differences. HWA broke aestival diapause approximately 1 month later than HWA in Victoria, B.C. (Zilahi-Balogh et al. 2003c), Connecticut (McClure 1987b), and elsewhere in Virginia (Gray and Salom 1996). It is likely that the sistentes broke aestivation earlier, but were below measurable levels. Either larger clippings or weekly sampling would have more accurately determined when aestivation ended. Sistentes began laying eggs approximately 1 month later than in Victoria, but at similar times in Connecticut, and Virginia, and sistentes stopped laying eggs 1 month earlier than in Victoria and Connecticut, but similar to Virginia. Progradientes adults and ovisacs appeared approximately 1 or 2 wk earlier than in Victoria, 1 wk earlier than Connecticut, and identical to Virginia sites.

Phenological synchrony is important for predator growth and reproduction. With synchrony between predator and prey, establishment and impact are possible. If *L. nigrinus* adults emerge before HWA breaks aestivation they would starve and if they emerge too late they would miss

HWA as soon as they are suitable as prey. The appearances of adult *L. nigrinus* and *L. rubidus* predators coincided with the resumption of HWA sistentes development following aestivation and were active through the presence of sistentes ovisacs as in Victoria, B.C. (Zilahi-Balogh et al. 2003c). Although we did not monitor emergence from the soil, *Laricobius* spp. are known to pupate and aestivate there, so we assume that the predators started emerging just before the first collection date (Franz 1958b). The decline in soil temperatures before the beetles appeared on the trees was in line with the temperature changes used to break aestivation in the lab (Lamb et al. 2007). *Laricobius* spp. eggs and larvae presence overlapped with HWA oviposition very well, as in Victoria, B.C. (Zilahi-Balogh et al. 2003c). The shorter duration of presence of sistentes at Kentland Farm than at Victoria corresponded with the period of egg laying by *Laricobius* spp. and subsequent larval development. Virginia has a continental climate and is warmer in the spring than the PNW maritime climate. This likely shortened the sistentes oviposition in Virginia relative to the PNW.

Biological control of the balsam woolly adelgid (BWA), *Adelges piceae* Rosenhauer with *L. erichsonii* was not successful perhaps because this predator has a relatively wide host range and is imperfectly synchronized with BWA (Franz 1958b). This suggests that BWA is not the primary host of *L. erichsonii*. *Laricobius nigrinus* feeds primarily on HWA and is synchronized with its prey, which is quite different from that of *L. erichsonii*. By successfully adapting to a different climate and tree species in Virginia, *L. nigrinus* shows potential for biological control of HWA. The primary host of *L. rubidus* is not HWA and perhaps this explains why it has not been effective at suppressing HWA (Wallace and Hain 2000). *Laricobius rubidus* has probably recently adapted to HWA phenology in the eastern US and may become an important natural enemy in the future. Other native and previously released predators were at low densities, which is consistent with previous descriptions (Montgomery and Lyon 1996, Wallace and Hain 2000). There was moderate overlap of generalists (*i.e.* *H. axyridis*) with immature *Laricobius* spp. in the spring that may reduce their growth and survival but this has not been observed in other studies (Flowers et al. 2005, 2006).

### Cage exclusion impact study

Adult *L. nigrinus* and *L. rubidus* preyed on HWA from fall through spring and their offspring preyed on eggs in sistentes ovisacs in the spring. Our results are conservative, because the cages were removed approximately 3 wk before *Laricobius* spp. larval predation on HWA ovisacs ended. There was a significant impact of these predators on HWA sistentes in this study probably because of the *L. nigrinus* release 2 yr earlier on an early infestation of HWA. This was not the case in the cage exclusion study of Wallace and Hain (2000) where they studied progredientes in native forests. The highly significant numerical response relationship between *Laricobius* spp. and prey indicates that the predators respond strongly to HWA ovisac density by increasing their density.

There was no significant difference in initial HWA density among treatments at the time the cages were erected, but absolute densities in descending order were, closed cage > open cage > no cage (see Table 3.2). It is possible that the trend in initial HWA density caused the significant differences in ovisac density among treatments recorded at the end of the experiment. However, we marked branches with equivalent HWA densities by tree at the beginning of the experiment and allocated the treatments at random. We believe the trend resulted from HWA disappearing from the open and no cage treatments due to predation, similar to a cage experiment with *Pineus pini* (Gmelin) (Hemiptera: Adelgidae) (Mailu et al. 1980). We could not measure this because we examined HWA remaining on the branch at the end of the experiment. We assumed that HWA remain firmly attached to the branch with their stylet and *Laricobius* spp. leave an exoskeleton after feeding. Franz (1958b) assessed predation by examining *L. erichsonii* gut contents, and determined that they swallowed whole adelgids in addition to chewing and sucking out their contents and this may cause HWA to disappear. This should be investigated in *L. rubidus* and *L. nigrinus*.

Unfortunately, the high native *L. rubidus* populations confound the true impact of *L. nigrinus*. The abundance of *L. rubidus*, due to the PBA infested white pine plantation, may not be an unusual occurrence at this site as newly HWA-infested hemlock forests usually have PBA infested white pines in the area that support *L. rubidus* populations (Montgomery and Lyon 1996, Wallace and Hain 2000). Their abundance on HWA infested trees is likely increasing as they utilize and adapt to this new abundant host (also see Chapter 2). This study shows that

*Laricobius* spp. play a dominant role in affecting HWA in the fall, winter, and spring. Syrphidae, *H. axyridis*, and other predators became active near the end of the experiment and their influence on the experiment is minor compared with *Laricobius* spp. They may have an important synergistic impact on the progredientes generations in the spring and summer (Flowers et al. 2006).

Biological control of BWA with *L. erichsonii* may not have been successful because the predator does not have an ability to suppress heavy BWA infestations as described in Europe and North America (Franz 1958b, Buffam 1962). Similarly, *L. nigrinus* has the potential to control HWA, but pest suppression will not be rapid, spectacular, or highly effective when released in heavily infested forests or damaged forests after an outbreak. Each beetle's consumption rate of 3-6 HWA per day (Lamb et al. 2005b) would not be sufficient to suppress a rapidly growing HWA infestation. Evans and Gregoire (2007b) described a heavy infestation very eloquently, "populations can grow so large that there are nearly as many HWA as needles on a tree." Tree health declines after outbreaks and trees become moribund very rapidly, especially in the southern US, which will probably not give the predator adequate time to build up populations that can control HWA. In this study on average, N2 - adult HWA mortality was only 15% greater when exposed to adult *L. nigrinus* and *L. rubidus* than when protected from them. A total of 35% of HWA ovisacs were disturbed in the no cage treatment, which represents *Laricobius* spp. larvae feeding on eggs and abiotic factors. Because fewer ovisacs were disturbed in the open cage (12%), crown abrasion during heavy winds, rain, snow, ice, birds, or unknown factors in the no cage treatment could explain some of the disturbed ovisacs. The open cage treatment could have also influenced normal predator behavior and oviposition. The cage effect on predator behavior is probable because many disturbed ovisacs in the no cage treatment had circumstantial evidence of predation. *Laricobius* spp. suck out the contents of eggs and leave a characteristic spoon shaped remnant that differs subtly from the chorion of a hatched egg (Franz 1958b). It is common for late instars to not consume all eggs in an ovisac but to crawl along a shoot and eat from fresh ovisacs with large clusters of eggs. This results in loosened masses of ovisacs following predator feeding without any specific signs of their presence.

Higher mortality rates will be required to effectively suppress heavy HWA populations and this may be possible in time. However, predators could prevent HWA outbreaks if released when pest densities are low. The biological control approach we advocate for *L. nigrinus* is through small releases during the early stages of HWA establishment and population growth at the stand- and landscape-level. We envision predator releases on several atypically heavily infested trees in an overall lightly infested stand to aid establishment and subsequent population growth, as described here. As such, predator : prey ratios are not extremely low and the predator has time to build up the numbers to suppress HWA before serious tree decline and death.

**Table 3.1.** Overlap values for the temporal occurrence of the predators *Laricobius nigrinus*, *Laricobius rubidus*, and their prey the hemlock woolly adelgid at Kentland Farm, Blacksburg, Virginia.

Occurrence of	Aestivating sistentes	Sistentes N2- Adult	Sistentes ovisacs	Progredi- entes N1- adult	Progre- dientes ovisacs
<i>L. nigrinus</i> adults	0.00*	0.71	0.46	0.11	0.00
<i>L. rubidus</i> adults	0.00	0.75	0.47	0.26	0.00
<i>Laricobius</i> eggs	0.00	0.14	0.88	0.23	0.00
<i>Laricobius</i> larvae	0.00	0.05	0.55	0.46	0.00

\* Pairwise comparisons were calculated based on data collected from Sep 2005-Aug 2006.

Values range from 0 to 1, with 1 being a perfect match.

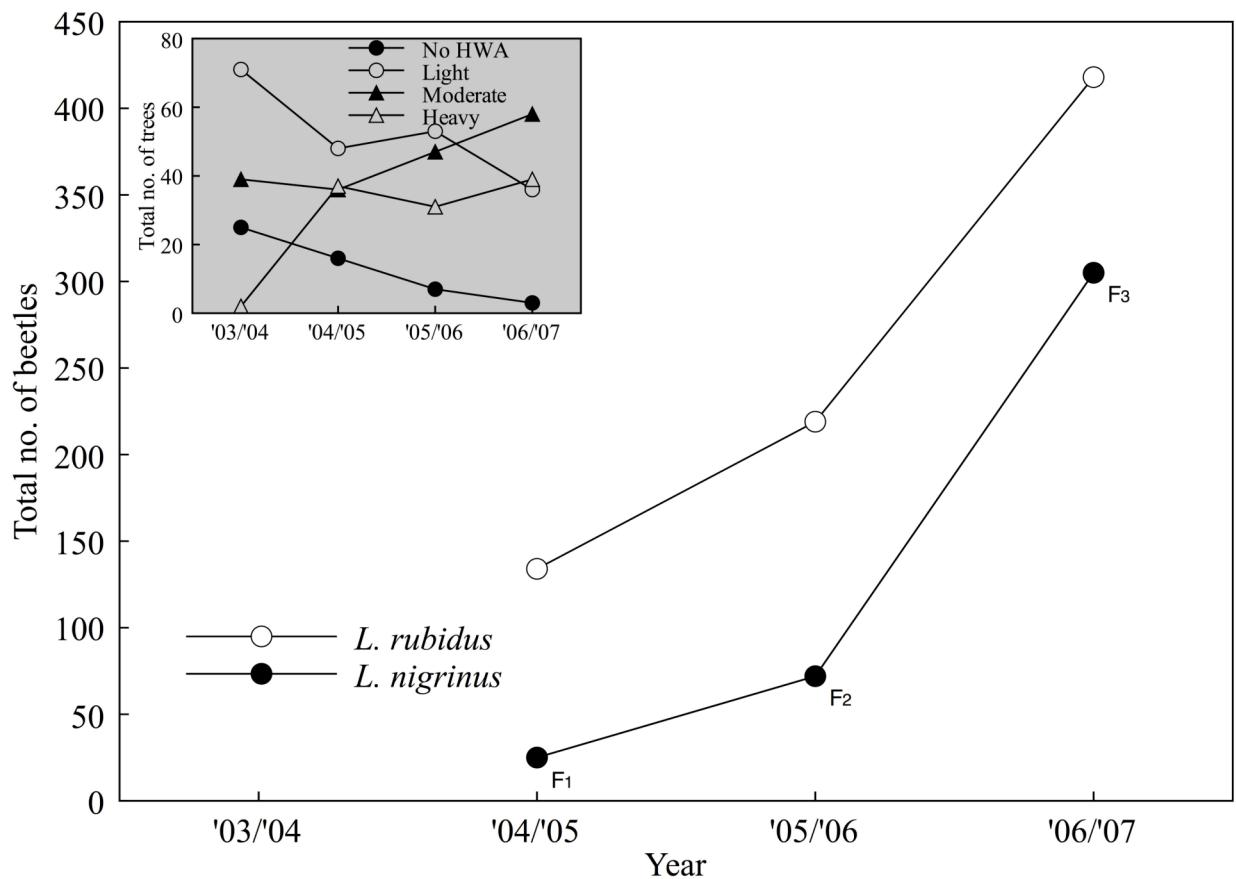
**Table 3.2.** Initial hemlock woolly adelgid sistentes density, HWA survival, predator density, and ovisac disturbance factors in a cage exclusion impact study at Kentland Farm, Blacksburg, Virginia 2 yr after release of the predator *Laricobius nigrinus*.

Parameter	Exclusion treatment		
	Closed cage*	Open cage	No cage
Initial HWA density/cm twig	2.1 ± 0.6 a <sup>1</sup>	1.3 ± 0.2 a	1.0 ± 0.2 a
% HWA survival	71.2 ± 3.2 a	58.8 ± 2.6 b	56.2 ± 3.9 b
No. <i>Laricobius</i> spp./cm twig <sup>2</sup>	0	0.03 ± 0.01 a	0.02 ± 0.01 a
No. Syrphidae spp./cm twig <sup>2</sup>	0	0.001 ± 0.0008 a	0.002 ± 0.0008 a
% of disturbed ovisacs due to:			
<i>Laricobius</i> spp.	0	19.8 ± 8.1 a	19.5 ± 4.2 a
Syrphidae	0	0.5 ± 0.5 a	0.8 ± 0.6 a
Unidentified	100	72.0 ± 9.8 a	79.8 ± 4.1 a

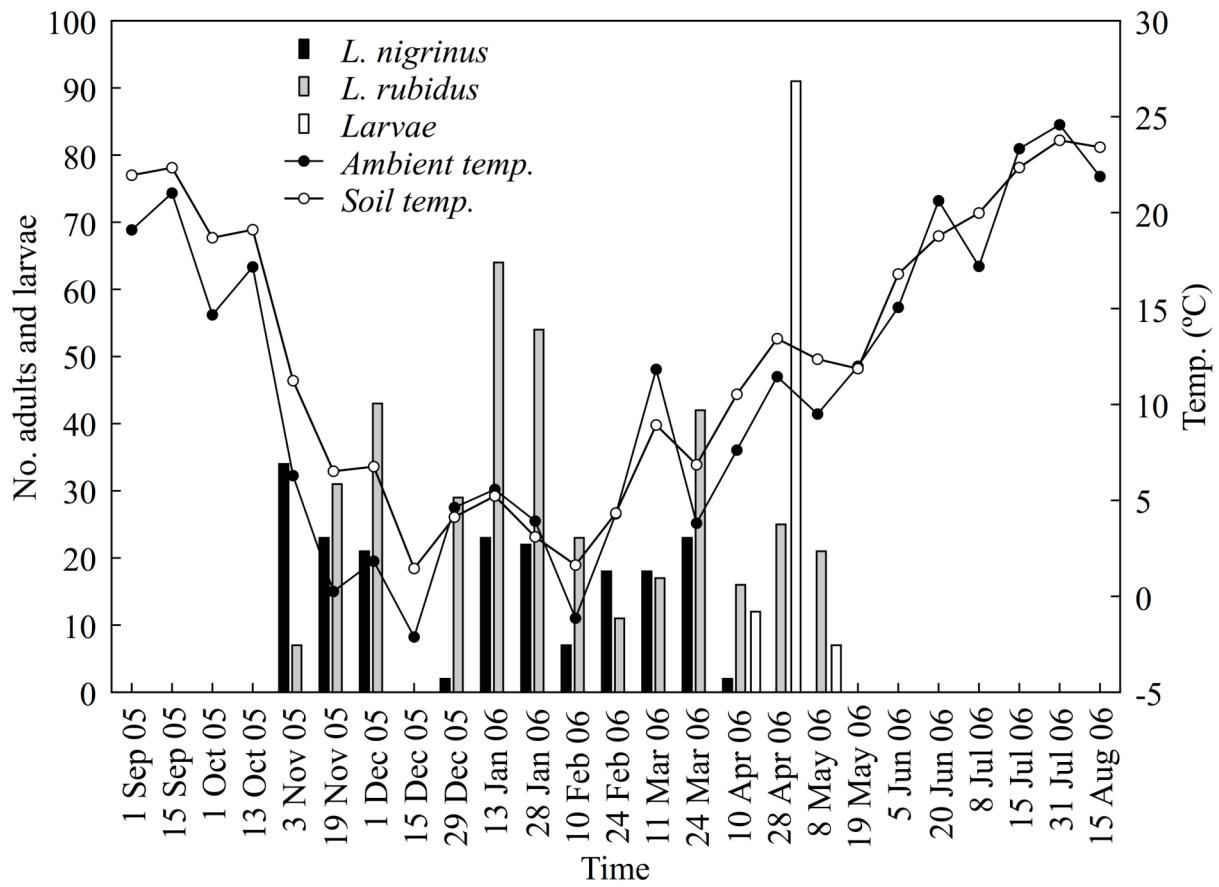
\* Cages were set up in Nov 2005 and dismantled in Apr 2006.

<sup>1</sup> Averages are the mean ± standard error ( $n = 14$ ). Treatments with the same letter within a row are not significantly different by Tukey's HSD after a two-way ANOVA or paired t-test ( $\alpha = 0.05$ ).

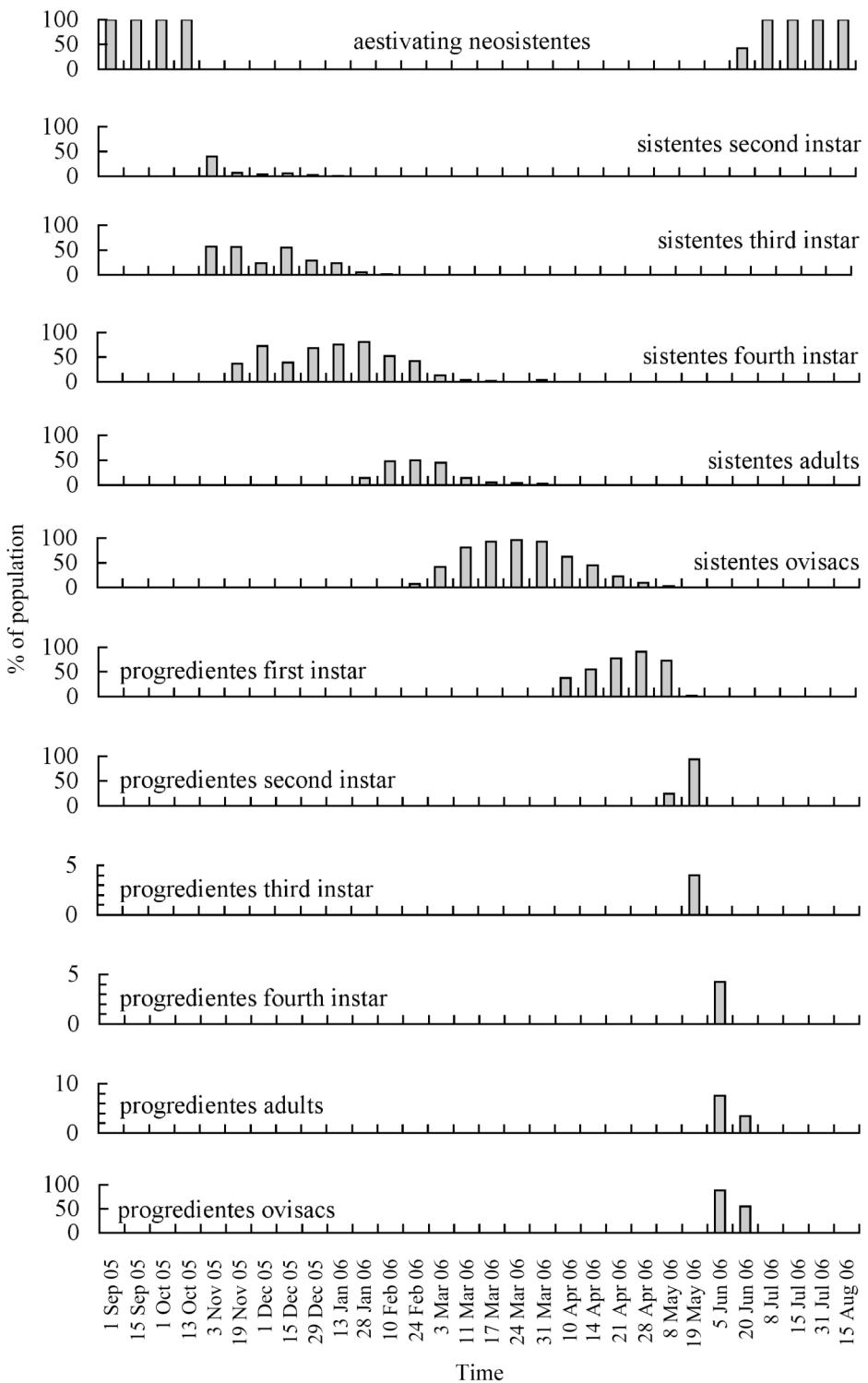
<sup>2</sup> Eggs & larvae only.



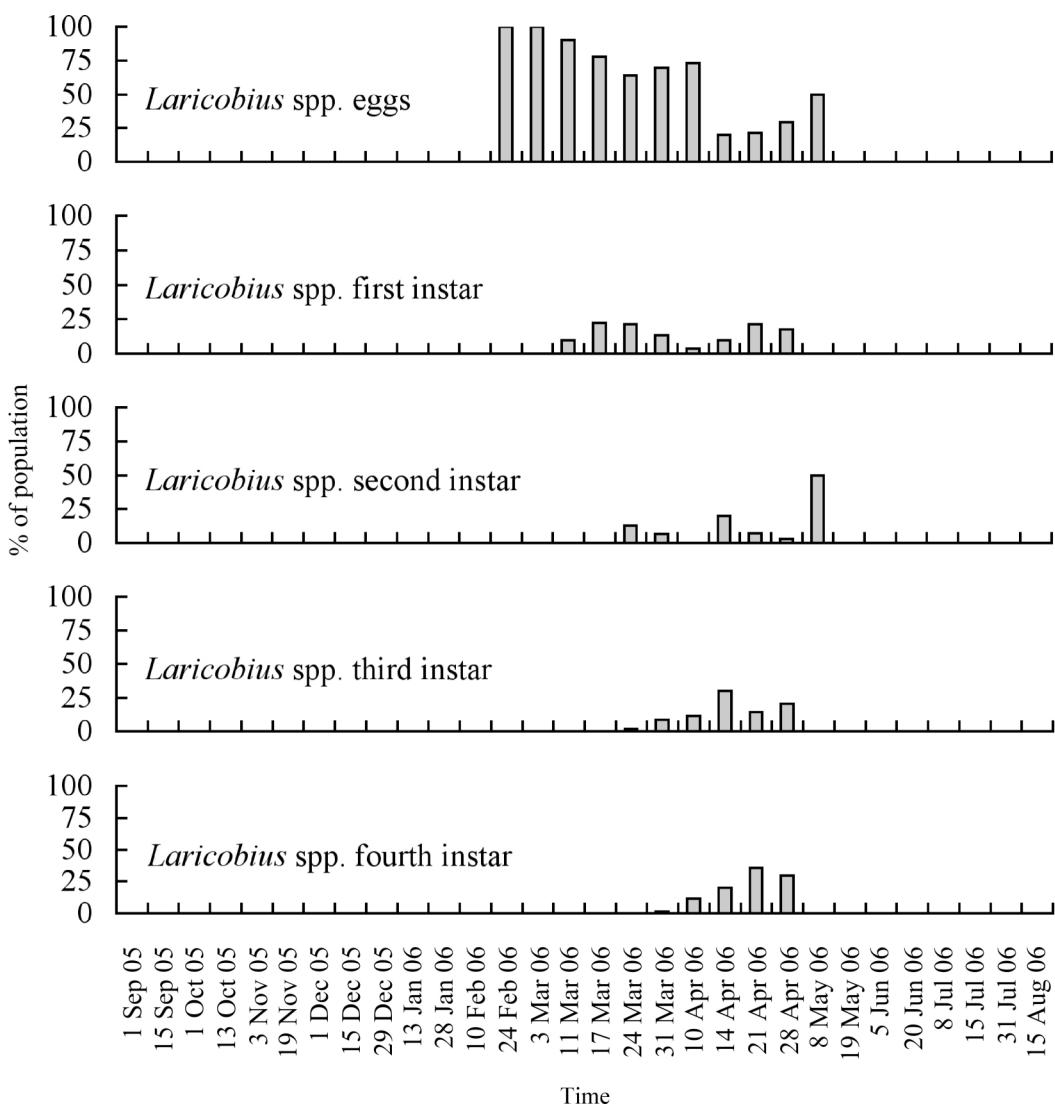
**Fig. 3.1.** Abundance of *Laricobius nigrinus* and *Laricobius rubidus* adults over time and hemlock woolly adelgid infestation levels on eastern hemlock trees (inset) at a field insectary in Virginia. In Nov 2003, 258 *L. nigrinus* adults were released at the site.



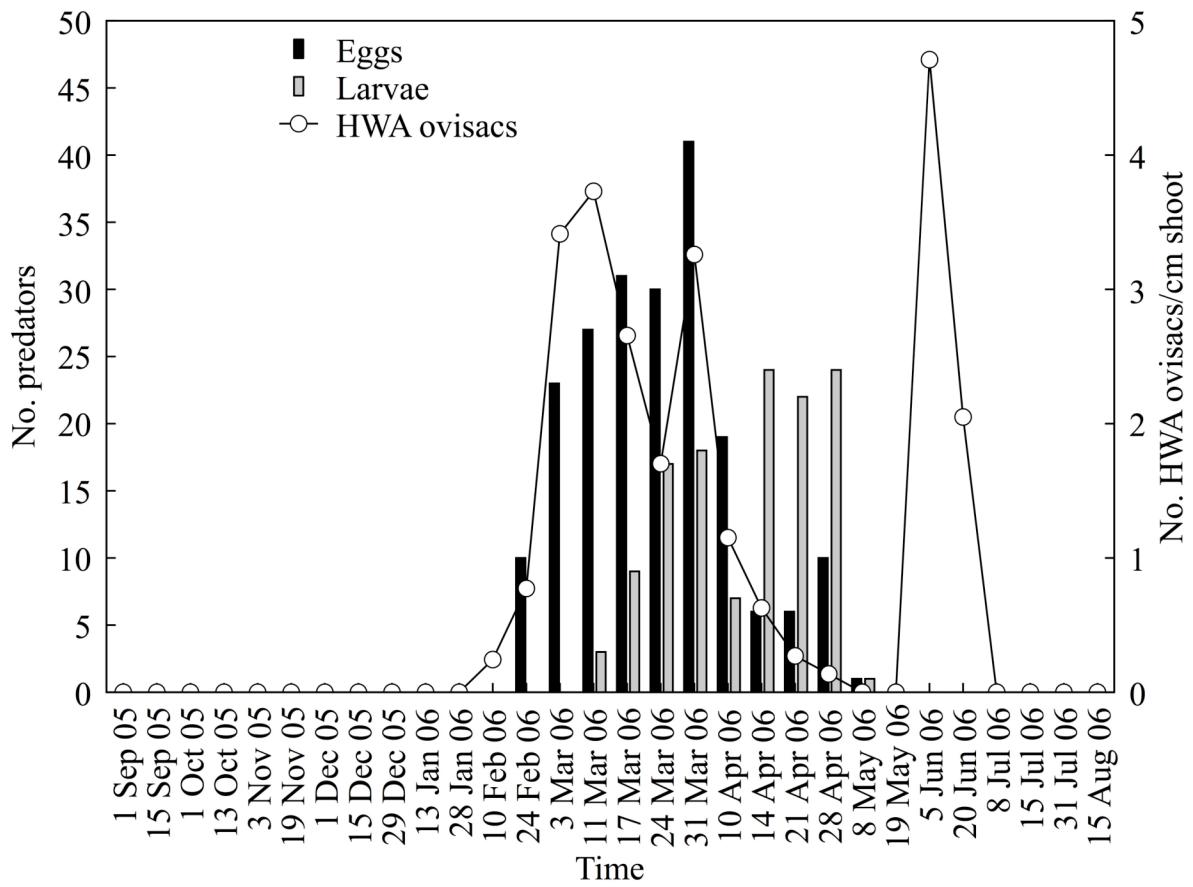
**Fig. 3.2.** Total numbers per sample date of *Laricobius nigrinus* and *Laricobius rubidus* adults and late instars (both species pooled) collected with beat sheets from eastern hemlock trees at a field insectary in Virginia. Mean daily ambient and soil temperatures were obtained from a weather station 1.4 km from the site.



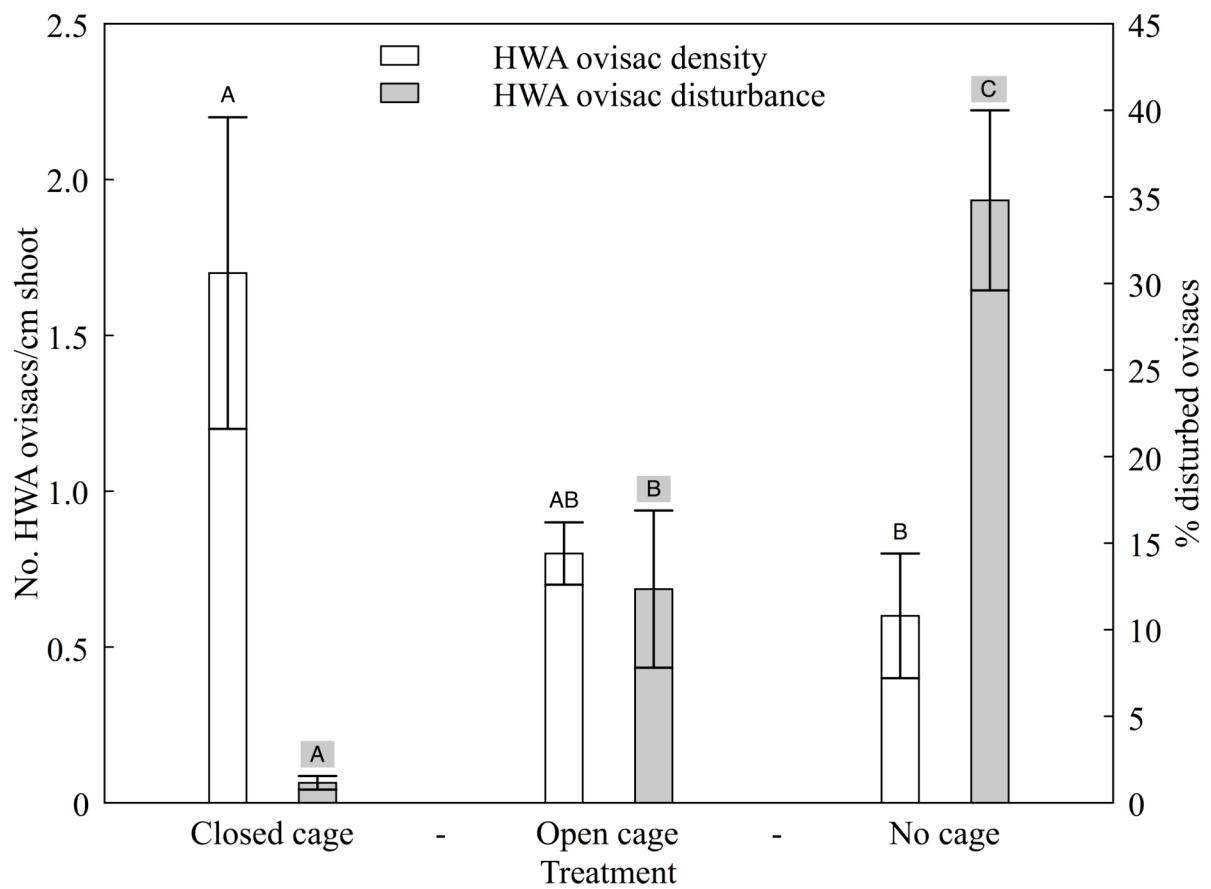
**Fig. 3.3.** Percentages of the hemlock woolly adelgid population in each life-stage per sample date at a field insectary in Virginia.



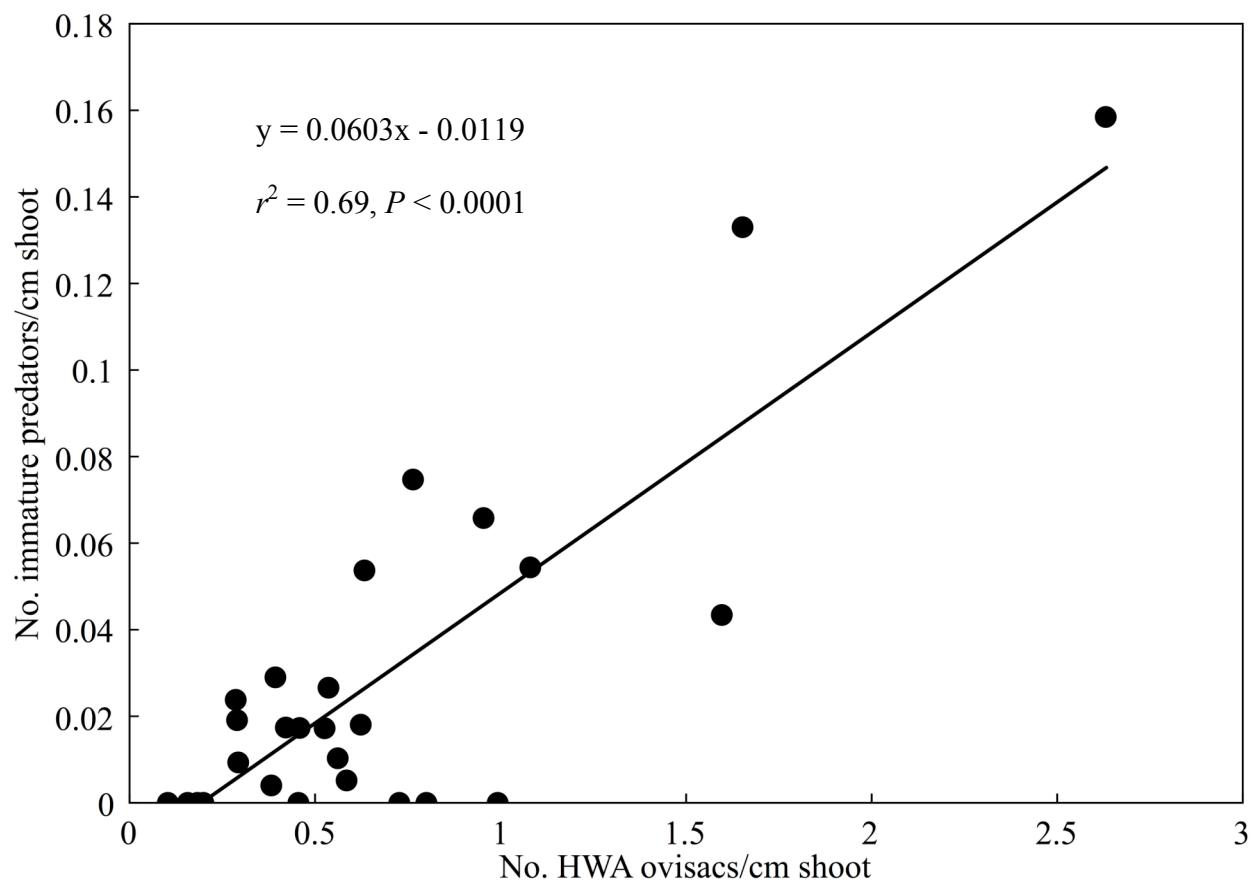
**Fig. 3.4.** Percentages of immature *Laricobius nigrinus* and *Laricobius rubidus* (pooled) populations in each life-stage per sample date at a field insectary in Virginia.



**Fig. 3.5.** Total numbers of *Laricobius nigrinus* and *Laricobius rubidus* eggs and larvae (pooled L1-L4 instars) and hemlock woolly adelgid sistentes and progredientes ovisac density per sample date at a field insectary in Virginia.



**Fig. 3.6.** *Laricobius nigrinus* and *Laricobius rubidus* impact on hemlock woolly adelgid ovisac density and ovisac disturbance by larval feeding or unspecified disturbance (mean  $\pm$  sem) in a cage exclusion impact study at a field insectary in Virginia.



**Fig. 3.7.** Relationship between immature *Laricobius nigrinus* and *Laricobius rubidus* density (pooled) and hemlock woolly adelgid ovisac density at a field insectary in Virginia.

## **Chapter 4 Comparison of *Laricobius nigrinus* (Coleoptera: Derodontidae) adult and larval sampling methods in native and introduced habitats**

### **Abstract**

We tested the relative efficacy of sampling for the hemlock woolly adelgid (HWA) *Adelges tsugae* Annand natural enemy *Laricobius nigrinus* Fender with beat sheets for adults or branch clipping for immatures in Seattle, Washington (native habitat) and the southern Appalachians (introduced habitat). In the Appalachians, beatsheet sampling resulted in false negatives as larvae were collected by branch clipping. In Seattle, both methods detected *L. nigrinus* because the predator was common. However, it was recovered in significantly greater numbers by branch clipping. Predator : prey ratios were high at heavily infested sites in Seattle and low in the eastern US, where it has been released recently. Adult diel variation in beat sheet samples and the frequency of *L. nigrinus* immatures in small and large ovisacs were determined to refine each sampling method. In winter, beatsheets recovered more adults in the afternoon when temperatures were high and in the spring, adults were collected at all times of day. Immature predators were more frequently found in large HWA ovisacs (2 - 3 mm) than small ovisacs (1 - 2 mm). Results suggest that for low predator populations, branch clipping should be used in the eastern US. If beat sheet sampling is used in the fall or spring, it should be followed by branch clipping in the spring if no adults are collected. For high predator populations, we recommend use of beatsheets.

**Keywords:** Biological control, post-release monitoring, beat sheet, branch clipping

## 4.1 Introduction

*Laricobius nigrinus* Fender (Coleoptera: Derodontidae) has been introduced from the Pacific Northwest to the eastern US since 2003 for classical biological control (Cheah et al. 2004, Lamb et al. 2006) of the highly invasive hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) (McClure 1991a, Orwig and Foster 1998). For forest health specialists attempting to determine establishment, impact, and overall success of the project at an operational scale, adequate sampling techniques are needed, as done for spotted knapweed biological control (Clark et al. 2001a).

*Laricobius nigrinus* is univoltine and adults feed on HWA on hemlock shoots during fall - spring (Zilahi-Balogh et al. 2003c). Typically, one egg is laid within the “woolly” HWA ovisacs in the early spring and larvae develop through four instars within the wool (Zilahi-Balogh et al. 2003a). After larvae complete feeding on HWA eggs, they drop to the soil, where they pupate and aestivate for several months before emergence and movement back to hemlock. Two common techniques used to monitor the predator include beat sheet sampling for adults during fall - spring and branch clipping for immatures in the spring (Zilahi-Balogh et al. 2003c). These two tree canopy sampling techniques are being used because other methods (Ozanne 2005) are not appropriate for biological control, cost prohibitive, dangerous, or inapplicable given the current state of knowledge about the predator. For example, chemical fogging may kill the natural enemies. Bucket trucks, tree-climbers, or shot-guns for sampling the canopy are restricted to where they are used, how many trees can be sampled, and special training is required. Active traps are not available because a pheromone has yet to be discovered (Broeckling and Salom 2002, 2003b). Passive malaise or sticky traps have a low probability of capturing recently released and rare biological control agents. Soil emergence traps were unsuccessful at recovery of F1 adults (Lamb et al. 2006).

Branch clipping and beat sheets each have several advantages and disadvantages. Branch clipping targets immatures, which are naturally more abundant than adults are in the spring. Eggs remain firmly glued to HWA ovisacs (Franz 1958b) and larvae are embedded in HWA wool regardless of the weather, time of day, and disturbances such as human activity, wind, or

rain. Branches can be clipped up to ~10 m height with a pole-pruner, but mature eastern hemlocks are some of the tallest trees in the eastern US. Dominant trees are typically 30 m tall (Burns and Honkala. 1990). The major disadvantage of branch clipping is that dissection of HWA ovisacs or Berlese funnels (Lamb et al. 2005a) are required to count the immatures. Furthermore, immatures of the native congener *Laricobius rubidus* Leconte appear to be morphologically indistinguishable from *L. nigrinus* and must be reared to adults to confirm the species or identified by PCR-RFLP [N. Havill, D. Mausel (Virginia Tech), A. Caccone, Yale Univ., unpub. report]. Advantages of beat sheets are that they are simple, adults are easily identified in the field, and the sampling season (fall - spring) is long. The major fault of beat sheets is that they are limited to use from the ground without the aid of tree-climbers and results from their use is sensitive to weather.

Beat sheet sampling from the ground has been relied on heavily for another predator of HWA, *Sasajiscymnus* (=*Pseudoscymnus*) *tsugae* Sasaji & McClure (Coleoptera: Coccinellidae), with often disappointing results (Casagrande et al. 2002, Asaro et al. 2005, Montgomery et al. 2007) while beating the upper canopy and branch clipping has been more successful (Cheah and McClure 2000, Cheah et al. 2004). We tested the relative efficacy of sampling for *L. nigrinus* adults with beat sheets from the ground or immatures by branch clipping in Seattle, Washington (native habitat) and the southern Appalachians (introduced habitat) to assure that the sampling techniques are adequate. Diel variation in *L. nigrinus* adult collections with beat sheets and the frequency of *L. nigrinus* immatures in small and large ovisacs were determined. A greater probability of recovering adults at a certain time of day or immatures in ovisacs of different size could further improve sampling efficacy. The goal of this study is to provide field personnel with an alternative sampling procedure to beat sheets and determine the frequency of *L. nigrinus* occurrence and predator : prey ratios in its native and introduced habitat.

## 4.2 Materials and methods

Beat sheet and branch clipping methods were compared for determining predator presence/absence at five southern Appalachian sites where *L. nigrinus* was rare due to its recent introduction (2003/2004), two natural forest sites with low HWA densities and two urban forest

sites with high HWA densities in Seattle, WA. Ten eastern hemlock, *Tsuga canadensis* L. Carrière, trees that predators were released upon were sampled in the Appalachians. At the VA-Big stony site, only seven trees were sampled. In the Seattle natural forest sites (Discovery and Fauntleroy Park), each had 10 lightly infested western hemlock, *Tsuga heterophylla* (Raf.) Sargent, trees randomly selected with the restriction that all trees have low crowns to enable beat sheet sampling. At two urban forests [Washington Park Arboretum (WPA) and Woodland Park], the sampling methods were compared for determining predator abundance. Twenty-five heavily infested trees were randomly selected at each site. At WPA, the 25 replications included four mature and dominant eastern hemlock trees that were planted in the 1950's.

### **Comparing beat sheet and branch clipping sampling**

In spring 2005, a 71 cm<sup>2</sup> canvas beat sheet (BioQuip, Rancho Dominguez, CA) was used to collect adult *L. nigrinus* from a HWA infested branch at each of the four cardinal points of the lower crown. If branches were missing from a cardinal point, then the next clockwise branch was chosen. Sampling was conducted from 10:00 - 16:00 during ambient temperatures of 6 - 22 °C. The most infested sections of each branch were tapped with a stick 10 times, adult *Laricobius* spp. collected with an aspirator, identified in the field, and the total collected was recorded per tree. *Laricobius nigrinus* were not returned to the tree but shipped to Virginia Tech for insectary propagation and release in the eastern US. Reference specimens were confirmed with the use of keys (Hatch 1962) and deposited into the Virginia Tech Department of Entomology Insect Collection, Blacksburg, VA.

Immediately after the beat sheet sampling, a pole-pruner was used to clip four terminal branches (30 cm length) from the mid/upper canopy of the release trees in the Appalachians. In Seattle, branches were clipped from the same lower crown branches that were sampled with the beat sheet and placed in a 3.8 L plastic bag. Branch beating does not affect results from branch clipping because *Laricobius* spp. eggs are glued to adelgid flocculence (Franz 1958b) and larvae are not dislodged unless they are mobile late instars, which were not present at the time we sampled. HWA sistentes oviposition of progredientes had peaked at WPA and Woodland Park and crawler eclosion had just begun. The dissection process began with the removal of 10 of the largest ovisacs from each branch to obtain a total of 40 ovisacs per tree. A strip of electrical tape

was placed sticky side up on a piece of Styrofoam with pins, and ovisacs were gently teased off the hemlock branch with a dissecting tool and placed on the tape. Ethanol (70%) was dripped on the ovisac to make the wool transparent and then teased apart under a dissecting microscope to count the number of immature *L. nigrinus*. *Laricobius* spp. exuvia and chorions were frequently found in the samples but were not considered in the analyses although they are useful for determining presence or absence. The percentage of ovisacs with immatures and the predator : prey ratios were calculated on a site and individual tree basis. The amount of time for a two-person crew to beat sheet and branch clip sample in Seattle was recorded. For beat sheets, the time it took to beat, search the canvas, and count beetles was recorded to the nearest minute. The time required for branch clipping was the sum of clipping four branches in the field plus dissecting ovisacs in the laboratory.

### **Sampling for diel activity**

At WPA, 30 HWA infested trees were labeled and five were randomly assigned a time-period to be sampled (2:00, 6:00, 10:00, 14:00, 18:00, or 22:00 h). Eight branches were beat per tree and a camp light was used at night to locate branches, aspirate beetles, identify, and record the number collected. The predators were returned to the trees after sampling. Ambient temperature, relative humidity, and wind speed at the time of sampling were acquired from the University of Washington, Department of Atmospheric Sciences weather station 1.3 km from the site. The study was conducted on 10/11 Dec 2005 and repeated on the same trees but re-randomized on 19/20 Mar 2006.

### **Relationship of ovisac size and presence of *L. nigrinus***

To investigate the frequency of *L. nigrinus* immature stages in HWA ovisacs of different size, two 30 cm branches were collected from three heavily infested western hemlock trees at Woodland Park that had high densities of HWA. Ovisac diameter was measured on the branch with a dissecting scope and eyepiece micrometer and categorized as small or large (1 - 2 or >2 mm). Each ovisac was dissected and the presence/absence of *L. nigrinus* immatures in 370 ovisacs of 1 - 2 mm and 686 ovisacs of >2 mm were recorded.

## **Statistical analysis**

We compared the number of trees with predators and predator abundance by ANOVA and Tukey's multiple comparison tests (Appalachians) or t-tests (Discovery and Fauntleroy; WPA and Woodland). We compared the percentage of ovisacs with predators among all of the sites by ANOVA. The effect of the two sampling methods at Discovery and Fauntleroy Park were analyzed by a chi-square contingency table with Yate's correction for continuity. Heterogeneity testing indicated that the sites were justifiably pooled. Paired t-tests were used to compare the abundance of *L. nigrinus* between the two methods at WPA and Woodland Park in Seattle, separately. The mean  $\pm$  sem amount of time it took for a two person crew to sample with each method was calculated from the 70 trees sampled in Seattle. The diel pattern of adult abundance from beat sheet sampling was analyzed by a chi-square goodness of fit test. Simple correlation coefficients were calculated between the weather conditions and adult abundance during the diel sampling. The effect of ovisac diameter on the occurrence of *L. nigrinus* immatures was analyzed by chi-square contingency tables and Yate's correction for continuity.

## **4.3 Results**

### **Comparing beat sheet and branch clipping sampling**

The effect of the two sampling methods on determining the presence/absence of *L. nigrinus* on the trees at the Appalachian sites were not statistically compared because the beat sheet results were constant at zero predators caught. Branch clipping showed the presence of *Laricobius* spp. at 4 of 5 Appalachian sites (Table 4.1). There were more trees with predator presence at VA-North Fork than at the other sites and VA-Big Stony was intermediate ( $\chi^2 = 13.4$ , df = 4,  $P = 0.009$ ), but the abundance of predators per tree was not different ( $F = 2.2$ , df = 4,42,  $P = 0.08$ ). At the Washington sites, both methods of sampling showed presence. At Discovery and Fauntleroy parks, there was no difference in predator detection between the methods ( $\chi^2 = 2.6$ , df = 1,  $P = 0.10$ ). There was no difference in larval presence/absence ( $\chi^2 = 0.0$ , df = 1,  $P = 1.0$ ) by tree or abundance/tree ( $t = 0.6$ , df = 18,  $P = 0.6$ ) between the sites. At WPA and Woodland Parks, branch clipping recovered significantly more *L. nigrinus* than beat sheets (WPA,  $t = 7.25$ , df = 24,  $P < 0.0001$ ; Woodland,  $t = 5.208$ , df = 24,  $P < 0.0001$ ). There was no difference in larval presence/absence ( $\chi^2 = 0.0$ , df = 1,  $P = 1.0$ ) or abundance ( $t = 0.8$ , df = 48,  $P = 0.4$ )

between the sites. Two *L. laticollis* adults were collected out of 300 *L. nigrinus* in Seattle, but no *L. rubidus* or *L. nigrinus* adults were recovered in the Appalachians. Beat sheet sampling took an average of  $4 \pm 1$  minute per tree and branch clipping and dissection took eight times longer ( $33 \pm 9$  minutes per tree).

As the numbers recovered and presence of immature *Laricobius* spp. were not different at four of five Appalachian sites, they were pooled. Predators were present on 11% (4 of 37) of the trees sampled (Table 4.1). The frequency of predators in ovisacs by site (*i.e.* 400 ovisacs) were less than 1.2%. The frequency of predators in ovisacs by tree (*i.e.* 40 ovisacs) ranged from a low of 0% to a high of 12.5%. At VA-North Fork, predators were present on 60% of the trees, 4% of the ovisacs/site, and from 0 to 20% of the ovisacs/tree. At the lightly infested Discovery and Fauntleroy Parks in Seattle (pooled), predators were present on 40% (8 of 20) of the trees sampled. The frequency of predators in ovisacs by site (*i.e.* 400 ovisacs) was 7% for Discovery and 4.5% for Fauntleroy Park. The frequency of predators in ovisacs by tree (*i.e.* 40 ovisacs) ranged from a low of 0% to a high of 30%. At the heavily infested WPA and Woodland Parks (pooled), immature *L. nigrinus* were present on 100% (50 of 50) of the trees. The frequency of predators in ovisacs by site (*i.e.* 1,000 ovisacs) was 43% at WPA and 38% at Woodland Park. The frequency of predators in ovisacs by tree (*i.e.* 40 ovisacs) ranged from 10% to a high of 100% (Fig 4.1). Predators were present in 21% of the ovisacs dissected from the four eastern hemlock trees. There were more immatures recovered per tree in the urban parks, WPA and Woodland than in the forested Discovery and Fauntleroy Parks ( $F = 19.3$ ,  $df = 66$ ,  $P < 0.0001$ ).

### **Sampling for diel activity**

In the winter, there was no significant difference in the numbers of *L. nigrinus* adults during a 24 h period ( $\chi^2 = 8.1$ ,  $df = 5$ ,  $P = 0.15$ ) but more were caught in the afternoon (Fig. 4.2). Adult abundance was significantly and positively correlated with ambient temperature ( $r^2 = 70\%$ ). Relative humidity and wind speed were not correlated with adults captured, but wind was very light during the sampling. In the spring, there was a significant difference in the numbers of *L. nigrinus* adults during a 24 h period ( $\chi^2 = 16.7$ ,  $df = 5$   $P = 0.005$ ). The majority of adults were caught at 02:00 due to one outlier tree and abundance was not correlated with any weather condition (Fig. 4.3).

### **Relationship of ovisac size and presence of *L. nigrinus***

The probability of finding *L. nigrinus* immatures in ovisacs was related to ovisac diameter ( $\chi^2 = 138$ , df = 1,  $P < 0.0001$ ). Immatures were found in small ovisacs (*i.e.* 1 - 2 mm) 7% of the time and in large ovisacs (*i.e.* 2 - 3 mm) 41% of the time.

## **4.4 Discussion**

### **Comparing beat sheet and branch clipping sampling**

In the Appalachians, beat sheets failed to collect *L. nigrinus*, which is consistent with the many false negative results generated during the monitoring of 22 experimental releases in the eastern US (see Chapter 2) (Table 4.2). Beat sheet sampling has a low “sensitivity” to *L. nigrinus* presence, which is common when sampling for rare individuals in general (Venette et al. 2002). Branch clipping was more sensitive but generated one false negative at a site of known predator presence. At NC-Hemlock Hill, branch clipping failed in this study, but during operational monitoring at this site, *L. nigrinus* was recovered in large quantities using both methods (see Chapter 2). This probably occurred because a greater number of branches were clipped during operational monitoring and beat sheets were used on several occasions. We placed thousands of ovisacs in Berlese funnels to rear out predators as opposed to dissecting hundreds of ovisacs. In laboratory tests, female *L. nigrinus* were observed to oviposit up to 396 eggs (mean = 101), which are typically placed singly within HWA ovisacs (Zilahi-Balogh et al. 2003a). The larvae are mostly sedentary within the wool, feeding on HWA and therefore cannot be easily dislodged or evade capture. Based on this, the collection of immatures has several advantages and disadvantages relative to beat sheet sampling (Table 4.3). The major disadvantage is that *L. rubidus* eggs and larvae are commonly collected from HWA ovisacs (see Chapter 2) and the assumption that all immatures are *L. nigrinus* is not valid. Rearing or DNA testing must be carried out to confirm the species. Nevertheless, the chances of collecting *L. nigrinus* were greater with branch clipping. In Seattle, we assume that the recovered immatures were *L. nigrinus* because *L. laticollis* was relatively rare. *Laricobius nigrinus* was common and each sampling method was effective at collecting the predator. Branch clipping revealed that very high predator : prey ratios were present and *L. nigrinus* likely plays an important role in HWA

population dynamics (Mausel 2005, Kohler 2007). The ratios and predator abundance were low at Discovery and Fauntleroy Parks partly because the life-stage development of HWA and *L. nigrinus* was earlier than in the WPA and Woodland Parks. HWA ovisacs were visibly smaller and as such the predators may not have laid as many eggs as at the other sites at the time of sampling. In addition, HWA densities were observed to be much lower than the urban parks, and therefore fewer predators were present. The predator : prey ratios observed in the Appalachians were very low and to suppress HWA populations, *L. nigrinus* must at least approach the densities observed in Seattle.

It is likely that HWA predators are more common at the tops of trees than at the bottom. HWA has been shown to be more common in the tops of trees when HWA densities are low (Evans and Gregoire 2007a). Trees in decline typically only have new growth at the tops of the trees because they allocate carbohydrates to height growth before lower crown branches. Where there is new growth, HWA is more abundant (McClure 1991c). Another predator, *S. tsugae* was collected from the top of a hemlock tree with the use of a bucket truck but not from the ground of the same tree (Cheah and McClure 2002). *Laricobius nigrinus* adults are frequently absent from the lower crown and immatures are commonly collected from the mid/upper crown, suggesting that adults are common there. We observed more HWA and *L. nigrinus* at the tops of trees at a field insectary (albeit the trees were small), which was used to guide mass collection of the beetles for re-distribution (see Chapter 3). More research into the vertical distribution of the predator at different prey densities could shed more light on ideal crown locations to sample in forests.

### **Sampling for diel activity**

Patterns of diel activity in insects are common and expressed in many behaviors such as flight (Daterman et al. 1965), walking, feeding, or resting (VanLaerhoven et al. 2003). In winter, it is likely that *L. nigrinus* seeks shelter during cold temperatures and spend less time walking, feeding, or resting on hemlock shoots. In spring, *L. nigrinus* was collected at all hours of the day because temperatures were warmer. The predator continuously feeds, rests, and searches for HWA on branches (Flowers et al. 2007) and although resting was more common in the day and

searching at night, sampling should not be affected unless ambient temperatures are below 0.0 °C (Zilahi-Balogh et al. 2003c).

### **Relationship of ovisac size and presence of *L. nigrinus***

*Laricobius nigrinus* lays more eggs in large rather than small ovisacs to provision larvae with large amounts of eggs for their growth and development. It is not known how female adults determine the size of ovisacs, but this may be judged through vision or tactile cues such as touching the ovisac with antennae or tarsi.

### **Sampling recommendations**

A satisfactory post-release monitoring program is valuable for determining establishment (Clark et al. 2001a) and to justify continued releases of natural enemies. A natural enemy could escape detection if monitoring is attempted with inadequate sampling procedures. This could explain how some natural enemies suddenly appear many years post-release when they become more abundant and/or interest is renewed (Humble 1994). Some may be present at such low densities in the early post-release years, but there is no effective sampling method for detection. For *L. nigrinus*, we recommend beat sheet sampling in the fall or spring and if this fails, branch clipping sampling of immatures with pole-pruners ( $\geq 10$  m) should be added in the spring. Beat sheet sampling was found to be less effective in the winter, as more adults were collected in the fall and spring than in winter (Zilahi-Balogh et al. 2003c), probably due to generally warmer temperatures and more predator activity. Beat sheet sampling may be most effective in the afternoon during the winter if temperatures rise above zero or anytime during the fall or spring. When populations are low, branch clipping should be the choice sampling method. For branch clipping and dissecting HWA ovisacs for *Laricobius* spp. immatures, emphasis should be on HWA ovisacs greater than 2 mm in diameter, or clipping foliage when ovisacs are large and use of Berlese funnels to rear out larvae (Lamb et al. 2005a). Phenological indicators of large ovisac presence used to time collection of immatures included the conspicuous flowering eastern redbud, *Cercis canadensis* L. (see Chapter 2). Actual dates that release sites were sampled in the eastern US are listed in Appendix 2.

**Table 4.1.** Total number of *Laricobius nigrinus* adults collected with beat sheets or immatures with branch clipping from hemlock woolly adelgid infested hemlocks in the southern Appalachians (introduced habitat) and Seattle, WA (native habitat).

Site	Habitat	Sample date 2005	n	Total no. <i>L. nigrinus</i>		% Ovisacs with predators
				Beat sheet*	Branch clipping <sup>1</sup>	
<b>Appalachians</b>						
VA-North Fork	Forest	19 Apr	10	0	16	4.0
VA-Big Stony	Forest	19 Apr	7	0	2	0.7
VA-Hurricane	Forest	18 Apr	10	0	1	0.2
NC-Hemlock hill	Forest	6 Apr	10	0	0	0.0
TN-Laurel Ck.	Forest	4 Apr	10	0	5	1.2
<b>Seattle, Washington</b>						
WA-Discovery	Forest	26 Feb	10	11	28	7.0
WA-Fauntleroy	Forest	26 Feb	10	1	18	4.5
WA-WPA	Urban forest	22 Feb	25	140	428	43.0
WA-Woodland	Urban forest	27 Feb	25	148	382	38.0

\* = Total number of adults collected from four branches per tree.

<sup>1</sup> = Total number of eggs/larvae from 40 HWA sistentes ovisacs per tree.

**Table 4.2.** Relationship between *Laricobius nigrinus* presence/absence by site as determined by branch clipping sampling and by beat sheet sampling at experimental releases in the eastern US (A) and at the southern Appalachian sites in this study (B). The gray highlighting indicates the number of false negative results by beat sheet sampling.

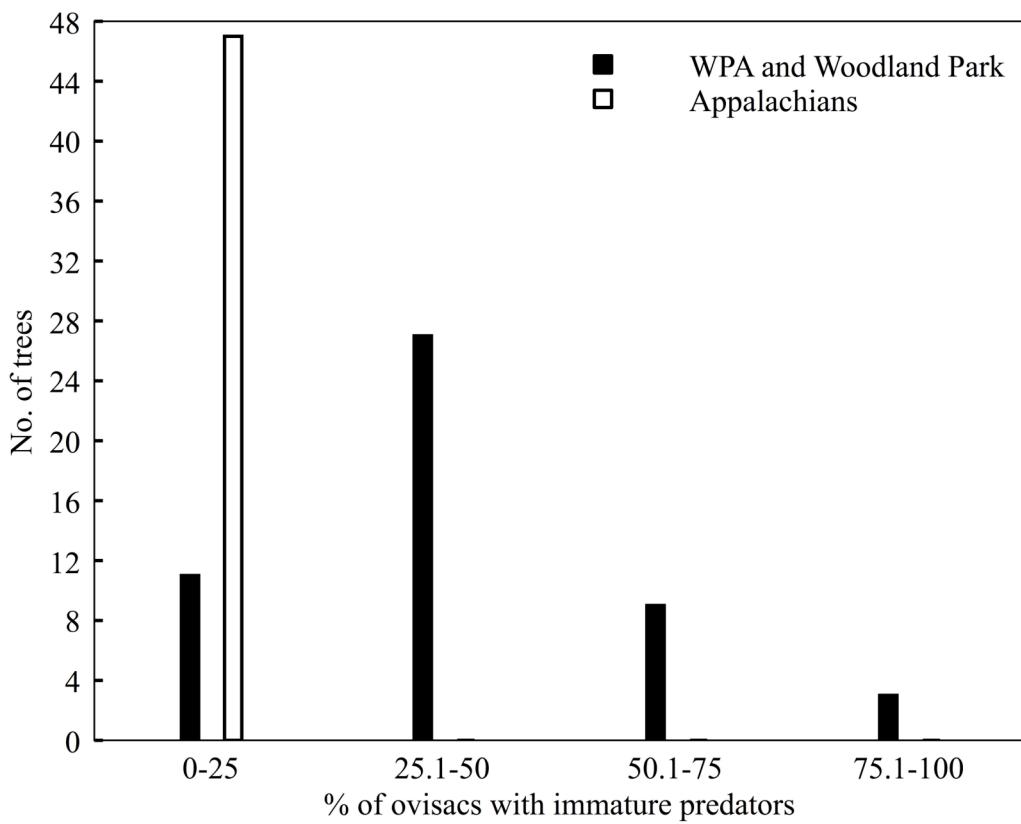
A*		Results from branch clipping	
Results from beat sheets		Present	Absent
Present		9	0
Absent		9	14

\* see Chapter 2, results from three years of sampling at 22 sites

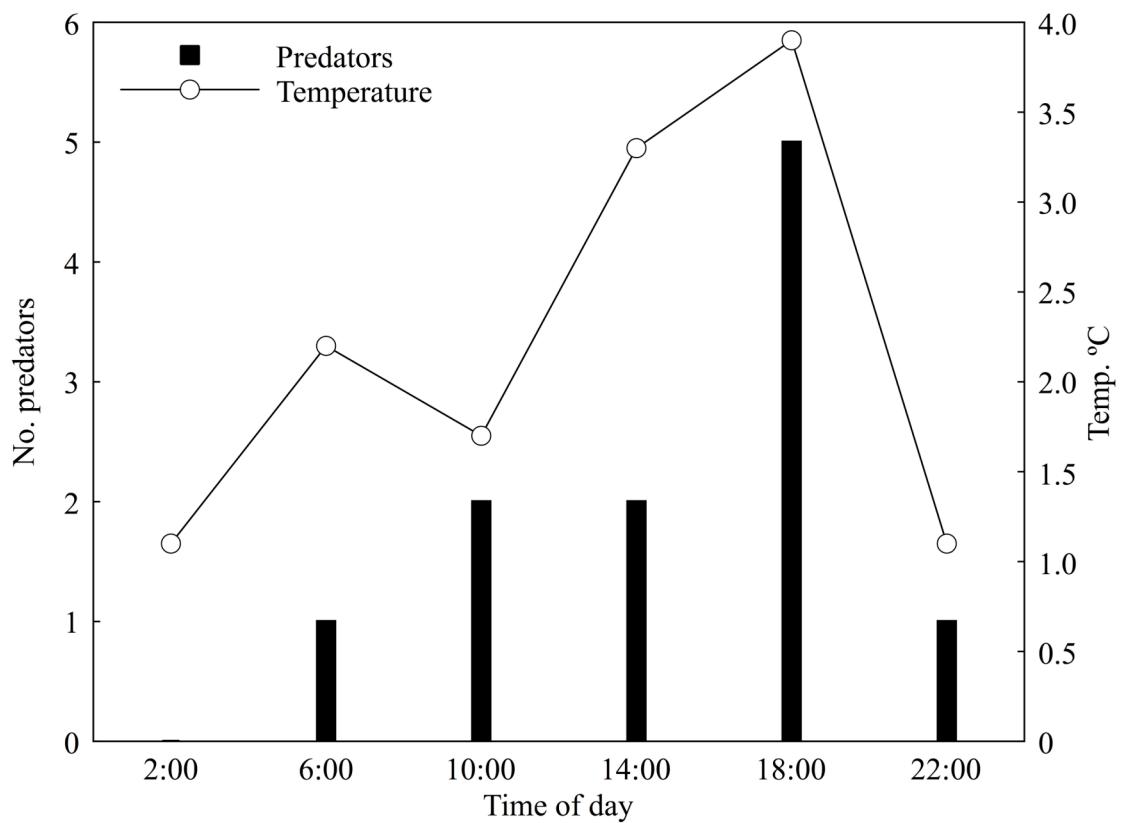
B		Results from branch clipping	
Results from beat sheets		Present	Absent
Present		0	0
Absent		4	1

**Table 4.3.** Relative advantages and disadvantages of beat sheet (adults) and branch clipping (immature) sampling methods for the collection of *Laricobius nigrinus*.

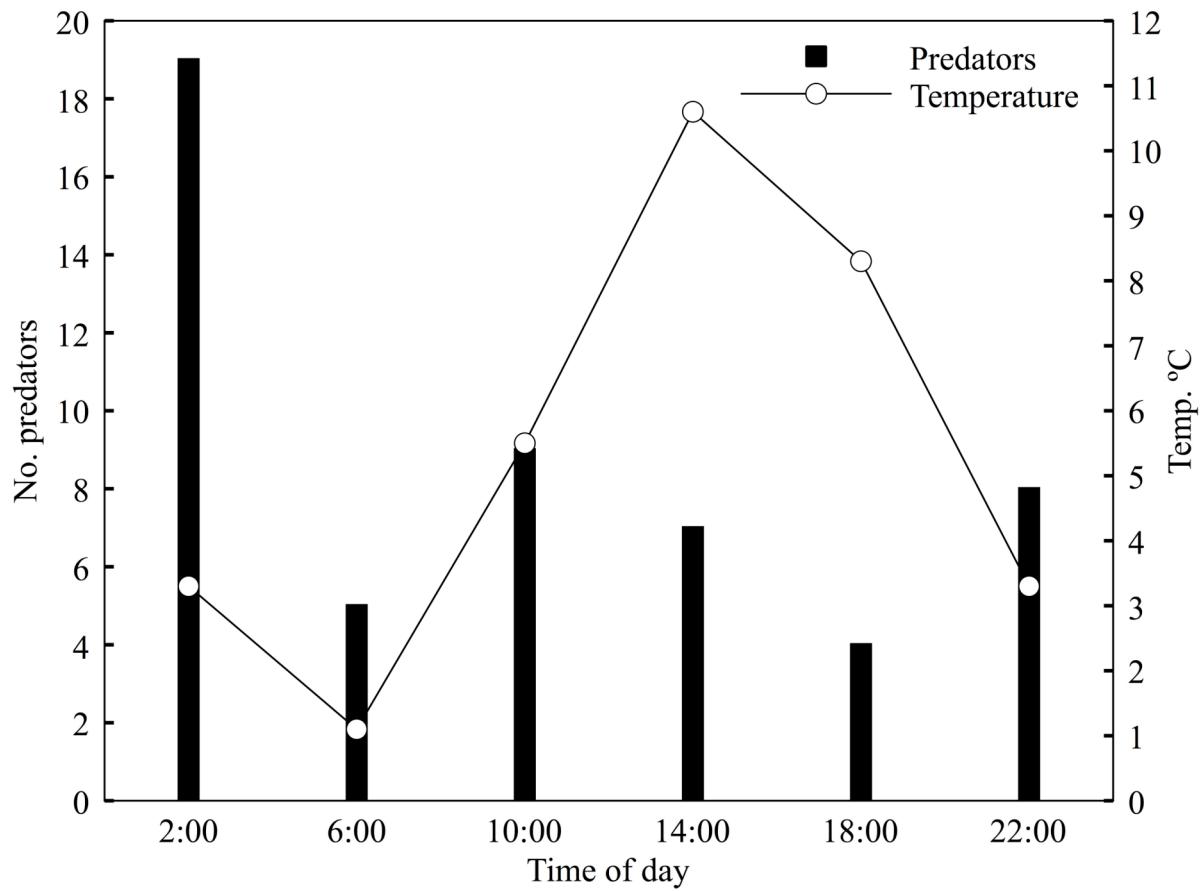
	Advantages	Disadvantages
Beat sheets	Time efficient Fall/spring sampling Simple Instant results	Weather sensitive Time of day sensitive Restricted to lower canopy False negatives common Not suitable for low populations
Branch clipping	Not weather sensitive Not time of day sensitive Can sample up to 10 m False negatives rare Better for low populations	Less time efficient Spring sampling More complex Delayed results Confusion with <i>L. rubidus</i>



**Fig. 4.1.** The number of hemlock trees at the Washington Park Arboretum (WPA) and Woodland Park ( $n = 50$ ) and Appalachian release sites ( $n = 47$ ) within four categories of the percentage of ovisacs with immature *Laricobius nigrinus*.



**Fig. 4.2.** Diel variation in temperature and total *Laricobius nigrinus* collected with beat sheets from hemlock woolly adelgid infested western hemlock trees at the Washington Park Arboretum in Seattle, WA on 10/11 Dec 2005.



**Fig. 4.3.** Diel variation in temperature and total *Laricobius nigrinus* collected with beat sheets from hemlock woolly adelgid infested western hemlock at the Washington Park Arboretum in Seattle, WA on 19/20 Mar 2005.

## **Chapter 5 Observations on hemlock woolly adelgid mortality and *Laricobius nigrinus* (Coleoptera: Derodontidae) predation on western hemlock trees of Seattle, Washington**

### **Abstract**

Mortality of the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) sistentes from the second instar through the ovisac stage were observed at four sites for 2 yr and partial life tables were constructed. Unspecified causes of nymph and adult mortality were important and *Laricobius nigrinus* Fender was the dominant predator of ovisacs at low- and high-HWA densities. Overall, HWA survival was variable and typically less than 50%. *Laricobius nigrinus* : HWA ratios ranged from 0.05 to 0.4 larvae/HWA and averaged 0.2 larvae/HWA. To observe the aggregation and numerical response of *L. nigrinus* in the field, 30 trees with a range of HWA density were sampled at each of two sites. Adult *L. nigrinus* abundance was positively related with HWA density and immature abundance was positively related with ovisac density. The predator had a density-dependent response to HWA and was prevalent at low- and high-HWA density trees. These results confirm that *L. nigrinus* was a good selection from the western North American range of HWA, and continued release in the eastern US is justified.

**Keywords:** Classical biological control, invasive species, *Adelges tsugae*, predator, *Tsuga canadensis*, *Tsuga caroliniana*, partial life tables, aggregation, numerical response

## 5.1 Introduction

The hemlock woolly adelgid, (HWA) *Adelges tsugae* Annand (Hemiptera: Adelgidae), is native to China, Tibet, Bhutan, northwestern India, Japan, Taiwan, and the Pacific Northwest (PNW) (Cheah et al. 2004). DNA evidence from HWA suggests the origin for the invasive eastern US population is around Osaka, Japan (Havill et al. 2006). It was first reported at Maymont Park, Richmond, Virginia in the early 1950's on planted eastern hemlock [*Tsuga canadensis* (L.) Carrière] (Miller 1988). It is a primary pest of eastern and Carolina hemlock (*Tsuga caroliniana* Engelmann) and kills all ages and sizes of trees (Orwig and Foster 1998, McClure and Cheah 1999, McClure et al. 2001, Orwig et al. 2002). Currently, HWA infests 18 states, which represents a major portion of eastern hemlocks (Morin et al. 2005) and 100% of Carolina hemlocks range in the US (Anonymous 2007a). In the PNW, HWA does not damage western hemlock [*Tsuga heterophylla* (Raf.) Sargent] or mountain hemlock [*Tsuga mertensiana* (Bong.) Carrière] forests (Furniss and Carolin 1977). Few descriptions exist of HWA being a pest in these regions (Collman 1972, McClure 1992). Work on HWA has recently begun in this region and its life cycle (Zilahi-Balogh et al. 2003c), fecundity (Mausel 2005), and natural enemies (Kohler 2007) have been described. The sistentes hatch in the late spring, aestivate in the summer, resume development in the fall, and lay progredientes eggs in the late winter/early spring (Zilahi-Balogh et al. 2003c) similar to the eastern US (McClure 1987b, McClure 1989, Gray and Salom 1996). The progredientes hatch in the early spring, develop, and lay sistentes eggs in late spring.

The predator, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), is native to the PNW (Fender 1945) and has been released against HWA since 2003 in the eastern US (Lamb et al. 2006). It has established at sites from Pennsylvania to Tennessee (see Chapter 2). Its native distribution ranges from northern California to coastal British Columbia and also in interior British Columbia, Alberta, and northern Idaho (Hatch 1962, Furniss and Carolin 1977, Tait et al. 1985, Lawrence 1989, Bright 1991). Thorough studies on the predator's biology in quarantine and in the field in the eastern US were done before its release (Lamb et al. 2002, Zilahi-Balogh et al. 2002, Zilahi-Balogh et al. 2003a, b, Flowers et al. 2005, Lamb et al. 2005b, Lamb et al. 2005a, Lamb 2005, Flowers et al. 2006, Lamb et al. 2006, Zilahi-Balogh et al. 2006).

Meanwhile, field studies in the PNW have increased our understanding of its biology and potential as an HWA biological control agent. *Laricobius nigrinus* life cycle is phenologically synchronized with HWA in Victoria, British Columbia, Canada (Zilahi-Balogh et al. 2003c). It feeds on sistentes and progredientes eggs when few other predators are active, oviposits within sistentes ovisacs, and can reach considerable predator : prey ratios (Mausel 2005). It has low temperature thresholds (Zilahi-Balogh et al. 2003b) that are similar to HWA (Salom et al. 2002). Kohler (2007) recently identified 55 predator species from HWA infested western hemlock and three species appear to be exceptional HWA predators. Two species of *Leucopis* (Diptera: Chamaemyiidae) were the most abundant in the summer. *Laricobius nigrinus* was the most widespread and abundant at low- to high-HWA densities from fall through spring. These predators' abundance were positively correlated with HWA abundance.

It is critical in modern classical biological control of insects to select the best and safest natural enemies in an exotics native range for release in its introduced range (Van Driesche and Bellows 1996). Before expensive and time consuming host-range testing in quarantine was standard operating procedure, one approach was to select and release many natural enemies with the expectation that one would be successful (Burgess and Crossman 1929, Mitchell and Wright 1967, Ehler 1990). Another method is an empirical approach where foreign collectors attempt to select the most important natural enemies during a sojourn to the native country. This is difficult because pests are usually not well studied, which results in even less knowledge about their natural enemies (*i.e.* many are new to science). Limited time, funding, and duration of trips often make these natural enemy choices arbitrary however, they may be deliberately based on abundance, impact, host-specificity, and successful taxa (Waage 1990). They also may appear to be less important in native habitats than they turned out to be in the introduced habitat.

More recent approaches involve the study of natural enemies in their native range (Gerling et al. 2004), in the lab (Lester and Harmsen 2002), or by mathematical modeling (Waage 1990) to predict the likelihood of success or failure before release. Though possible, predicting success is unlikely (Ehler 1990, Debach and Rosen 1991, Waage and Mills 1992, Van Driesche and Bellows 1996, Kimberling 2004). An intermediate approach, where the empirical and predictive methods are used simultaneously, for severely damaging exotic pests that require rapid natural

enemy introductions is logical. As an empirically chosen natural enemy is undergoing host-range testing, rearing, and release, studies should be ongoing in its native range to attempt to predict its success or failure. A holistic approach utilizes life table studies of the pest, discovery of natural enemies that regulate a pest at endemic levels, selection of agents that complement each other, and fill empty niches (Waage 1990, Waage and Mills 1992). Studies on interactions of natural enemies could indicate factors such as competition, predation, or parasitism that obscure the potential for successful biological control of some species (Ehler 1990, Waage and Mills 1992, Gerling et al. 2004).

In this study, we made detailed observations on HWA and *L. nigrinus* in their native range in an attempt to predict its biological control success or failure in the eastern US. Specifically, age-specific partial life tables for the sistentes stages attacked by the predator were constructed, predator prevalence, and predator : prey ratios at low- and high-HWA density sites were described. The aggregation and reproductive components of *L. nigrinus* numerical response were observed. The goal of this research was to determine if the predator should continue to be released.

## 5.2 Materials and methods

### Partial life tables, predator and prey parameters

Four sites known as the Washington Park Arboretum (WPA), Woodland Park, Discovery Park, and Carkeek Park were selected in Seattle, Washington. Five HWA infested trees were randomly chosen on 28 Feb 2005 from a population of infested trees within each park (Table 5.1). Each tree appeared healthy and had no observable HWA symptoms in spite of high populations in some cases. The predominant HWA stage on the sample dates 28 Feb 2005 and 3 Mar 2006 was sistentes ovisacs. From each of the four cardinal points of each tree's lower crown 30 cm long branches were cut and placed in 3.8 L plastic bags. The trees were sampled again on 3 Mar 2006. The bags were frozen (-15 to -18 °C) for 24 - 48 hours in Seattle, shipped overnight to Virginia Tech's Beneficial Insects Quarantine Laboratory, and re-frozen until dissection. After the examinations, all the material was autoclaved and discarded.

The four branches per tree were sub-sampled by clipping the previous growing season's shoots from a randomly selected lateral half of the branch and measured to the nearest 0.1 cm. Each shoot was examined with a dissecting microscope (12 - 20X) and the total number of sistentes ovisacs, total numbers of predators, live and dead sistentes HWA in each instar N1 - N4 (N = nymphs), and adult stage were determined. As it was impossible to determine the generation to which a dead N1 belonged (McClure 1989), we did not include them in the life table. Most dead N1 were likely sistentes that died during settling, aestivation, or shortly after. Late hatching progredientes settle on shoots that sistentes also settle on in late spring, due to generation overlap, when the current year shoots are green and expanding. The dead N1 stage could have also been progredientes because some individuals settle, feed, and possibly ingest toxic terpenoids on this growth, as described for eastern hemlock (Lagalante et al. 2006). Alternatively, sistentes settle, enter a non-feeding aestivation state, and avoid the toxic compounds. Dead N1 density per tree was calculated as the total number of N1 divided by the total length of examined shoots and averaged over the five trees per site.

The HWA instar at the time of death was determined by adding one to the number of molts by counting exuvia embedded within the flocculence on the shoot. Dead HWA and their exuvia remain attached to branches for approximately one generation but exuvia are occasionally lost. Any dead HWA nymph or adult that could not be reliably identified as to its stage, was classified as unknown. If the HWA was live and exuvia counts were not reliable, we measured its body length with an eyepiece micrometer to determine the stage (McClure 1989). Sistentes were considered dead at the time of freezing if their body was hard, abnormally shaped, lacked hemolymph, or the hemolymph was cloudy brown or black when probed with a dissecting probe. They were considered live if the body was normal shaped, rebounded to touch with a probe, and had dark purple colored hemolymph (McClure 1991c, Palmer and Sheppard 2002). The number of HWA entering each life stage was summed for each site. Similarly, the numbers of progredientes eggs, *L. nigrinus* eggs and larvae (hereafter called immatures), and other predators' immatures were recorded from the ovisacs and summed for the five trees per site. A strip of electrical tape was placed sticky-side up on a piece of styrofoam and secured in place with pins. Ovisacs were removed from the hemlock shoot with a dissecting tool and placed on the electrical tape. A drop of 70% ethanol was placed on the ovisac to make the flocculence

transparent and probed under a dissecting microscope. Syrphidae and Chamaemyiidae predators were rare and pooled. Occasionally, progredientes eggs were left on the shoot following ovisac removal as were L3 and L4 (L = larvae) *L. nigrinus*. We added these numbers to those recorded in the ovisacs.

Age-specific partial life tables for each site were constructed for 2005 and 2006 (Teetes et al. 1992, Bellows and Van Driesche 1999, Pustejovsky and Smith 2006). We justify this because our intention was to study the stages *L. nigrinus* feeds on (the sistentes N2 - N4, adults, and ovisacs with progredientes eggs). Sistentes ovisac mortality (*i.e.* progredientes eggs) was classified as caused by *L. nigrinus* or other predator larvae. All nymphal and adult mortality was classified as from an unspecified cause because determining the cause of death was impossible as in other adelgid life-table studies (Mailu et al. 1980). Mortality likely occurred from predation (Kohler 2007) or host defenses (McClure 1992, Lagalante and Montgomery 2003, Montgomery et al. 2005, Lagalante et al. 2006). Mortality due to cold temperature in Seattle is unlikely because the climate is mild and freezing temperatures are rarely reached. Each immature predator was assumed to cause 100% egg mortality of the ovisac it was recorded within.

We calculated the finite survival rate of sistentes HWA (hereafter survival) from fall (N2 sistentes) to early spring (sistentes ovisacs) (Krebs 1989). Survival equaled the number of live sistentes and ovisacs without predators (*i.e.* the number that survived) divided by the number of live and dead sistentes and total ovisacs. HWA density (no./cm) was calculated as the total number of live sistentes and total ovisacs divided by the total shoot length. *Laricobius nigrinus* density was calculated as the total number of immatures divided by the total shoot length. The *L. nigrinus*: HWA ratio was calculated as the total number of predator immatures divided by the total number of live sistentes and total ovisacs per tree. The number of eggs per ovisac was calculated as the total number of eggs divided by the total number of ovisacs per tree. All the parameters were calculated per tree and averaged over the five trees per site. A *t*-test was used to compare the parameters between 2005 and 2006 at each site using SPSS® version 10.0 at the 0.05 alpha-level. The number of eggs per ovisac was not compared statistically. All averages are presented as means  $\pm$  standard error.

### **Aggregation and numerical response**

To observe the aggregation response of adult *L. nigrinus* to different prey densities in the field, 30 trees with a range of HWA density were selected at each of two sites. Trees at the Woodland Park and Washington Park Arboretum were selected and sampled on 22 Jan 2006 and 12 Feb 2006, respectively. Each tree's crown and branches appeared healthy and had no HWA damage symptoms. Four branches were sampled with beatsheets at the cardinal points of each tree. The branch was tapped ~10 times with a stick above a 71 cm<sup>2</sup> canvas beatsheet (Bio-quip, Rancho Dominguez, CA). Weather data were acquired from the University of Washington, Department of Atmospheric Sciences weather station 1.3 km and 3 km from the Arboretum and Woodland, respectively (Anonymous 2007b). Temperature and maximum wind speed were recorded by the min. and the average over the sampling period was calculated. The total numbers of *L. nigrinus* and *L. laticollis* (a native congener) adults per tree were recorded and returned to the trees. A 30 cm long branch sample was clipped from each of the four branches sampled with the beatsheet. The branches were placed into a 3.8 L plastic bag and frozen until examination with a dissecting microscope (12 - 20X). Each branch was thawed, shoots 2 yr old or younger were cut off, and placed into a brown paper bag. We used 2 yr old growth in addition to 1 yr old growth because HWA were common on these nodes and contributed to the prey density on the branches. Shoots were randomly sub-sampled and the HWA (N2 - adults and ovisacs) were probed and recorded as live or dead, as described previously. Sub-sampling continued until 100 total HWA were examined. The total length of the examined shoots was measured to calculate the live HWA density per tree. A linear regression was conducted to determine if the numbers of *L. nigrinus* adults were correlated with HWA density using SPSS® version 10.0.

To observe how HWA ovisac density influences *L. nigrinus* oviposition in the field, the 30 trees at Woodland Park and the Washington Park Arboretum were sampled again on 11 and 12 Mar 2006, respectively. Four 30 cm long branch samples were clipped from the trees, frozen, and sub-sampled for microscopic examination, as described. The sistentes ovisac density and total number of immature *L. nigrinus* were determined for each tree. The electrical tape method of dissecting and counting the ovisacs and immature predators was used (see Chapter 4). A linear regression was used to describe the relationship between the numbers of predators and ovisac densities. HWA ovisac densities were square root transformed to correct for nonlinearity for

both sites (Zar 1999). At the Arboretum, predator numbers were square root transformed to correct for non-constant variance and non-normality. All averages are presented as the mean  $\pm$  standard error.

### 5.3 Results

#### Partial life tables, predator, and prey parameters

Overall, the dead N1 density of either the sistentes or progredientes generation was high and averaged, in decreasing order:  $0.6 \pm 0.3/\text{cm}$  (min-max;  $0.01\text{-}2.8/\text{cm}$ ) at Woodland Park,  $0.4 \pm 0.1/\text{cm}$  ( $0.02\text{-}1.1/\text{cm}$ ) at the Washington Park Arboretum,  $0.4 \pm 0.2/\text{cm}$  ( $0.005\text{-}1.4/\text{cm}$ ) at Carkeek Park, and  $0.3 \pm 0.1/\text{cm}$  ( $0.0\text{-}0.9/\text{cm}$ ) at Discovery Park. Although we cannot reliably attribute the N1 mortality to either generation, we believe most of them to be sistentes and as such, their mortality was high.

The life tables for WPA and Woodland Park indicate that the greatest age-specific HWA mortality in both years was *L. nigrinus* predation of sistentes ovisacs (Table 5.2). Other species were relatively uncommon predators of ovisacs compared to *L. nigrinus* and unspecified mortality of sistentes nymphs and adults was considerable, especially when summed. At Discovery and Carkeek Park the greatest mortality occurred in the nymphal stages. Ovisac predation was minor and *L. nigrinus* caused greater mortality than other predators, as in WPA and Woodland Parks. At Carkeek Park in 2006, ovisac predation was considerable.

The mean sistentes survival was low to moderate and not significantly different between 2005 and 2006 at WPA and Woodland (Table 5.4). AT WPA, HWA density and *L. nigrinus* significantly decreased from 2005 than 2006 and predator : prey ratios did not change. At Woodland, no parameters changed between years. At Discovery and Carkeek Park, the HWA survival rate declined significantly from 2005 to 2006 and the other parameters did not change.

#### Aggregation and numerical responses

The abundance of *L. nigrinus* adults was positively related with sistentes density at the Arboretum ( $F = 16.2$ ,  $\text{df} = 1,28$ ,  $P < 0.001$ ) but not at Woodland ( $F = 1.6$ ,  $\text{df} = 1,28$ ,  $P < 0.22$ )

(Fig. 5.1). The number of adults collected per tree at the Arboretum averaged  $5.4 \pm 0.9$ . Adults were collected from 90% of the trees. At Woodland, an average of  $1.5 \pm 0.5$  adults were collected per tree. Adults were collected from 50% of the trees. The Arboretum was sampled at 11:15-15:30, under clear skies, mean temp.  $6.3^{\circ}\text{C}$ , and mean maximum wind speed of 9.6 km/hr. Woodland was sampled at 11:30-16:30, under partly cloudy skies, mean temp.  $6.8^{\circ}\text{C}$ , and mean maximum wind speed of 21.1 km/hr. At the Arboretum, one *L. laticollis* was collected.

The abundance of *L. nigrinus* immatures was positively related to ovisac density at the Arboretum ( $F = 17.3$ ,  $\text{df} = 1,28$ ,  $P < 0.0001$ ) and at Woodland ( $F = 13.7$ ,  $\text{df} = 1,28$ ,  $P = 0.001$ ) (Fig. 5.2). An abnormally high-density sample (2.6 ovisacs/cm, 18 *L. nigrinus*) at Woodland influenced the regression and was removed from the analysis. When it was included, the regression remained significant ( $y = 11.44x + 3.59$ ,  $r^2 = 0.35$ ,  $F = 15.09$ ,  $\text{df} = 1,28$ ,  $P = 0.001$ ). The numbers of immatures collected per tree at the Arboretum averaged  $13.1 \pm 1.7$ . Immatures were collected from 100% of the trees. The number of immatures collected per tree at Woodland averaged  $8.9 \pm 1.0$ . Immatures were also collected from 100% of the trees.

## 5.4 Discussion

### Partial life tables, predator, and prey parameters

Like most r-adapted species, N1 HWA mortality was considerable and were likely mostly sistentes. This could be an important source of HWA mortality and should be investigated further. The N1 carcasses appeared physically intact and often were not desiccated, which suggests that predators did not chew or suck them dry. The unknown cause of mortality in the sistentes nymphal and adult stages was likely due to predation. HWA has no parasitoids (Montgomery and Lyon 1996) and adult *L. nigrinus* are active in the fall, winter, and spring, which overlaps the N2 through adult stages (Zilahi-Balogh et al. 2003c). *Leucopis argenticollis* has been observed to be active in the fall as well (Kohler 2007). Winters are mild in Seattle and not sufficiently cold to cause mortality as described in some eastern US locations (Parker et al. 1999, Skinner et al. 2003). Pathogens of HWA in the PNW have not been described. In addition, the health of the trees was good, which implies that poor host quality is not causing the mortality (McClure 1991c). HWA mortality could be due to western hemlock “resistance” to

HWA, but this has not been demonstrated conclusively (McClure 1992, Lagalante and Montgomery 2003, Mausel 2005). The HWA densities reported in this paper demonstrate that high HWA populations and high survival rates are possible on western hemlock in a variety of growing conditions. If we assume a portion of the unspecified mortality of nymphs and adults was from *L. nigrinus* adults, their contribution to overall mortality is larger than it appears. The predator adults have pointed mandibles that pierce the exoskeleton of HWA sistentes nymphs and adults and ingest the hemolymph, from fall through spring (Zilahi-Balogh et al. 2003a, Flowers et al. 2007). In the spring, larvae similarly feed on HWA progredientes eggs preferentially but also feed on nymphs, adults, and crawlers.

Since we sampled in late February and early March, our estimates of ovisac predation are underestimated. There were several weeks remaining of *L. nigrinus* oviposition, larval feeding, and the activity of dipteran predators was perhaps beginning as well. If the samples were taken later in the spring, it is probable that mortality of ovisacs due to predation would be larger than what we describe. Nevertheless, ovisac predation was considerable at most sites and the low amounts of eggs/ovisac could have been due to predation. We observed signs of egg predation and *L. erichsonii* adults have been shown to swallow entire eggs a majority of the time and leave no evidence of predation (Franz 1958b), which may be common in *L. nigrinus*. HWA may not have completed oviposition, as well.

The predominant predator of HWA ovisacs was *L. nigrinus* larvae at all the sites. Predator : prey ratios were large in many cases and were greater than that observed in the successful biological control of the Eurasian Pine Adelgid by *Leucopis obscura* Haliday (i.e. 0.04) (Culliney et al. 1988). At Woodland in 2006, Discovery in both years, and Carkeek in 2005 ovisac predation was low. The numbers of surviving ovisacs was generally high at all sites, which is likely due to the early sample date. The early sample dates were chosen because late instar *L. nigrinus* larvae are mobile and voracious consumers of eggs in ovisacs. This feeding loosens the flocculence and eggs, which causes them to become an indiscrete mass that is often dislodged by wind and rain. If samples were taken later, it would be impossible to determine how many ovisacs were there initially. The solution to these problems is to take systematic samples throughout the year and construct complete life tables (Bellows and Van Driesche 1999). This would be an excellent

way to gauge the effectiveness of introduced natural enemies of HWA in the eastern US, as done for the larch casebearer in Oregon (Ryan 1990). In the PNW, it would be valuable to repeat this study for the progredientes generation to see which predators are the most important for this generation and could be potentially released in the East. Progredientes survival is likely reduced by a diverse natural enemy community (Kohler 2007).

**Washington Park Arboretum.** High populations of HWA and *L. nigrinus* were found in this park. HWA survival was moderate in 2005 and low in 2006, but not significantly different. Accordingly, HWA and *L. nigrinus* density declined in 2006, but the predator : prey ratios and ovisac mortality remained high. Nymph and adult mortality in 2006 were high, suggesting that adult predators were feeding heavily on these branches in the fall and winter, which reduced the number of ovisacs considerably. With reduced amounts of ovisacs, predator abundance probably declined and ovisac mortality in 2006 was similar to 2005.

**Woodland Park.** Woodland Park was a site with high HWA and *L. nigrinus* densities and few predator or prey parameters changed from 2005 to 2006. This site was different from the others because the sistentes population increased slightly from 2005 to 2006. Perhaps, this can be explained by the low amounts of nymph and adult mortality in 2005. In 2006, nymph and adult mortality increased but ovisac mortality decreased.

**Discovery Park.** Discovery Park had high HWA densities but low predator densities. Sistentes survival was high in 2005 because of low nymph, adult, and ovisac mortality, which resulted in a large number of ovisacs. *Laricobius nigrinus* and other predator populations were low as shown by their low densities and predator : prey ratios. This may explain the high HWA survival. In 2006, HWA survival declined significantly to moderate levels but predator populations did not increase significantly and the reason for HWA collapse is not clear. Adult predators were probably abundant in the fall preying on nymphs and dispersed in the spring. N2 and unknown stage mortality was high and ovisac mortality increased in 2006, but the predator : prey ratios stayed the same.

**Carkeek Park.** Carkeek Park was different from the other sites because it was a natural forest that had low HWA populations and most trees in the area were uninfested. Sistenes survival was moderate in 2005, perhaps because predators were at low density and hence not collected with our methods. HWA survival was significantly lower in 2006, *L. nigrinus* was recovered, and other predators were not. HWA had high mortality in the nymph and ovisac stages in 2006. *Laricobius nigrinus* presence and impact at this site demonstrates its ability to persist and be an important natural enemy when HWA densities are low, a desirable quality of a natural enemy. A host-specific predator present at low prey densities must have good searching abilities, as well. The surviving HWA were frequently located under bud scales at the base of shoots, which offered a refuge from predation. Similar lightly infested sites in the eastern US are probably ideal locations to release the predator for biological control of HWA before outbreaks occur and HWA populations are too large to be significantly impacted by *L. nigrinus*.

### **Aggregation and numerical response**

Adult *L. nigrinus* abundance was positively related with HWA density at the Washington Park Arboretum demonstrating its density dependence relationship with HWA. This may occur by adult attraction to high-density patches (Sabelis 1992), more time spent in these patches (Hassell and May 1974, Beddington et al. 1976), or a numerical response of the previous generation(s). Attraction to HWA or infested plant odors has not been demonstrated but is possible (Broeckling and Salom 2003b). Aggregation is important for biological control because predation rates, oviposition rates, and survival will likely increase and considerable mortality on HWA can be inflicted when pest populations are growing. This result was in line with the results of Kohler (2007) who described increasing predator abundance with increasing prey densities. Laboratory behavioral studies showed that *L. nigrinus* promptly flew from uninfested seedlings and did not fly from heavily infested ones (See chapter 6). The lack of aggregation at Woodland may be because of a lag between HWA buildup and *L. nigrinus* response. Another explanation is that we sampled in January when the predators are less active due to generally cooler temperatures (Zilahi-Balogh et al. 2003c), even though the temperature at the time of sampling was similar. The number of adults recovered was clearly lower than at the Arboretum. Another plausible explanation is that windy conditions caused the predators to drift away from the beatsheets.

*Laricobius nigrinus* oviposition increased with increasing ovisac density demonstrating its density dependence relationship with HWA. As described previously, the adults aggregated at high densities and individuals likely increased their ovipositional rate and lifetime fecundity on heavily infested branches. This is an important attribute predators use to raise their population rate of increase in response to growing prey populations (Beddington et al. 1976). Alternatively, when HWA populations were low, *L. nigrinus* oviposited less, which may prevent intraspecific competition, demonstrated by Flowers *et al.* (2005, 2006), and maximize survival. In both of these studies, *L. nigrinus* was ubiquitous and abundant on trees with a wide-range of HWA-infestation. *Laricobius nigrinus* is likely an important factor in regulating HWA populations.

The *L. nigrinus* release program against HWA has been preceded by the balsam woolly adelgid (BWA), *Adelges piceae* (Ratzeburg), program with *Laricobius erichsonii* Rosenhauer, which established in North America but was not successful in suppressing BWA. Early in that program Franz (1958b) recognized that *L. erichsonii* was not likely a good natural enemy. It had many adelgid hosts and was not synchronized with BWA in Europe, implying that BWA was not its primary host. The prey specificity and synchrony of *L. nigrinus* focuses its impact on HWA. Its presence at low-density sites indicates that it has good searching ability, and wide geographic range in the PNW indicate that it has wide climatic tolerance and adaptability. Its dominance at low- and high-prey densities and density-dependant responsiveness suggests that it has the capability to regulate HWA. On the other hand, HWA was introduced from Japan not the PNW, *L. nigrinus* has fewer generations than HWA, is difficult to mass rear, and needs to be effective in forests. Some of these traits have proven unsuccessful in the history of biological control (Kimberling 2004). We cannot predict the outcome *L. nigrinus* in the eastern US with certainty, but if the investment in its proper release continues, there is a good chance for it to provide at least partial biological control of HWA.

**Table 5.1.** Characteristics of the study sites in Seattle, Washington where hemlock woolly adelgid mortality and *Laricobius nigrinus* predation were studied on western hemlock trees.

<b>Field site</b>	<b>Area</b>		<b>UTM Coordinates</b>		<b>Elev.</b> (m a.s.l)	<b>Tree (min-max)</b>	
	<b>(ha)</b>	<b>Habitat</b>	<b>Easting</b>	<b>Northing</b>		<b>DBH(cm)</b>	<b>Height(m)</b>
W.P. Arboretum	93	Urban forest	553054	5276056	25	15.0-40.4	4.6-18.3
Woodland Park	37	Urban forest	549278	5279573	70	53.8-82.8	12.2-21.3
Discovery Park	216	Urban forest	544671	5278344	70	8.1-77.0	3.0-21.3
Carkeek Park	75	Forest	546920	5284361	25	15.0-40.4	7.6-21.3

**Table 5.2.** Age-specific partial life tables for *sistentes* hemlock woolly adelgid on western hemlock at four sites in Seattle, Washington in 2005 and 2006.

Life stage (x)	No. entering each stage ( $l_x$ )	Factor responsible for $d_x$	No. dying per factor ( $d_x$ )	No. dying per stage ( $d_x$ )	Marginal death rate
<b>W.P. Arboretum 2005</b>					
N2	327	unspecified*	17	17	0.05
N3	310	unspecified	18	18	0.06
N4	292	unspecified	16	16	0.05
Adult	276	unspecified	9	9	0.03
Unknown	267	unspecified	38	38	0.14
Ovisac	229			71	
		<i>L. nigrinus</i> larvae	65		0.28
		Other predators	6		0.03
Intact Ovisac	158				
<b>W.P. Arboretum 2006</b>					
N2	242	unspecified	57	57	0.24
N3	185	unspecified	39	39	0.21
N4	146	unspecified	19	19	0.13
Adult	127	unspecified	3	3	0.02
Unknown	124	unspecified	33	33	0.27
Ovisac	91			29	
		<i>L. nigrinus</i> larvae	27		0.30
		Other predators	2		0.02
Intact Ovisac	62				
<b>Woodland Park 2005</b>					
N2	371	unspecified	33	33	0.09
N3	338	unspecified	8	8	0.02
N4	330	unspecified	17	17	0.05
Adult	313	unspecified	10	10	0.03
Unknown	303	unspecified	26	26	0.08
Ovisac	277			82	
		<i>L. nigrinus</i> larvae	68		0.25
		Other predators	14		0.05
Intact Ovisac	195				
<b>Woodland Park 2006</b>					
N2	437	unspecified	58	58	0.13
N3	379	unspecified	33	33	0.09
N4	346	unspecified	12	12	0.03
Adult	334	unspecified	29	29	0.09
Unknown	305	unspecified	38	38	0.12
Ovisac	267			47	
		<i>L. nigrinus</i> larvae	39		0.15
		Other predators	8		0.03
Intact Ovisac	220				

**Table 5.2** (continued).

Life stage (x)	No. entering each stage (l <sub>x</sub> )	Factor responsible for d <sub>x</sub>	No. dying per factor (d <sub>x</sub> )	No. dying per stage (d <sub>x</sub> )	Marginal death rate
<b>Discovery Park 2005</b>					
N2	616	unspecified	13	13	0.02
N3	603	unspecified	21	21	0.03
N4	582	unspecified	20	20	0.03
Adult	562	unspecified	5	5	0.009
Unknown	557	unspecified	44	44	0.08
Ovisac	513			15	
		<i>L. nigrinus</i> larvae	11		0.02
		Other predators	4		0.008
Intact Ovisac	498				
<b>Discovery Park 2006</b>					
N2	349	unspecified	90	90	0.26
N3	259	unspecified	20	20	0.08
N4	239	unspecified	4	4	0.02
Adult	235	unspecified	6	6	0.03
Unknown	229	unspecified	36	36	0.16
Ovisac	193			20	
		<i>L. nigrinus</i> larvae	18		0.09
		Other predators	2		0.01
Intact Ovisac	173				
<b>Carkeek Park 2005</b>					
N2	78	unspecified	10	10	0.13
N3	68	unspecified	12	12	0.18
N4	56	unspecified	6	6	0.11
Adult	50	unspecified	2	2	0.04
Unknown	48	unspecified	6	6	0.12
Ovisac	42			0	0.00
		<i>L. nigrinus</i> larvae	0		
		Other predators	0		
Intact Ovisac	42				
<b>Carkeek Park 2006</b>					
N2	50	unspecified	15	15	0.30
N3	35	unspecified	6	6	0.17
N4	29	unspecified	4	4	0.14
Adult	25	unspecified	1	1	0.04
Unknown	24	unspecified	9	9	0.37
Ovisac	15			4	
		<i>L. nigrinus</i> larvae	4		0.27
		Other predators	0		0.00
Intact Ovisac	11				

\* Mortality likely occurred from *L. nigrinus* adult feeding (Kohler 2007) or host defenses (McClure 1992, Lagalante and Montgomery 2003, Montgomery et al. 2005, Lagalante et al. 2006).

**Table 5.3.** The finite survival rate of hemlock woolly adelgid (HWA) sistentes, HWA density, *Laricobius nigrinus* density, and predator : prey ratios on western hemlock at four sites in Seattle, Washington in 2005 and 2006.

Site								
	W.P. Arboretum				Woodland Park			
<u>Parameter</u>	<u>2005</u>	<u>2006</u>	<u>t</u> <sup>1</sup>	<u>sig.</u> <sup>2</sup>	<u>2005</u>	<u>2006</u>	<u>t</u>	<u>sig.</u>
HWA survival rate <sup>3</sup>	42.2 ± 11.3	23.3 ± 6.5	1.4	0.2	52.3 ± 6.0	42.6 ± 7.6	1.0	0.3
Live <sup>4</sup> HWA/cm	0.5 ± 0.1	0.1 ± 0.04	3.0	0.02*	0.3 ± 0.1	0.4 ± 0.3	0.07	0.9
<i>L. nigrinus</i> /cm <sup>5</sup>	0.1 ± 0.03	0.03 ± 0.01	3.6	0.01*	0.08 ± 0.03	0.05 ± 0.03	0.5	0.6
<i>L. nigrinus</i> /HWA <sup>5</sup>	0.4 ± 0.2	0.3 ± 0.1	0.8	0.4	0.2 ± 0.04	0.1 ± 0.04	1.1	0.3
Other predator/HWA <sup>5</sup>	0.02 ± 0.01	0.1 ± 0.1	1.0	0.4	0.04 ± 0.01	0.03 ± 0.01	0.4	0.7
	Discovery Park				Carkeek Park			
<u>Parameter</u>	<u>2005</u>	<u>2006</u>	<u>t</u>	<u>sig.</u>	<u>2005</u>	<u>2006</u>	<u>t</u>	<u>Sig.</u>
HWA survival rate	78.2 ± 4.0	43.7 ± 10.1	3.2	0.01*	50.0 ± 5.0	26.8 ± 6.7	2.8	0.02*
live HWA/cm	0.7 ± 0.2	0.4 ± 0.3	0.6	0.5	0.07 ± 0.02	0.03 ± 0.02	1.1	0.3
<i>L. nigrinus</i> /cm	0.02 ± 0.01	0.04 ± 0.04	0.6	0.6	0.0 a	0.008 ± 0.005	1.6	0.1
<i>L. nigrinus</i> /HWA	0.07 ± 0.05	0.05 ± 0.02	0.3	0.8	-- <sup>6</sup>	0.1 ± 0.07	--	--
Other predator/HWA	0.008 ± 0.006	0.03 ± 0.03	0.7	0.5	--	--	--	--

<sup>1</sup> Parameter means±standard error between years at each site were compared with a t-test ( $\alpha=0.05$ , 2-tailed, DF =8).

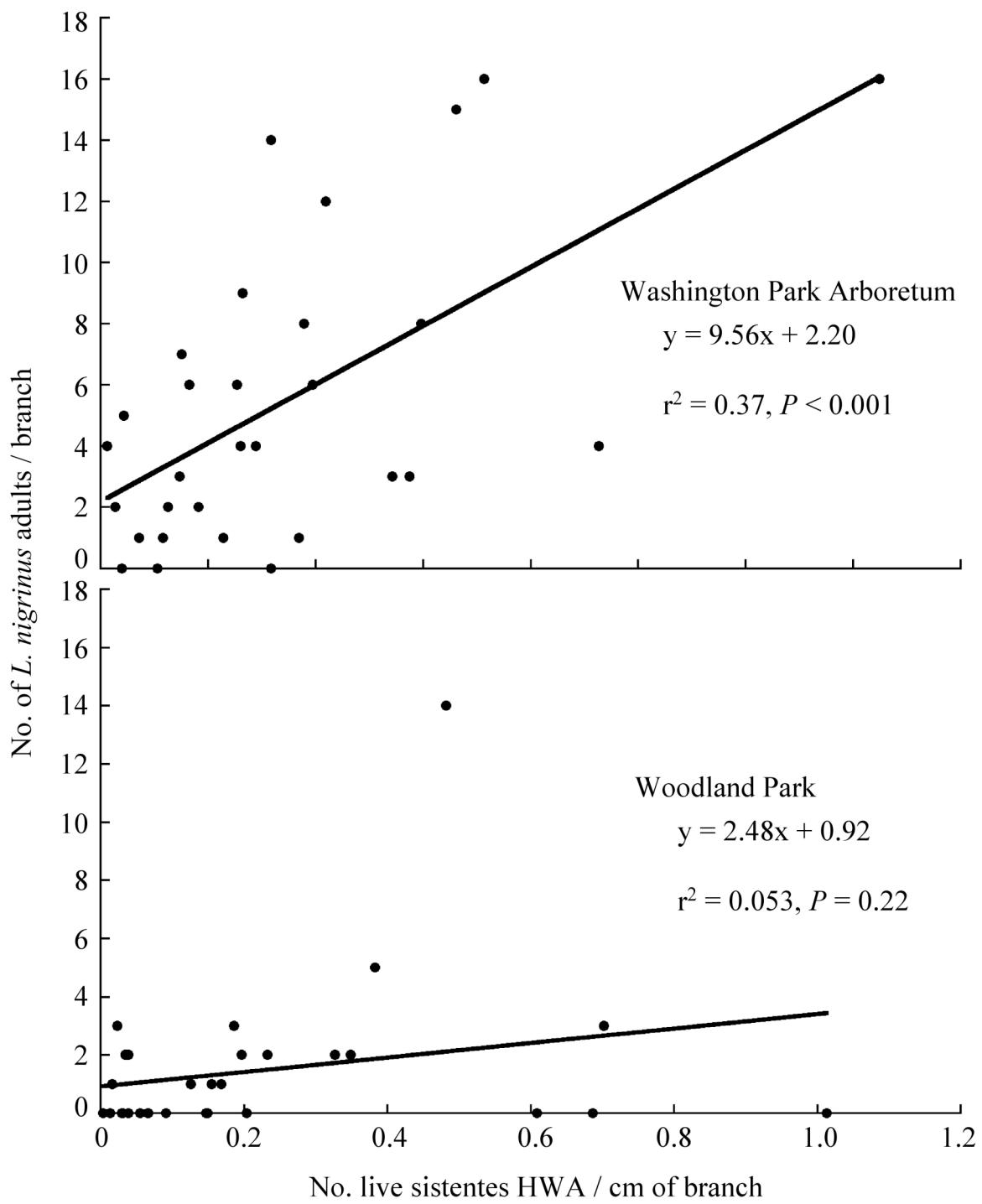
<sup>2</sup> \* indicates a significant difference

<sup>3</sup> Survival of sistentes N2-ovisacs (i.e. ovisacs with predators were not considered survivors)

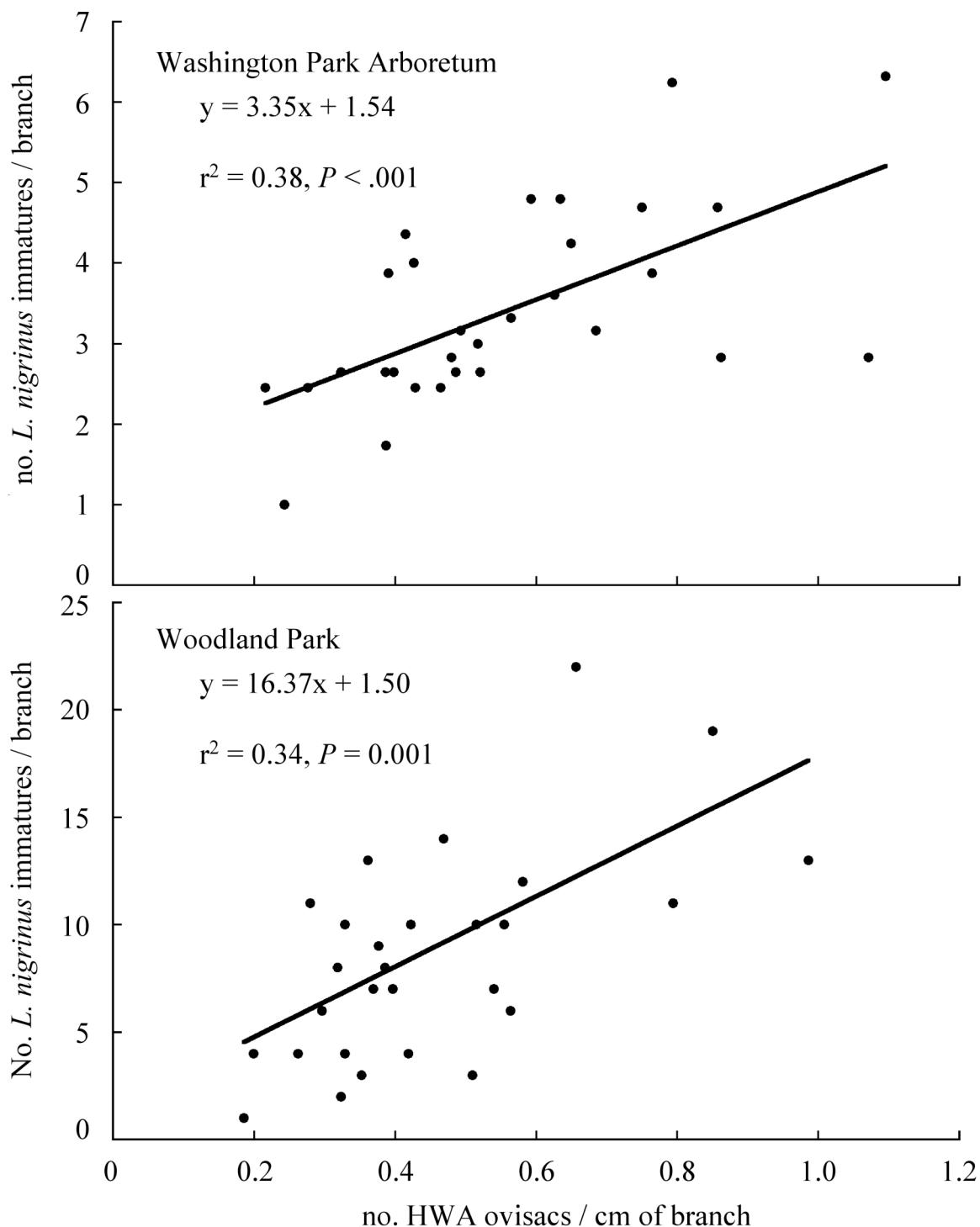
<sup>4</sup> Live HWA includes N2-ovisacs without predators

<sup>5</sup> Predator eggs and larvae only

<sup>6</sup> Indicates that no predators were recovered or no statistical tests were used



**Fig. 5.1.** Relationship between the abundance of *Laricobius nigrinus* adults and sistentes hemlock woolly adelgid nymph and adult density at the Washington Park Arboretum and Woodland Park in Seattle, Washington ( $n = 30$ ).



**Fig. 5.2.** Relationship between the abundance of *Laricobius nigrinus* eggs and larvae and sistentes hemlock woolly adelgid ovisac density (all square root transformed) at the Washington Park Arboretum and Woodland Park in Seattle, Washington ( $n = 30$ ).

# **Chapter 6 Host searching behavior and visual response of *Laricobius nigrinus* (Coleoptera: Derodontidae) on hemlock trees in the laboratory**

## **Abstract**

The host searching behavior of *Laricobius nigrinus* Fender was studied to understand how the predator locates a hemlock tree and its prey, the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand. A potted hemlock or artificial Christmas tree was used to test the ability of *L. nigrinus* to locate trees in light or darkness from the soil. It showed no preference for the artificial or hemlock trees and the hemlock and artificial trees were contacted and climbed in light significantly more than in darkness. The predator orientated to the tree visually as shown by the transecting path they rapidly took to the tree and the random undirected paths they took in darkness, which typically failed to locate the tree. An HWA-infested and uninfested potted hemlock was used to study *L. nigrinus* searching behavior by video recording the predator on the plant. Careful viewing of the video and creation of behavioral flow diagrams showed that common sequences of behaviors on infested or uninfested plants that led to feeding when the prey were present and flight dispersal from the tree when prey were absent. On an uninfested plant, the predators groomed, climbed the tree, and walked on needles more than on infested plants. They periodically scanned the environment by waving of the antennae, head, and tarsi to potentially detect olfactory, visual, or mechanical cues.

**Keywords:** Object orientation, extensive searching, intensive searching, *Tsuga canadensis*, *Adelges tsugae*

## 6.1 Introduction

The Derodontidae is a small family in the order Coleoptera that inhabits the temperate latitudes of the northern and southern hemispheres that are small, cryptic, and rarely observed (Lawrence and Hlavac 1979). They are considered a primitive family in the polyphaga sub-order of the Coleoptera and very little is known about their behavior. There are four genera with three being mycophagous and one, *Laricobius* Rosenhauer, that is predaceous on holarctic Adelgidae (Hemiptera) (Lawrence and Hlavac 1979, Lawrence 1989, Leschen 2000). There are 14 known species worldwide and three are native to North America (Zilahi-Balogh 2001, Zilahi-Balogh et al. 2007). The European *Laricobius erichsonii* Rosenhauer apparently established in North America on balsam woolly adelgid (BWA), *Adelges piceae* (Ratzeburg) (Mitchell and Wright 1967). *Laricobius* spp. are monophagous arboreal predators of adelgids, which feed on subcortical cells of conifers in the Pineaceae via a stylet (Lawrence and Hlavac 1979, Lawrence 1989, Downie and Arnett 1996).

*Laricobius nigrinus* behavior has been the subject of recent studies because it is an important natural enemy of the highly invasive hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (McClure 1991a) and a potential biological control agent (Cheah et al. 2004). Its ability to find its host habitat and host is important information in helping devise effective monitoring of the beetles presence. Antennal morphology suggests that *L. nigrinus* use host volatiles (Broeckling and Salom 2003b). The multiporous basiconic sensilla on *L. nigrinus* terminal antennal annuli are known to function for olfaction (Kiel 1999), but olfactory experiments were inconclusive (Broeckling and Salom 2002). Other sensilla for contact chemoreception on mouthparts, tarsi, or ovipositor have not been described. Behavioral studies on general behaviors, diel patterns, and interactions with conspecifics or potential heterospecific competitors in the laboratory showed that host searching was more common in the night and resting more common in the day (Flowers et al. 2007). The predator was noted to touch HWA ovisacs with their antennae during prey inspection, selectively feed and oviposit, and rest in confined locations such as shoot axis and between needles. Presence of another *L. nigrinus* increased searching and decreased resting.

In the study reported herein, we examined adult *L. nigrinus* behavior immediately after aestivation upon emergence from the soil. Laboratory conditions that simulated conditions a beetle may encounter when searching for a tree and HWA were used. Post-emergence is a critical time as the predator emerges from the soil, disperses, finds hemlock trees, and locates HWA for nourishment after several months of fasting. A basic understanding of host searching behavior may help sampling methods and superior sampling technologies to beat sheet sampling or branch clipping could improve monitoring of *L. nigrinus* establishment, impact, and dispersal.

## 6.2 Materials and methods

### *L. nigrinus* movement

**Locating a tree.** One potted eastern hemlock was used to investigate the ability of *L. nigrinus* to locate a tree by vision in a climate controlled cold room. A microcosm of the forest soil under a single tree's canopy was created to mimic what a predator experiences when first emerging from the soil. The pot was 30 cm tall and 15 cm wide with the seedling planted near the center of the pot. The seedling was 40 cm tall, 5 years old, and 0.5 cm diameter at the root crown. The soil surface "arena" was 15 cm in diameter and had an area of 177 cm<sup>2</sup>. On the surface of the soil in the pot, 3 cm of beige sand was added so the black predators could be observed while walking on the surface. A border of white paper (5 cm above the sand) was covered in fluon and placed along the perimeter of the arena to cover the black edge of the pot, to provide contrast to the seedling stem, and to keep the beetles within the arena (Fig. 6.1). The pot was placed within a large muslin cage to allow recovery of predators that flew from the seedling. A separate identical pot contained a "tree" made of artificial Christmas tree foliage wrapped around a plastic rod to imitate the hemlock's appearance. The silhouette of this artificial seedling was similar to the hemlock seedling in terms of height, diameter, and stem length. An incandescent lamp was placed above the plant and the laboratory room was lit with ceiling mounted fluorescent lights.

The predator's ability to find the trees from the soil surface were tested under four situations: hemlock under white light, hemlock under red light, artificial tree under white light, and artificial tree under red light. The four treatments represent the following potential sensory stimuli: vision and odor, odor but no vision, vision but no odor, and no vision and no odor, respectively. Most

insects perceive complete darkness under a red light, which enables observation of its behavior. Odors produced by the trees were not likely very strong or important in this study because the hemlock was uninfested with HWA, not wounded, and air movement was undirected. The artificial tree's odors were likely even less important. Each treatment was replicated 20 times under cool temperatures (9.7 - 12.9 °C) and moderate relative humidity (42 - 78%). Vigorous lab-reared beetles were randomly assigned to each treatment and starved for 24 h before each test (in isolation at 15 °C in a Petri dish on wetted filter paper). With a paintbrush, individual predators ( $n = 20$ ) were placed 7 cm from the tree on the sand surface and facing the general direction of the tree. The amount of time that passed until the predator contacted the stem and began climbing was recorded to the nearest second with a stopwatch. Timing began when the predator began walking and lasted up to 10 min. when the trial ended. There did not appear to be chemical cues left on substrates by the predators in other studies (Flowers et al. 2007), but as a precaution between tests, a minimum of 30 min. elapsed, the sand was turned, rewetted with distilled water, and smoothed out. The experiment was conducted in fall with freshly emerged predators (11 - 13 Nov 2005) and in winter (31 Jan - 1 Feb 2006) with predators that had been feeding on HWA-infested eastern hemlock in rearing containers for two months. The adults used in the winter tests were maintained at 6 °C day and 4 °C night and 12:12 (light: dark) before pre-test isolation. Tests were run between 9:00 and 17:00. A chi-square contingency table was used to compare the treatments and followed by partitioning. To compare the time taken for the predators to find the hemlock or artificial seedlings in white light, an independent samples *t*-test was used. There were no significant differences in time between the fall and winter assays for the hemlock tree or the artificial tree. Therefore, the data were pooled.

**HWA searching behavior on a tree.** Two potted eastern hemlocks as described previously were used to study *L. nigrinus* hemlock woolly adelgid searching behavior on a tree infested with or without HWA in a climate controlled cold room (10 - 12 °C) or laboratory (19 - 21°C) ( $n = 10$ ). The infested tree had approximately 75% of the needle bases infested. The experiment was conducted with adults immediately after emergence from aestivation sites in soil containers. Each unfed adult was tested after 24 h of isolation, but larvae had been reared on HWA on eastern hemlock the previous spring. Only vigorous individuals were used. The cold treatment was conducted in a walk in cold-room and the warm treatment in an adjacent laboratory. Other

conditions were created to make each room nearly identical. There was a temperature gradient from the bottom of the plant to the top due to heat released from the incandescent lamp to mimic sunlight in a forest.

Preliminary studies included observations on 13 subjects on the seedling treatments described previously. Based on a list of behaviors recorded, nine were selected for subsequent trials that were video recorded (Table 6.1). Individual lab-reared beetles dropped into the arena were continuously recorded with a Panasonic Digital Palmcorder Model PV-GS35 (Panasonic, Knoxville, TN). Trials were run for up to 30 min. between 9:00 and 17:00. A minimum of 30 min. was taken between replications and the sand was turned, re-wetted, and smoothed out. The Palmcorder was linked to a desktop computer to transfer the images, archive, and watch the video as described in detail by Flowers et. al. (2007). The Palmcorder's settings were optimized for this study by using the cinema mode, image stabilizer, LCD spotlight, and best quality settings. The zoom was used up to 6X. The camera was held by hand and when the predators became obscured by plant parts the tree was rotated and camera position was manipulated to minimize missing footage. After the trials on uninfested seedlings, the predators were placed on an HWA-infested twig and were noted to feed or not feed. The beetles' sex was determined after the trials and were not re-used.

The time that each of the nine behaviors began and ended was recorded from the video. The elapsed time until a behavior began ("latency"), the number of times the behavior occurred ("bout frequency"), and total bout length for each behavior was recorded to the nearest second. The timing of the trial began when the predator contacted the stem and ended when it flew off the seedling, ate HWA, or 30 min. expired. Four behavioral flow diagrams were constructed for the infested and uninfested trees in the cold and warm temperatures. The commonness of each behavior from 10 replications of one individual for each treatment was expressed as the number of times the behavior occurred divided by 10. Proportions greater than 1.0 indicate repetitive behavior. Prevailing behavioral sequences were represented in the diagram by gray block arrows and minor behaviors by black line arrows. The criterion for a prevailing behavior was that its occurrence was  $\geq 0.70$  for the unidirectional transition from one behavior to another. Bi-directional arrows indicate behaviors that were cyclical (*i.e.* climbing stem to grooming to

climbing stem to grooming, etc). For the prevailing cyclical behaviors, the proportion was calculated for each sequence of the cycle (*i.e.* both ends of the block arrow). The cyclical minor behaviors had only one proportion given, which is the sum of the two sequences. Pertinent parameters (*i.e.* latency, frequency, and total length) for each behavior were compared between cold or warm conditions or between uninfested and infested hemlock trees by independent samples t-tests or non-parametric Mann-Whitney *U* tests at the 0.05 alpha-level. All averages were presented as means  $\pm$  standard error.

### 6.3 Results

#### ***L. nigrinus* movement:**

**Locating a tree.** The number of times *L. nigrinus* found a hemlock or artificial tree was affected by light or darkness (Fall,  $\chi^2 = 43.6$ , df = 3,  $P < 0.0001$ ; winter  $\chi^2 = 26.5$ , df = 3,  $P < 0.0001$ ; Table 6.2, 6.3). Trees were contacted significantly more in light than in darkness, but the predators showed no preference for the artificial or hemlock trees. The time it took the predators to contact the artificial tree (fall and winter pooled) was not different from the hemlock tree in the light ( $t = 0.09$ , df = 70,  $P = 0.9$ ), averaging  $51 \pm 4$  sec. for the predators to contact the tree (both tree types pooled).

**HWA searching behavior on a tree.** The host searching behavior sequence of *Laricobius nigrinus* on the HWA-infested hemlock at 10 - 12 °C showed HWA feeding 100% of the time (Fig. 6.2). Cycles of grooming and climbing were common and the prevailing sequence was: climbing stems cycling with grooming; walking lateral branches; evaluating HWA; and feeding on HWA. There were numerous minor cycles. Walking on needles was a minor behavior but there was no flight behavior. Evaluations of HWA did not always result in immediate feeding although this was the prevailing sequence. On occasion, the predator would evaluate HWA and then walk on needles, laterals, or groom before re-launching the searching sequence. There were 5 females and 5 males tested in this treatment.

The host searching behavior sequence on the HWA-infested hemlock at 19 - 21 °C also showed feeding on HWA 100% of the time (Fig. 6.3). Cycles of grooming and climbing were minor and

there were many other minor cycles. A common cycle was walking lateral branches and walking needles. The prevailing sequence was: climbing stem; walking lateral branches cycling with walking needles; evaluating HWA; and feeding on HWA. Walking on needles was a common behavior and there was flight behavior. However, flight in all cases resulted in the predator landing back on the tree at a different needle, lateral, or stem. Evaluations of HWA also did not always result in immediate feeding. The predator would occasionally evaluate HWA and then walk on needles or laterals. There were 7 females and 3 males tested in this treatment.

The host searching behavior sequence on the uninfested hemlock at 10 - 12 °C resulted in flight off the tree 80% of the time (Fig. 6.4). Cycles of grooming and climbing stem, climbing stem and walking needles, and walking needles and grooming were common. A minor cycle was walking laterals and grooming. Because of several cyclical behaviors, the prevailing sequence was more complex: climbing stem; cycles of grooming and climbing, walking needles and climbing stem (or walking laterals cycling with needles back to grooming or climbing); walking needles; pre-flight exercise, and flight off the tree. One individual flew off and back on the tree, and off again. Pre-flight exercise did not always result in flight because individuals had difficulty taking off, unfolding their wings, or appeared to abandon the attempt. After failed attempts, they would walk on more needles, laterals, or climb the stem before reinitiating the searching sequence that led to flight off the tree. The predominant site for takeoff was from a needle tip. Two individuals searched the tree for 30 min. when video recording stopped. All predators fed on HWA in post-trail testing, indicating they were hungry and searching for food. There were 5 females and 5 males tested in this treatment.

The host searching behavior sequence on the uninfested hemlock at 19 - 21 °C resulted in flight off the tree 90% of the time (Fig. 6.5). Cycles of grooming and walking needles, and walking needles and laterals were common. Two minor cycles included walking needles and climbing stem, and grooming and walking laterals. Similar to above, the prevailing sequence was: climbing stem; walking laterals; walking needles cycling with walking laterals (or cycles with grooming, back to climbing, walking laterals, and needles); walking needles; pre-flight exercise; and flight off tree. Three individuals flew off and back on the tree, but eventually flew off the

tree. One individual did not fly off and searched for HWA for 30 min. All predators fed on HWA in post-trail testing. There were 7 females and 3 males tested in this treatment.

The total time spent searching by climbing stems, walking laterals, and needles (pooled) was longer on uninfested trees ( $669 \pm 94$  sec.,  $n = 10$ ) than on infested trees ( $278 \pm 60$  sec.,  $n = 10$ ) in the cold treatment ( $t = 3.5$ ,  $df = 18$ ,  $P = 0.003$ ). The results were similar in the warm treatment ( $t = 2.3$ ,  $df = 18$ ,  $P = 0.03$ ) and searching was shorter in duration (uninfested,  $291 \pm 54$  sec.,  $n = 10$ ; infested,  $143 \pm 34$  sec.,  $n = 10$ ) consistent with faster movement in warmer conditions. This result is logical because food was absent on the uninfested tree and they spent more time searching before flying away. Total frequency of needle walking was greater on uninfested ( $4.5 \pm 0.7$ ) than on infested trees ( $1.5 \pm 0.4$ ) ( $t = 3.5$ ,  $df = 38$ ,  $P = 0.001$ ) suggesting that this behavior is important. In addition, there were more bouts of stem climbing on uninfested trees ( $3.3 \pm 0.5$ ) than on infested trees ( $1.7 \pm 0.2$ ) ( $t = 2.8$ ,  $df = 38$ ,  $P = 0.007$ ), but there was no difference between walking on lateral branches ( $t = 1.4$ ,  $df = 38$ ,  $P = 0.17$ ). The predator groomed longer on uninfested trees ( $362 \pm 107$  sec.) than on infested trees ( $103 \pm 35$  sec.) ( $U = 71$ ,  $P = 0.03$ ). There were also more bouts of grooming on uninfested trees ( $2.7 \pm 0.5$ ) than on infested trees ( $1.3 \pm 0.3$ ) ( $U = 126$ ,  $P = 0.04$ ).

Predators frequently evaluated HWA on infested trees and there was no difference between the length of evaluations in the warm ( $256 \pm 78$  sec.,  $n = 10$ ) and cold ( $324 \pm 84$  sec.,  $n = 10$ ) conditions ( $t = 0.6$ ,  $df = 18$ ,  $P = 0.6$ ). Next, we compared the time spent searching (*i.e.* climbing stems, walking laterals, and needles,  $211 \pm 37$  sec.,  $n = 20$ ) to see how this compared with time spent evaluating HWA ( $290 \pm 56$  sec.,  $n = 20$ ) and there was no difference ( $t = 1.2$ ,  $df = 38$ ,  $P = 0.24$ ). The predators spent as much time evaluating HWA as they did searching for HWA.

The *L. nigrinus* feeding latency (time elapsed until feeding) on the infested tree did not differ in cold ( $686 \pm 161$  sec.,  $n = 10$ ) and warm conditions ( $471 \pm 120$  sec.,  $n = 10$ ) ( $t = 1.07$ ,  $df = 18$ ,  $P = 0.3$ ) probably because the trees were heavily infested and prey easy to locate. Flight latency from the uninfested tree was longer in cold ( $831 \pm 129$  sec.,  $n = 8$ ) than in warm conditions ( $455 \pm 103$  sec.,  $n = 9$ ) ( $t = 2.3$ ,  $df = 15$ ,  $P = 0.036$ ), consistent with the takeoff difficulty in the cold.

## 6.4 Discussion

### *L. nigrinus* movement:

**Locating a tree.** The arboreal *L. nigrinus* visually orientated to the stem's silhouette as shown by the transecting paths they rapidly took to locate it and the fact that they could not find it in darkness. Object orientation is believed to be the one of the first behaviors to have evolved in animals (Jander 1975) and is obvious in this primitive derodontid. It is likely that other behavioral modalities like olfaction (Bell 1990) (*i.e.* kairomones and other semiochemicals) are used to find infested hemlock trees from long distances, but this study was limited to short-range host cues. Tiger beetles are exceptional visual predators and also use other host searching modalities to capture prey in darkness (Riggins and Hoback 2005). *Thanasimus dubius* (Coleoptera: Cleridae) uses visual and kairomonal cues to locate its prey, the southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae) (Billings and Cameron 1984, Strom et al. 1999).

After being transferred with the paintbrush, *L. nigrinus* occasionally became disorientated and would walk away from the tree, make turns, and eventually return when the stem enters their field of view. Once seen, they determined the precise spatial location of the tree and found it. When the predator found the tree in darkness, it appeared to be by chance via random movement. A live trap design that catches beetles walking up the bole of the tree would be valuable for monitoring release sites or for mass collections of the predator in its native range. Similarly, multiple funnel traps are used to catch flying bark beetles, which respond to chemical attractants attached to the trap in concert with a black vertical silhouette that simulates a tree stem (Lindgren 1983).

**HWA searching behavior on a tree.** The primary finding of this study is that dispersal of *L. nigrinus*, like most species, is related to food abundance. When HWA is abundant, *L. nigrinus* does not disperse from the tree and when HWA is absent, it does. Thus releases of adults on infested trees would likely enhance establishment by limiting the dispersal of small numbers of beetles and reducing demographic Allee effects, such as mate finding difficulties. Beetles spend

more time searching on uninfested trees (more stem climbing and walking on needles) than on infested trees. Because of increased climbing, beetles may ascend the tree higher on uninfested than infested trees. In the field, there are indications that this predator is more common in higher areas of the canopy than from the ground (see Chapter 4). These searching behaviors are consistent with “extensive searching” described for other aphidophagous predators in the absence of prey (Bell 1990, Flowers et al. 2007). Needle walking behavior included waving of the head, antennae, and front tarsi in the air at the end of the needle as described in other predators: coccinellids, syrphids, and chrysopids (Bell 1991). *Laricobius nigrinus* would often alternate walking on needles on opposite sides of a lateral branch repeatedly, then climb the stem or walk on laterals, and return to needle walking. This periodic scanning cycle is probably used to detect olfactory cues from HWA or infested hemlock (Broeckling and Salom 2003a), visual cues from another tree, or mechanical cues like another needle. Experiments testing olfactory cues may require that the beetles be on a needle for it to react to odors normally.

Evaluation of HWA in this study is analogous to the “intensive searching” or “local-area concentrated searching” (Nakamuta 1985, Flowers et al. 2007). An evaluation of HWA without feeding and continued searching suggests that the predator has prey preferences such as live sistentes rather than dead ones. Touching the antennae to HWA wool, tree stems, and needles and waving in the air were observed. Generally these behaviors are used by insects to provide various information about their environment when searching by providing tactile and olfactory inputs (Bell 1990). Although contact chemoreceptors are not believed to be located on the antennae (Broeckling and Salom 2003b), they may be present on other structures on the tarsi and mouthparts that encounter HWA wool. Grooming is done to clean and optimize the function of sensory organs. Because grooming occurred early in the host searching sequence and was more common on uninfested trees, other potentially important sensory modalities in addition to vision may be involved. Predators may clean their sensory organs in the absence of prey cues to optimize their senses and re-evaluate the environment before they risk flight dispersal.

**Table 6.1.** Descriptions of the behaviors analyzed from video footage of *Laricobius nigrinus* host searching behavior on hemlock seedlings infested or uninfested with HWA, in the laboratory.

Behavior	Definition
Climbing stem	Walking vertically on the main stem of the seedling
Walking lateral branch	Walking on any woody lateral branch or shoot
Evaluating HWA	Antennating HWA, brief insertions of mouth parts in wool, slow walking, and frequent turning
Feeding on HWA	Sustained insertion of mouthparts in HWA wool and predator “digging” movements to reach sistentes through the wool
Grooming	Cleaning of antennae and legs
Walking on needles	Walking on needles on laterals or main stem, predators often walk from one needle to another (often the same needle) and wave legs, head, and antennae from the end of the needle.
Pre-flight exercise	Movements associated with warming of the indirect flight muscles, opening of the elytra, and unfolding wings
Flying off of tree	Takeoff from the tree and landing on muslin cage
Flying off and back on tree	Takeoff from the tree and landing back on the tree

**Table 6.2.** The effect of uninfested eastern hemlock or artificial trees under light or darkness (red light) on the ability of newly emerged *Laricobius nigrinus* in fall to contact and begin climbing the stem within a small arena.

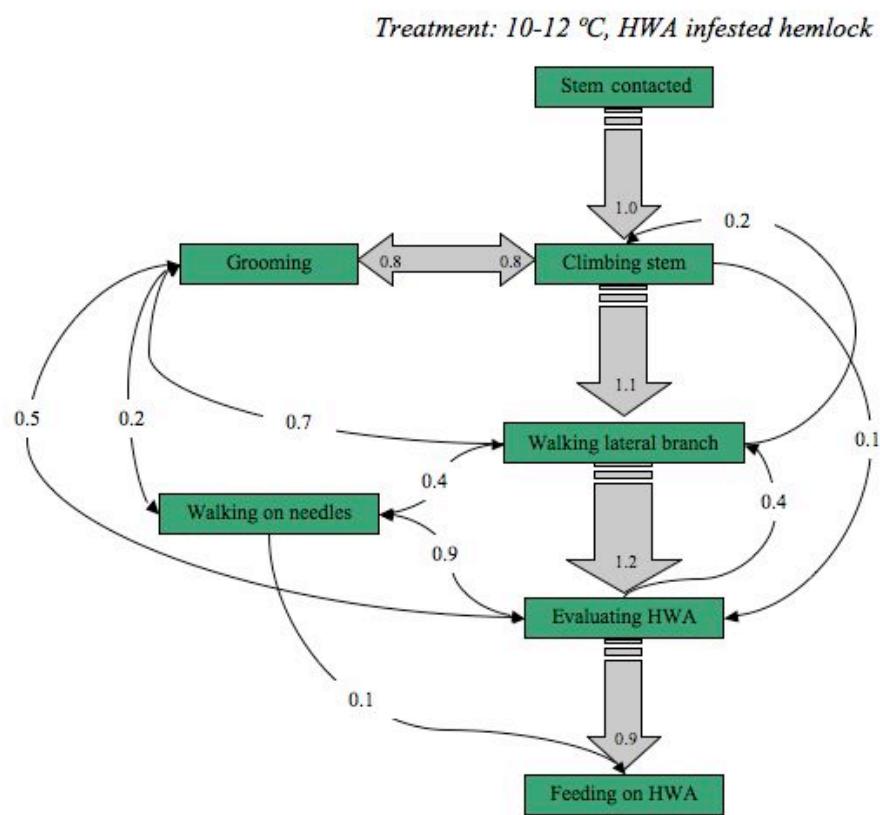
	Hemlock		Artificial tree	
	White light	Red light	White light	Red light
Contacted stem	17	5	19	2
Did not contact stem	3	15	1	18

**Table 6.3.** The effect of uninfested eastern hemlock or artificial trees under light or darkness (red light) on the ability of *Laricobius nigrinus* 2 months post-emergence in winter to contact and begin climbing the stem within a small arena.

	Hemlock		Artificial tree	
	White light	Red light	White light	Red light
Contacted stem	20	9	16	6
Did not contact stem	0	11	4	14

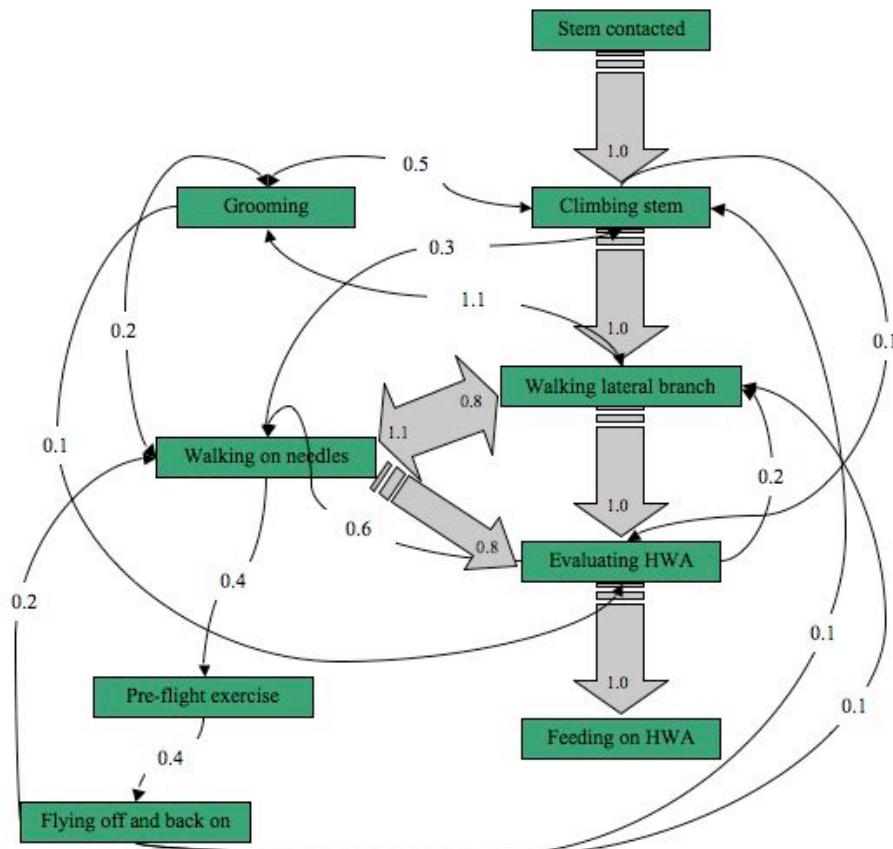


**Fig. 6.1.** The experimental setup used to study the ability of *Laricobius nigrinus* to locate a tree in light and darkness (red light). The artificial tree is on the left and eastern hemlock on the right.

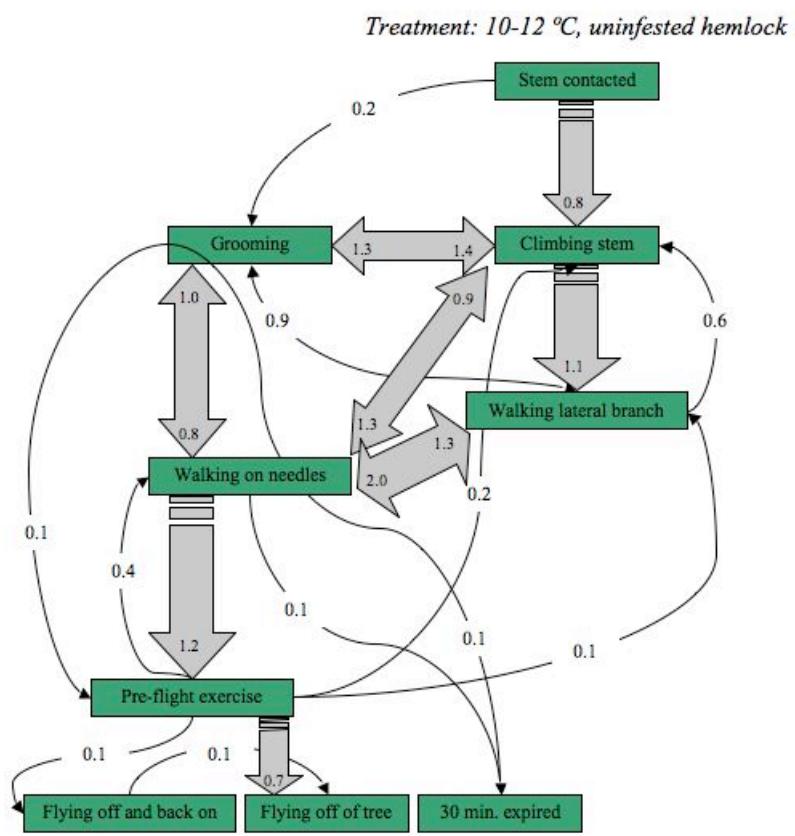


**Fig. 6.2.** Behavioral sequence diagram of *Laricobius nigrinus* host searching on a hemlock seedling infested with hemlock woolly adelgid in a climate controlled cold room (10-12 °C). Percentages show the frequency of occurrence of each behavioral sequence out of ten replications. Prevailing behaviors are shown by gray block arrows and minor behaviors by black line arrows. Bi-directional arrows indicate behaviors that cycle.

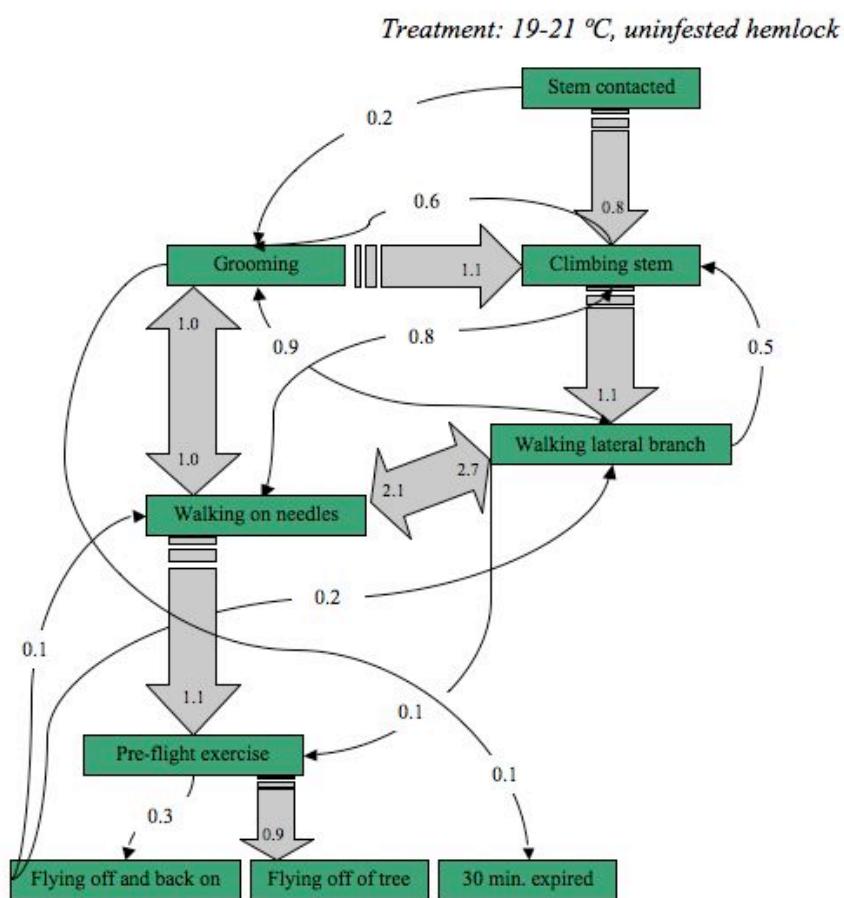
Treatment: 19-21 °C, HWA infested hemlock



**Fig. 6.3.** Behavioral sequence diagram of *Laricobius nigrinus* host searching on a hemlock seedling infested with hemlock woolly adelgid in a laboratory (19-21 °C). Percentages show the frequency of occurrence of each behavioral sequence out of ten replications. Prevailing behaviors are shown by gray block arrows and minor behaviors by black line arrows. Bi-directional arrows indicate behaviors that cycle.



**Fig. 6.4.** Behavioral sequence diagram of *Laricobius nigrinus* host searching on an uninfested hemlock seedling in a climate controlled cold room (10-12 °C). Percentages show the frequency of occurrence of each behavioral sequence out of ten replications. Prevailing behaviors are shown by gray block arrows and minor behaviors by black line arrows. Bi-directional arrows indicate behaviors that cycle.



**Fig. 6.5.** Behavioral sequence diagram of *Laricobius nigrinus* host searching on an uninfested hemlock seedling in a laboratory (19-21 °C). Percentages show the frequency of occurrence of each behavioral sequence out of ten replications. Prevailing behaviors are shown by gray block arrows and minor behaviors by black line arrows. Bi-directional arrows indicate behaviors that cycle.

## Chapter 7 Summary

The hemlock woolly adelgid (HWA) is a primary pest of eastern and Carolina hemlock and kills healthy trees at any age. The long-term existence of both species is threatened and negative impacts on biodiversity are already apparent across large areas of the eastern US. Without effective suppression, HWA will probably eliminate the southern and mid-latitude range of eastern hemlock and potentially threaten Carolina hemlock with extinction. The scale of suppression needed for HWA has focused research on classical biological control with the goal of complete success over a wide geographical area. One of several promising species is *Laricobius nigrinus* and the large-scale release and monitoring phase for this species began with the research described here. The overall goal of this research was to release *L. nigrinus* in the eastern US and to determine if it can establish, reduce HWA density below physiologically damaging levels, and keep hemlocks alive.

Chapter 2 describes different release locations, numbers of predators, and timing of release at 22 sites between 2003 and 2005 in eight states. Recoveries of F<sub>3</sub> generation and increasing abundance over time indicate establishment at 59% of the sites. Release location was most related with predator establishment and abundance, HWA density, and hemlock vigor index. Cold locations were related with poor establishment or low abundance, declines in HWA density, and increases in hemlock vigor. Paired release and control sites detected a predator impact on HWA density, but HWA densities remained high and hemlock vigor declined sharply. Investment in releasing *L. nigrinus* appears worthwhile and collections from the coastal regions of the Pacific Northwest may prove most effective by focusing releases in hardiness zones 6b, 6a, which will increase the overall establishment rate and potential impact. Releases in zone 5b appear marginal for establishment but releases should continue in these locations. Small releases of 75 adults/site should be considered by forest health specialists for stands at an early stage of infestation at widely dispersed sites. Releases in the spring and sequentially (*i.e.* fall or winter and spring) may increase establishment and impact on HWA.

In chapter 3, a field insectary for propagating *L. nigrinus* was established by planting hemlocks, infesting them with HWA, and introducing *L. nigrinus* onto the trees. Beginning in 2004, HWA

and *Laricobius* spp. populations increased annually from which 305 adult *L. nigrinus* were collected and re-distributed to forests in 2007. Phenology and cage impact studies of *L. nigrinus*, *L. rubidus* LeConte, and HWA were studied and the presence of adults and immatures were synchronized with HWA. HWA survival and density were higher in the closed cage than the open cage and no cage treatments. HWA ovisac predation was less in the closed cage than the open cage and no cage. The ease of establishment, impact, and adaptability of *L. nigrinus* to a new environment and co-existence with *L. rubidus* indicate that these predators have potential for controlling HWA.

In chapter 4, we compared sampling of *L. nigrinus* with beat sheets for adults or branch clipping for immatures. In the Appalachians, beat sheet sampling resulted in false negatives as larvae were collected by branch clipping. In Seattle, both methods detected *L. nigrinus* because the predator was common. Predator : prey ratios were high at heavily infested sites in Seattle and low in the eastern US, where it has been released recently. In winter, beatsheets recovered more adults in the afternoon when temperatures were high and in the spring, adults were collected at all times of day. Immature predators were more frequently found in large HWA ovisacs (2-3 mm) than small ovisacs (1-2 mm). Results suggest that for low predator populations, branch clipping should be used in the eastern US. If beat sheet sampling is used in the fall or spring, it should be followed by branch clipping in the spring if no adults are collected. For high predator populations, we recommend use of beatsheets.

In chapter 5, partial life tables were constructed for HWA sistentes at four sites for 2 yr in Seattle. Unspecified causes of nymph and adult mortality were important and *L. nigrinus* was the dominant predator of ovisacs. Adult *L. nigrinus* abundance was positively related to HWA density and immature abundance was related to ovisac density. These results confirm that *L. nigrinus* was a good selection from the western North American range of HWA, and continued release in the eastern US is justified.

In chapter 6, the host searching behavior of *L. nigrinus* was studied to understand how it locates a tree and HWA. The predator orientated to a tree visually, fed when prey were present, and flew when absent. They periodically scanned the environment on uninfested plants by waving of

the antennae, head, and tarsi to potentially detect olfactory, visual, or mechanical cues. On an uninfested plant, the predators groomed, climbed the tree, and walked on needles more than on infested plants.

### **Future work**

To determine if *L. nigrinus* can successfully suppress HWA, long-term monitoring of release sites will need to continue. In addition, further studies on release methodology could be valuable. At sites where *L. nigrinus* established and control sites (see chapter 2), HWA sistentes density should be monitored over the long-term (10-20 yr) at 2 yr intervals to see if *L. nigrinus* can reduce the pest to below physiologically damaging levels. These surveys should include tree health measures and photos to relate to baseline data. At new release sites made subsequent to this study, cage exclusion experiments could be conducted without the need for control sites. Complete HWA life-table studies could be done at several eastern US sites, to better understand the population dynamics and impact of predation, winter mortality, and other factors. Comparisons of wild versus lab beetle releases on establishment and impact could improve the effectiveness of the program as well as the release of more cold adapted beetles from the northern Rockies.

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## Appendices

**Appendix 1.** Location of *Laricobius nigrinus* adult releases in 22 hemlock forests in the eastern US between November 2003 and March 2005.

Site no.	Location (State-site name)	UTM Coordinates	
		easting	northing
1	MA-Mt. Tom	0694037	4680509
2	PA-Rothrock	0268595	4504040
3	PA-Bear Run	0308873	4530115
4	MD-Finzel	0673537	4395792
5	MD-Rocky Gap	0699934	4397022
6	MD-Frederick	0287372	4378989
7	WV-Seneca	0594329	4239369
8	WV-Watoga	0579409	4217462
9	WV-Monongahela	0586510	4219612
10	VA-North Fork	0542872	4144202
11	VA-Big Stony	0538027	4141138
12	VA-Hurricane	0456464	4064121
13	VA-Highland	0453808	4060842
14	VA-Lick Ck.	0461976	4096147
15	VA-Dickey Ck.	0461389	4065773
16	NC-Hemlock Hill	0421416	4001859
17	NC-Holloway	0431471	4000032
18	NC-Ivy Ck.	0377050	3962858
19	NC-Locust Ck.	0390540	3964523
20	NC-Middle Ck.	0389830	3961853
21	TN-Laurel Ck.	0250526	3943370
22	GA-Overflow Ck.	0299391	3869325

**Appendix 2.** Branch clipping sampling dates at *Laricobius nigrinus* release sites to collect eggs and larvae.

Location	2005	2006	2007
MA-Mt. Tom	--	10 May	6 May
PA-Rothrock	3 May	4 May	--
PA-Bear Run	--	4 May	29 Apr
MD-Finzel	6 May	3 May	--
MD-Rocky Gap	--	3 May	30 Apr
MD-Frederick	--	3 May	27 Apr
WV-Seneca	25 Apr	27 Apr	--
WV-Watoga	26 Apr	27 Apr	--
WV-Monongahela	26 Apr	14 May	--
VA-North Fork	19 Apr	15 Apr	--
VA-Big Stony	19 Apr	15 Apr	--
VA-Hurricane	18 Apr	20 Apr	--
VA-Highland	--	24 Apr	18 Apr
VA-Lick Creek	--	18 Apr	18 Apr
VA-Dickey Creek	--	20 Apr	18 Apr
NC-Hemlock Hill	6 Apr	16 Apr	--
NC-Holloway	--	16 Apr	2 Apr
NC-Ivy Creek	--	16 Apr	1 Apr
NC-Locust Creek	--	16 Apr	1 Apr
NC-Middle Creek	--	16 Apr	31 Mar
TN-Laurel Creek	4 Apr	9 Apr	--
GA-Overflow Creek	--	9 Apr	23 Mar

**Appendix 3.** Hemlock vigor index (HVI) values are determined by taking the index value for each parameter and then summing them. Values range from 0 to 100 and a higher HVI indicates a healthier tree.

No. new shoots:	No. dead tips:	Crown transparency:	Live crown ratio:
100.1+	30	0-2	95.1-99%
90.1-100	27	2.1-4	90.1-95%
80.1-90	24	4.1-6	85.1-90%
70.1-80	21	6.1-8	80.1-85%
60.1-70	18	8.1-10	75.1-80%
50.1-60	15	10.1+	70.1-75%
40.1-50	12		65.1-70%
30.1-40	9		60.1-65%
20.1-30	6	<45%	55.1-60%
10.1-20	3	45.1-50	50.1-55%
0-10	0	50.1-55	45.1-50%
		55.1-60	40.1-45%
		60.1-65	35.1-40
		>65.1	30.1-35
			<30
			0

**Appendix 4.** Changes in hemlock woolly adelgid density and eastern hemlock vigor parameters (mean  $\pm$  standard error) over time at *Laricobius nigrinus* release sites and paired control sites in the eastern US.

Location	Release site				Control site			
	2003/2004	2004/2005	2005/2006	2006/2007	2003/2004	2004/2005	2005/2006	2006/2007
<b>MA-Mt. Tom</b>								
% shoots infested	--	40.0 $\pm$ 11.0	37.5 $\pm$ 13.8	66.2 $\pm$ 19.0	--	0.1 $\pm$ 0.1	0 $\pm$ 0	0.3 $\pm$ 0.2
No. new shoots	--	42.8 $\pm$ 22.2	70.9 $\pm$ 23.8	59.1 $\pm$ 24.3	--	96.0 $\pm$ 9.7	111.9 $\pm$ 7.1	128.9 $\pm$ 7.6
No. dead tips	--	6.5 $\pm$ 2.4	7.0 $\pm$ 2.5	9.7 $\pm$ 3.7	--	2.9 $\pm$ 0.9	1.8 $\pm$ 0.6	3.0 $\pm$ 0.7
Live crown ratio	--	99.0 $\pm$ 0	98.2 $\pm$ 0.8	98.2 $\pm$ 0.8	--	99.0 $\pm$ 0	99.0 $\pm$ 0	99.0 $\pm$ 0
Transparency	--	62.0 $\pm$ 3.4	68.0 $\pm$ 5.4	67.0 $\pm$ 8.1	--	52.0 $\pm$ 0	54.0 $\pm$ 1.0	51.0 $\pm$ 1.9
Decline class	--	2.6 $\pm$ 0.6	3.2 $\pm$ 0.6	3.2 $\pm$ 0.6	--	1.0 $\pm$ 0	1.0 $\pm$ 0	1.0 $\pm$ 0
<b>PA-Rothrock</b>								
% shoots infested	19.5 $\pm$ 3.2	35.8 $\pm$ 8.3	53.4 $\pm$ 8.8	25.0 $\pm$ 8.0	3.2 $\pm$ 1.9	10.2 $\pm$ 4.9	14.6 $\pm$ 5.9	30.0 $\pm$ 11.1
No. new shoots	57.6 $\pm$ 5.4	20.6 $\pm$ 5.0	18.8 $\pm$ 5.6	17.7 $\pm$ 7.4	67.1 $\pm$ 4.5	54.0 $\pm$ 7.1	62.6 $\pm$ 9.9	85.3 $\pm$ 14.2
No. dead tips	2.1 $\pm$ 0.3	5.3 $\pm$ 0.9	8.5 $\pm$ 1.1	10.4 $\pm$ 1.9	1.7 $\pm$ 0.3	3.5 $\pm$ 0.5	3.0 $\pm$ 0.7	4.8 $\pm$ 1.1
Live crown ratio	96.5 $\pm$ 1.0	97.7 $\pm$ 0.9	97.4 $\pm$ 0.7	97.8 $\pm$ 0.6	98.6 $\pm$ 0.4	98.6 $\pm$ 0.4	95.0 $\pm$ 1.8	97.7 $\pm$ 0.9
Transparency	47.0 $\pm$ 1.9	51.5 $\pm$ 1.8	50.5 $\pm$ 2.0	50.0 $\pm$ 2.1	47.5 $\pm$ 1.7	55.0 $\pm$ 1.5	55.0 $\pm$ 0.7	51.5 $\pm$ 1.3
Decline class	1.3 $\pm$ 0.1	1.6 $\pm$ 0.2	2.2 $\pm$ 0.2	2.4 $\pm$ 0.2	1.0 $\pm$ 0	1.7 $\pm$ 0.3	1.4 $\pm$ 0.2	1.5 $\pm$ 0.2
<b>PA-Bear Run</b>								
% shoots infested	--	16.9 $\pm$ 4.6	47.2 $\pm$ 6.0	32.4 $\pm$ 4.7	--	5.1 $\pm$ 2.7	14.3 $\pm$ 5.0	31.2 $\pm$ 6.1
No. new shoots	--	76.4 $\pm$ 11.3	44.3 $\pm$ 8.3	16.5 $\pm$ 5.5	--	97.0 $\pm$ 9.4	88.0 $\pm$ 11.6	61.0 $\pm$ 11.7
No. dead tips	--	2.3 $\pm$ 0.6	3.7 $\pm$ 0.7	7.2 $\pm$ 1.7	--	0.9 $\pm$ 0.2	1.4 $\pm$ 0.4	3.0 $\pm$ 0.8
Live crown ratio	--	96.1 $\pm$ 0.9	94.2 $\pm$ 1.2	95.1 $\pm$ 1.2	--	99.0 $\pm$ 0	99.0 $\pm$ 0.4	99.0 $\pm$ 0
Transparency	--	53.5 $\pm$ 1.3	55.5 $\pm$ 1.6	52.5 $\pm$ 2.1	--	51.0 $\pm$ 1.2	54.0 $\pm$ 1.5	52.5 $\pm$ 1.3
Decline class	--	1.5 $\pm$ 0.2	2.2 $\pm$ 0.3	2.6 $\pm$ 0.2	--	1.0 $\pm$ 0	1.4 $\pm$ 0.2	1.4 $\pm$ 0.2
<b>MD-Finzel</b>								
% shoots infested	42.3 $\pm$ 7.2	25.7 $\pm$ 8.6	44.0 $\pm$ 6.6	28.5 $\pm$ 6.6	3.9 $\pm$ 2.6	0.8 $\pm$ 0.4	11.6 $\pm$ 4.7	6.03 $\pm$ 3.4
No. new shoots	49.5 $\pm$ 15.3	56.3 $\pm$ 16.9	49.7 $\pm$ 11.7	51.4 $\pm$ 8.6	94.2 $\pm$ 6.8	143.7 $\pm$ 16.4	65.6 $\pm$ 7.8	85.0 $\pm$ 4.7
No. dead tips	2.5 $\pm$ 0.7	7.2 $\pm$ 1.4	12.0 $\pm$ 2.7	4.7 $\pm$ 1.5	2.1 $\pm$ 0.5	3.7 $\pm$ 0.8	3.9 $\pm$ 1.0	2.6 $\pm$ 0.6
Live crown ratio	94.3 $\pm$ 1.1	93.8 $\pm$ 1.1	91.4 $\pm$ 1.2	90.4 $\pm$ 2.0	97.3 $\pm$ 1.0	97.7 $\pm$ 0.9	97.4 $\pm$ 0.7	98.2 $\pm$ 0.5
Transparency	50.5 $\pm$ 0.9	51.0 $\pm$ 1.5	43.5 $\pm$ 2.0	46.5 $\pm$ 1.1	53.5 $\pm$ 0.8	48.0 $\pm$ 1.1	51.0 $\pm$ 1.2	46.5 $\pm$ 1.1
Decline class	1.0 $\pm$ 0	1.9 $\pm$ 0.3	1.3 $\pm$ 0.1	1.0 $\pm$ 0	1.0 $\pm$ 0	1.0 $\pm$ 0	1.1 $\pm$ 0.1	1.2 $\pm$ 0.2

**Appendix 4. (Continued)**

Location	Release site				Control site			
	2003/2004	2004/2005	2005/2006	2006/2007	2003/2004	2004/2005	2005/2006	2006/2007
<b>MD-Rocky Gap</b>								
% shoots infested	--	38.4 ± 6.2	39.7 ± 7.8	44.4 ± 11.1	--	45.7 ± 7.3	78.3 ± 7.0	95.1 ± 2.5
No. new shoots	--	55.1 ± 6.1	44.4 ± 7.9	4.4 ± 1.7	--	49.3 ± 7.3	34.8 ± 7.7	7.7 ± 4.3
No. dead tips	--	1.7 ± 0.3	10.2 ± 1.5	7.0 ± 0.9	--	1.1 ± 0.4	6.3 ± 1.4	9.8 ± 1.8
Live crown ratio	--	98.4 ± 0.6	98.1 ± 0.6	99.0 ± 0	--	98.7 ± 0.3	97.9 ± 0.7	99.0 ± 0
Transparency	--	56.0 ± 0.5	61.0 ± 1.4	63.3 ± 1.9	--	56.7 ± 1.3	64.0 ± 2.0	71.0 ± 1.9
Decline class	--	2.4 ± 0.1	3.3 ± 0.2	3.1 ± 0.2	--	2.1 ± 0.2	3.3 ± 0.3	3.7 ± 0.2
<b>MD-Frederick</b>								
% shoots infested	--	18.2 ± 1.7	80.7 ± 2.1	100 ± 0	--	4.1 ± 4.1	2.0 ± 2.0	25.8 ± 11.6
No. new shoots	--	92.7 ± 42.2	108.6 ± 16.9	4.2 ± 2.7	--	41.4 ± 0.9	107.2 ± 28.7	61.1 ± 21.4
No. dead tips	--	5.9 ± 0.4	1.8 ± 1.2	14.7 ± 6.3	--	1.9 ± 0.9	0.7 ± 0.2	1.6 ± 0.6
Live crown ratio	--	99.0 ± 0	99.0 ± 0	97.0 ± 2.0	--	99.0 ± 0	99.0 ± 0	97.0 ± 2.0
Transparency	--	67.5 ± 2.5	72.5 ± 2.5	75.0 ± 0	--	72.5 ± 2.5	72.5 ± 2.5	72.5 ± 2.5
Decline class	--	4.0 ± 0	4.0 ± 0	4.0 ± 0	--	4.0 ± 0	4.0 ± 0	4.0 ± 0
<b>WV-Seneca</b>								
% shoots infested	18.8 ± 5.2	63.6 ± 13.0	26.5 ± 10.0	44.8 ± 12.4	14.3 ± 3.2	92.3 ± 6.1	17.9 ± 7.2	39.6 ± 7.0
No. new shoots	60.0 ± 3.6	21.9 ± 7.7	21.0 ± 8.1	25.3 ± 7.6	67.3 ± 8.2	8.7 ± 4.0	10.2 ± 2.4	37.9 ± 5.7
No. dead tips	3.5 ± 0.9	8.0 ± 2.0	7.4 ± 1.6	7.8 ± 2.3	10.1 ± 1.6	10.4 ± 1.3	9.9 ± 1.4	7.8 ± 1.3
Live crown ratio	93.8 ± 1.7	92.8 ± 1.8	94.8 ± 0.9	94.4 ± 1.1	78.5 ± 5.1	85.0 ± 3.1	82.0 ± 2.9	86.0 ± 2.6
Transparency	54.5 ± 1.2	60.0 ± 1.0	60.5 ± 2.2	60.5 ± 3.3	59.5 ± 1.7	65.0 ± 1.7	69.0 ± 1.8	65.5 ± 2.2
Decline class	1.0 ± 0	3.1 ± 0.3	3.2 ± 0.4	3.0 ± 0.3	1.8 ± 0.3	3.7 ± 0.2	3.7 ± 0.2	3.4 ± 0.2
<b>WV-Watoga</b>								
% shoots infested	18.9 ± 5.5	61.4 ± 8.1	53.2 ± 15.9	60.2 ± 8.7	1.9 ± 1.1	20.5 ± 9.3	10.9 ± 4.3	24.4 ± 7.4
No. new shoots	71.2 ± 5.6	41.1 ± 8.6	14.9 ± 6.9	36.4 ± 6.9	83.9 ± 5.5	33.8 ± 5.2	30.7 ± 6.1	48.9 ± 6.1
No. dead tips	5.1 ± 1.4	8.4 ± 2.0	6.0 ± 0.9	9.5 ± 2.2	3.6 ± 0.7	6.1 ± 1.3	6.1 ± 1.1	7.9 ± 6.1
Live crown ratio	92.7 ± 1.7	95.0 ± 1.6	93.3 ± 1.4	93.7 ± 1.3	95.0 ± 1.9	96.4 ± 1.2	94.1 ± 1.8	93.6 ± 1.9
Transparency	50.0 ± 1.0	60.5 ± 1.7	62.0 ± 1.5	60.5 ± 1.4	54.0 ± 1.2	54.0 ± 1.0	58.5 ± 2.2	54.5 ± 1.7
Decline class	1.0 ± 0	2.8 ± 0.1	3.7 ± 0.1	3.0 ± 0.2	1.0 ± 0	1.5 ± 0.2	2.1 ± 0.4	1.7 ± 0.2

**Appendix 4. (Continued)**

Location	Release site				Control site			
	2003/2004	2004/2005	2005/2006	2006/2007	2003/2004	2004/2005	2005/2006	2006/2007
<b>WV-Monongahela</b>								
% shoots infested	19.0 ± 3.5	48.4 ± 9.3	26.9 ± 6.8	43.9 ± 9.9	5.8 ± 1.8	31.3 ± 8.3	38.3 ± 13.7	45.3 ± 12.1
No. new shoots	61.5 ± 5.0	21.7 ± 5.2	20.1 ± 6.3	59.3 ± 14.7	61.9 ± 3.9	37.9 ± 4.5	17.5 ± 5.1	27.5 ± 10.4
No. dead tips	1.9 ± 0.4	11.4 ± 1.5	9.3 ± 1.2	10.3 ± 1.6	1.0 ± 0.3	6.0 ± 1.2	7.6 ± 1.2	8.2 ± 10.4
Live crown ratio	91.1 ± 2.4	91.1 ± 2.4	91.0 ± 1.6	93.2 ± 1.5	99.0 ± 0	99.0 ± 0	96.0 ± 1.2	92.0 ± 5.9
Transparency	56.0 ± 1.2	62.0 ± 2.0	64.0 ± 2.2	61.5 ± 2.2	55.0 ± 1.3	58.5 ± 1.8	58.0 ± 2.1	58.5 ± 2.9
Decline class	1.0 ± 0	2.6 ± 0.2	3.3 ± 0.3	2.8 ± 0.4	1.0 ± 0	1.9 ± 0.2	2.7 ± 0.3	2.7 ± 0.3
<b>VA-North Fork</b>								
% shoots infested	43.1 ± 5.8	58.3 ± 8.7	19.1 ± 7.1	37.9 ± 5.4	41.1 ± 8.1	59.9 ± 11.0	23.8 ± 4.7	47.8 ± 8.0
No. new shoots	77.5 ± 10.1	28.7 ± 9.0	69.9 ± 11.0	126.3 ± 12.3	83.7 ± 9.8	46.5 ± 13.4	54.2 ± 9.6	110.2 ± 19.7
No. dead tips	6.4 ± 1.3	7.4 ± 1.9	6.2 ± 1.4	7.0 ± 1.1	1.1 ± 0.2	3.3 ± 0.9	4.4 ± 1.2	6.8 ± 1.4
Live crown ratio	98.6 ± 0.4	98.6 ± 0.4	98.6 ± 0.4	98.2 ± 0.5	99.0 ± 0	99.0 ± 0	99.0 ± 0	99.0 ± 0
Transparency	52.0 ± 1.1	55.5 ± 1.4	60.0 ± 2.0	53.5 ± 2.2	52.0 ± 0.8	58.0 ± 1.7	58.5 ± 2.7	56.5 ± 2.6
Decline class	1.3 ± 0.1	3.0 ± 0.2	2.5 ± 0.3	2.6 ± 0.3	1.1 ± 0.1	2.4 ± 0.3	1.8 ± 0.3	2.1 ± 0.3
<b>VA-Big Stony</b>								
% shoots infested	23.7 ± 3.2	29.4 ± 5.0	6.1 ± 2.4	22.4 ± 4.6	41.1 ± 8.1	59.9 ± 11.0	23.8 ± 4.7	47.8 ± 8.0
No. new shoots	78.3 ± 8.4	27.7 ± 5.4	110.1 ± 8.4	111.5 ± 17.0	83.7 ± 9.8	46.5 ± 13.4	54.2 ± 9.6	110.2 ± 19.7
No. dead tips	3.5 ± 0.5	3.3 ± 0.6	3.1 ± 0.5	2.2 ± 0.3	1.1 ± 0.2	3.3 ± 0.9	4.4 ± 1.2	6.8 ± 1.4
Live crown ratio	99.0 ± 0	99.0 ± 0	99.0 ± 0	99.0 ± 0	99.0 ± 0	99.0 ± 0	99.0 ± 0	99.0 ± 0
Transparency	51.0 ± 1.2	59.0 ± 1.8	58.5 ± 2.1	55.0 ± 1.5	52.0 ± 0.8	58.0 ± 1.7	58.5 ± 2.7	56.5 ± 2.6
Decline class	1.2 ± 0.1	3.0 ± 0.3	2.5 ± 0.3	2.1 ± 0.3	1.1 ± 0.1	2.4 ± 0.3	1.8 ± 0.3	2.1 ± 0.3
<b>VA-Hurricane</b>								
% shoots infested	41.7 ± 7.6	53.5 ± 10.8	29.6 ± 9.2	80.7 ± 3.9	25.0 ± 5.1	48.1 ± 6.8	23.0 ± 6.9	55.9 ± 20.2
No. new shoots	80.6 ± 9.8	41.6 ± 12.5	19.1 ± 12.9	25.4 ± 6.8	67.3 ± 8.0	42.4 ± 8.8	35.7 ± 6.2	97.0 ± 18.3
No. dead tips	2.5 ± 0.6	4.3 ± 0.8	7.6 ± 1.0	8.2 ± 0.8	2.1 ± 0.6	5.6 ± 2.1	5.6 ± 1.7	6.3 ± 0.9
Live crown ratio	97.0 ± 1.0	98.0 ± 0.9	97.0 ± 1.3	98.0 ± 1.3	97.7 ± 0.9	98.1 ± 0.9	96.7 ± 1.6	96.4 ± 1.2
Transparency	53.0 ± 1.7	57.0 ± 1.7	59.3 ± 2.0	66.4 ± 2.1	55.5 ± 0.9	59.5 ± 2.0	62.0 ± 2.5	65.0 ± 2.1
Decline class	1.1 ± 0.1	2.3 ± 0.3	2.9 ± 0.3	3.7 ± 0.2	1.2 ± 0.1	2.5 ± 0.3	3.1 ± 0.2	2.9 ± 0.3

**Appendix 4. (Continued)**

Location	Release site				Control site			
	2003/2004	2004/2005	2005/2006	2006/2007	2003/2004	2004/2005	2005/2006	2006/2007
<b>VA-Highland</b>								
% shoots infested	--	58.0 ± 6.9	42.6 ± 8.4	59.8 ± 6.0	--	14.4 ± 5.1	6.5 ± 2.6	28.9 ± 5.6
No. new shoots	--	60.2 ± 7.8	23.1 ± 6.3	49.3 ± 10.3	--	61.4 ± 5.8	45.6 ± 4.9	72.5 ± 7.1
No. dead tips	--	7.3 ± 1.7	5.5 ± 1.2	12.9 ± 3.0	--	3.8 ± 0.7	5.6 ± 1.2	10.3 ± 1.8
Live crown ratio	--	98.5 ± 0.4	97.3 ± 0.7	98.2 ± 0.4	--	95.0 ± 1.0	95.3 ± 0.9	96.6 ± 0.5
Transparency	--	53.3 ± 1.5	54.7 ± 2.0	59.3 ± 1.9	--	55.7 ± 1.0	57.7 ± 1.1	61.0 ± 1.4
Decline class	--	1.9 ± 0.2	2.5 ± 0.2	2.9 ± 0.2	--	2.0 ± 0.1	2.3 ± 0.2	2.7 ± 0.2
<b>VA-Lick Creek</b>								
% shoots infested	--	34.6 ± 13.3	65.9 ± 10.2	54.8 ± 13.9	--	31.6 ± 10.4	43.5 ± 7.4	23.3 ± 7.5
No. new shoots	--	36.4 ± 8.1	30.0 ± 13.2	3.9 ± 2.4	--	39.1 ± 15.9	41.3 ± 17.4	34.8 ± 18.6
No. dead tips	--	9.5 ± 0.9	9.0 ± 1.2	23.1 ± 2.6	--	6.8 ± 2.4	6.5 ± 2.4	14.0 ± 4.8
Live crown ratio	--	99.0 ± 0	96.6 ± 1.0	97.2 ± 1.8	--	97.2 ± 1.8	97.2 ± 1.8	97.2 ± 1.8
Transparency	--	56.0 ± 2.4	59.0 ± 1.9	63.0 ± 3.0	--	58.0 ± 3.0	58.0 ± 2.5	65.0 ± 4.2
Decline class	--	2.2 ± 0.2	2.4 ± 0.2	3.2 ± 0.4	--	2.0 ± .04	2.8 ± 0.4	2.8 ± 0.6
<b>VA-Dickey Creek</b>								
% shoots infested	--	61.8 ± 29.9	71.0 ± 4.3	55.0 ± 11.6	--	63.9 ± 13.3	75.0 ± 0	31.2 ± 16.5
No. new shoots	--	53.8 ± 5.8	5.9 ± 4.4	16.7 ± 8.7	--	33.9 ± 11.4	2.2 ± 2.2	6.5 ± 0.5
No. dead tips	--	5.9 ± 0.4	15.6 ± 9.9	14.0 ± 5.0	--	6.1 ± 0.4	8.8 ± 1.2	9.3 ± 1.3
Live crown ratio	--	99.0 ± 0	99.0 ± 0	97.0 ± 2.0	--	97.0 ± 2.0	95.0 ± 0	95.0 ± 0
Transparency	--	55.0 ± 0	60.0 ± 5.0	67.5 ± 2.5	--	57.5 ± 2.5	65.0 ± 0	67.5 ± 2.5
Decline class	--	2.5 ± 0.5	3.0 ± 1.0	3.5 ± 0.5	--	2.5 ± 0.5	4.0 ± 0	4.0 ± 0
<b>NC-Hemlock Hill</b>								
% shoots infested	33.6 ± 9.3	85.7 ± 5.0	29.8 ± 16.4	68.8 ± 20.2	8.8 ± 6.9	36.8 ± 7.0	33.6 ± 11.1	65.7 ± 7.4
No. new shoots	120.6 ± 13.3	39.9 ± 12.2	1.4 ± 0.5	18.7 ± 4.4	124.7 ± 9.8	66.3 ± 14.1	16.6 ± 4.8	29.9 ± 8.1
No. dead tips	5.1 ± 0.9	13.3 ± 5.8	15.6 ± 5.0	16.4 ± 3.8	6.0 ± 0.8	6.5 ± 0.9	5.8 ± 1.2	7.3 ± 1.5
Live crown ratio	94.1 ± 2.3	91.2 ± 2.7	89.3 ± 2.4	90.4 ± 1.7	98.6 ± 0.4	95.2 ± 2.5	91.7 ± 2.4	97.0 ± 0.7
Transparency	54.5 ± 1.4	62.0 ± 2.4	69.5 ± 2.0	76.0 ± 0.7	53.0 ± 0.8	55.0 ± 0.7	61.0 ± 1.6	64.0 ± 1.9
Decline class	1.9 ± 0.2	3.2 ± 0.4	3.9 ± .01	4.0 ± 0	1.0 ± 0	1.2 ± 0.1	3.5 ± 0.3	3.6 ± 0.3

**Appendix 4.** (Continued)

Location	Release site				Control site			
	2003/2004	2004/2005	2005/2006	2006/2007	2003/2004	2004/2005	2005/2006	2006/2007
<b>NC-Holloway Gap</b>								
% shoots infested	--	32.9 ± 14.2	47.4 ± 10.2	53.3 ± 7.5	--	15.0 ± 6.7	24.1 ± 4.7	46.2 ± 10.0
No. new shoots	--	81.0 ± 12.1	19.2 ± 8.6	37.7 ± 14.0	--	82.9 ± 10.4	51.9 ± 4.7	72.6 ± 17.6
No. dead tips	--	4.9 ± 1.5	8.3 ± 2.4	17.4 ± 3.8	--	2.7 ± 2.0	2.4 ± 1.6	12.0 ± 7.2
Live crown ratio	--	96.4 ± 1.8	96.4 ± 1.8	96.4 ± 1.8	--	99.0 ± 0	99.0 ± 0	99.0 ± 0
Transparency	--	58.0 ± 1.2	65.0 ± 3.2	71.0 ± 5.8	--	57.0 ± 1.2	57.0 ± 1.2	60.0 ± 3.2
Decline class	--	2.4 ± 0.4	3.4 ± 0.4	3.8 ± 0.2	--	1.4 ± 0.4	1.6 ± 0.4	3.0 ± 0.4
<b>NC-Ivy Creek</b>								
% shoots infested	--	58.1 ± 8.1	70.7 ± 14.2	83.3 ± 16.7	--	18.5 ± 5.9	74.6 ± 6.6	57.2 ± 7.2
No. new shoots	--	59.0 ± 7.8	9.4 ± 6.2	0.1 ± 0.1	--	55.4 ± 3.1	24.1 ± 3.7	19.2 ± 5.8
No. dead tips	--	12.8 ± 2.2	12.5 ± 1.5	18.6 ± 1.5	--	2.3 ± 0.6	2.5 ± 0.7	10.3 ± 1.6
Live crown ratio	--	98.2 ± 0.4	93.4 ± 1.2	95.2 ± 1.2	--	97.9 ± 0.7	95.6 ± 1.0	97.3 ± 0.7
Transparency	--	59.0 ± 1.6	57.7 ± 1.7	70.0 ± 2.4	--	59.3 ± 1.0	59.7 ± 1.2	66.7 ± 1.2
Decline class	--	2.4 ± 0.3	3.1 ± 0.2	3.8 ± 0.1	--	2.8 ± 0.2	2.8 ± 0.2	3.5 ± 0.2
<b>NC-Locust Creek</b>								
% shoots infested	--	39.9 ± 2.3	100.0 ± 0	71.3 ± 1.0	--	12.9 ± 1.1	84.6 ± 5.4	56.0 ± 6.0
No. new shoots	--	78.6 ± 4.9	9.9 ± 9.9	13.1 ± 0.6	--	79.4 ± 2.6	4.9 ± 1.6	4.6 ± 2.6
No. dead tips	--	2.2 ± 2.2	4.5 ± 2.5	9.1 ± 0.4	--	1.0 ± 0	1.5 ± 0.2	8.9 ± 0.1
Live crown ratio	--	99.0 ± 0	99.0 ± 0	99.0 ± 0	--	99.0 ± 0	99.0 ± 0	99.0 ± 0
Transparency	--	47.5 ± 2.5	60.0 ± 5.0	62.5 ± 2.5	--	55.0 ± 0	50.0 ± 5.0	55.0 ± 0
Decline class	--	1.0 ± 0	2.5 ± 0.5	3.0 ± 0	--	1.0 ± 0	2.0 ± 0	2.0 ± 0
<b>NC-Middle Creek</b>								
% shoots infested	--	48.2 ± 8.9	88.3 ± 4.3	72.3 ± 7.5	--	13.0 ± 3.9	62.4 ± 7.6	15.1 ± 0
No. new shoots	--	61.4 ± 9.0	0.9 ± 0.5	13.9 ± 3.5	--	78.9 ± 7.8	30.1 ± 7.0	2.0 ± 2.0
No. dead tips	--	1.6 ± 0.8	4.6 ± 1.6	5.1 ± 1.3	--	2.0 ± 0.5	4.1 ± 1.0	12.1 ± 1.7
Live crown ratio	--	99.0 ± 0	99.0 ± 0	98.2 ± 0.5	--	99.0 ± 0	97.6 ± 1.4	99.0 ± 0
Transparency	--	57.5 ± 1.3	63.5 ± 1.5	66.5 ± 2.4	--	48.5 ± 1.8	56.0 ± 1.9	54.5 ± 1.9
Decline class	--	1.9 ± 0.3	3.6 ± 0.2	3.6 ± 0.2	--	1.1 ± 0.1	2.1 ± 0.3	3.1 ± 0.2

**Appendix 4.** (Continued)

Location	Release site				Control site			
	2003/2004	2004/2005	2005/2006	2006/2007	2003/2004	2004/2005	2005/2006	2006/2007
<b>TN-Laurel Creek</b>								
% shoots infested	23.6 ± 6.2	75.5 ± 5.6	63.3 ± 16.8	56.5 ± 8.4	27.9 ± 8.4	74.3 ± 6.1	46.0 ± 15.2	70.1 ± 6.8
No. new shoots	84.0 ± 6.6	41.2 ± 8.7	1.5 ± 1.1	22.2 ± 3.5	74.2 ± 4.8	35.8 ± 8.6	2.8 ± 1.5	23.2 ± 4.2
No. dead tips	1.8 ± 0.3	3.2 ± 0.9	9.9 ± 1.0	11.9 ± 1.4	0.6 ± 0.1	4.8 ± 1.0	9.2 ± 2.3	9.4 ± 1.7
Live crown ratio	99.0 ± 0	99.0 ± 0	98.2 ± 0.5	95.9 ± 1.9	97.4 ± 0.7	99.0 ± 0	96.8 ± 1.2	96.0 ± 1.1
Transparency	49.5 ± 0.5	53.0 ± 0.8	60.5 ± 1.4	62.0 ± 2.6	50.5 ± 0.9	58.8 ± 2.1	64.5 ± 2.3	67.5 ± 2.8
Decline class	1.0 ± 0	1.6 ± 0.2	3.9 ± 0.1	3.4 ± 0.2	1.0 ± 0	2.7 ± 0.3	3.7 ± 0.2	3.6 ± 0.2
<b>GA-Overflow Creek</b>								
% shoots infested	--	44.1 ± 9.2	80.6 ± 4.6	93.3 ± 3.6	--	1.1 ± 0.7	70.8 ± 8.3	84.2 ± 10.2
No. new shoots	--	55.1 ± 10.0	10.9 ± 5.6	5.7 ± 3.9	--	51.1 ± 5.6	46.8 ± 10.4	3.8 ± 2.2
No. dead tips	--	2.3 ± 1.1	7.2 ± 2.7	9.0 ± 2.0	--	0.4 ± 0.2	5.6 ± 2.3	5.2 ± 0.9
Live crown ratio	--	97.4 ± 1.0	94.6 ± 2.6	95.4 ± 2.2	--	98.2 ± 0.8	97.4 ± 1.0	96.4 ± 1.8
Transparency	--	59.0 ± 2.9	60.0 ± 0	68.0 ± 4.1	--	54.0 ± 1.0	57.0 ± 2.0	65.0 ± 0
Decline class	--	1.4 ± 0.2	3.4 ± 0.2	4.0 ± 0	--	1.0 ± 0	1.8 ± 0.4	3.6 ± 0.2