

Pre-release Evaluation of *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae), a Potential Biological Control Agent for the Hemlock Woolly Adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), in the Eastern United States

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Abstract

Hemlock woolly adelgid, *Adelges tsugae* Annand, is an invasive pest threatening eastern (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*T. caroliniana* Englem.) forests in the eastern US. A new predator, *Laricobius osakensis* Montgomery and Shiyake, has been found in association with *A. tsugae* in Japan. *Laricobius osakensis* was evaluated in a series of pre-release studies to assess its potential as a biological control agent for *A. tsugae*. Host-range studies indicated that *L. osakensis* is a specific predator that feeds predominantly and reproduces only on *A. tsugae*. The functional response—prey consumption changes in response to changes in prey density—was similar for both *L. osakensis* and *Laricobius nigrinus* Fender adults. However, *L. osakensis* had a higher numerical response—changes in oviposition in response to changes in prey density—than *L. nigrinus*. *Laricobius osakensis* larvae had a higher functional response than *L. nigrinus* larvae. *Laricobius osakensis*' higher numerical and functional response indicates that this species can potentially be more effective than *L. nigrinus*. In the evaluation of *L. osakensis* in sleeve cages in the field from December to April high rates of adult survival, feeding, and reproduction were found. A pair of predators in a cage killed on average five adelgids/day. Peak oviposition occurred in March and April. Larvae from eggs placed in the cages reached maturity in 28-50 days, depending on the season, and only 6.7 % died before

reaching maturity. *Laricobius osakensis* was able to survive, feed, develop, and reproduce in USDA cold-hardiness zones 5b and 6a of southwest Virginia. Behavior of *L. osakensis* and *L. nigrinus* was qualitatively similar but varied quantitatively. *Laricobius osakensis* was more active and had a lower association with *T. canadensis*. Interactions between species were minimal and not detrimental to either. Intrasexual copulation attempts were observed between males and to a lesser extent between females; however, intrasexual interactions were less frequent than intersexual interactions between the two species. Otherwise activity, including oviposition, was not altered by the presence of the other species. These studies indicate that *L. osakensis* has the potential to be a valuable addition to the natural enemies complex against *A. tsugae*.

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“No Man is an island” John Donne (1572-1631)

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Chapter 1 Introduction

1.1 Importance of hemlocks in the eastern US

Hemlocks are coniferous in the family Pinacea trees belonging to the genus *Tsuga* Carrière. The number of species described in this genus has changed over time, but contemporary authors generally accept ten species (Farjon 1990; Fralish and Franklin 2002; Hosie 1969; Vidaković 1991). Of the ten species of hemlock occurring worldwide, six occur naturally in Asia and four in North America (Hosie 1969). The species present in the eastern US are the eastern (*Tsuga canadensis* (L.) Carrière) and Carolina (*Tsuga caroliniana* Englem.) hemlocks. The eastern hemlock has the widest distribution of the North America *Tsuga* species (Fig. 1.1). It occurs in east Canada, from Lake Superior to Cape Breton, and northeast US. Its distribution reaches as far west as southern Ontario, northern Michigan, Wisconsin and eastern Minnesota, and in the southeast occurs across the Appalachian Mountains to north Georgia and north Alabama. It is also scattered in some locations east of the Mississippi river (Fig. 1.1) (Farjon 1990; Hosie 1969). In the northern part of its distribution it can be found from sea level to 800 m, but in the south it only occurs above 250 m up to higher elevations of 1500 m. It occurs in a variety of soil types with medium to high moisture and good drainage (Cheyney 1942; Vidaković 1991). Eastern hemlock is a late-successional dominant species that can be found in pure stands but more often is found in co-dominance with other species (Peattie 1950). It is one of the most long-lived trees in North America and dominates about 1×10^6 ha of forest within its range (Godman and Lancaster 1990; McWilliams and Schmidt 2000).

In contrast with eastern hemlock, the Carolina hemlock has the smallest range of the North American *Tsuga* species. It is endemic to the southern Appalachian region, occurring mainly on the Blue Ridge and in scattered populations from southwest Virginia to northeast Georgia (Fig. 1.1) (Farjon 1990; Fralish and Franklin 2002; Peattie 1950). It has more specific ecological requirements than other *Tsuga* species. In addition to requiring high moisture, it only occurs in mountainous areas between 600 and 1500 m elevation, on rocky, north/east-facing slopes or ridges and along stream in ravines. It is a rare and scattered tree which usually grows in mixed stands or in small near pure groves of a few individuals (Farjon 1990). In some places, as on the walls of rocky gorges, the Carolina hemlock can be found in association with the eastern hemlock. Carolina hemlock is usually smaller than eastern hemlock with maximum height of 20 to 25 m for the former and 30 to 40 m for the latter species (Farjon 1990). Both species can also be readily differentiated by their leaf arrangement, leaf morphology, and cone size (Fralish and Franklin 2002; Grimm 2002). Carolina hemlock branchlets have a more bristly appearance than eastern hemlock due to a different needle arrangement—eastern hemlock needles are mainly in one plain (2-ranked) and the Carolina hemlock needles spread in all directions. Looking closely at the foliage, eastern hemlock needles have serrated margins while Carolina hemlock needles have entire margins. Eastern hemlock cones have an ovoid-cylindrical shape and are half the size of the ovoid-elliptical shaped cones of the Carolina hemlock (Farjon 1990; Grimm 2002).

There is a general recognition hemlock forests' great ecological value (Cleaves 2008). Hemlocks are frequently considered a foundation species in several forest settings in the eastern US. Foundation species are tree species which define the forest structure

and control ecosystem dynamics (Ellison et al. 2005a). Hemlocks commonly grow along headwater streams, greatly influencing stream characteristics, like temperature and nutrient cycling, as well as their communities. Hemlocks have such a distinct influence on freshwater dynamics that streams flowing through hemlock forests support unique assemblages of salamanders, fish, and freshwater invertebrates that are intolerant of seasonal drying (Morkeski 2007; Ross et al. 2003; Snyder et al. 2002a). Several species depend greatly on the environment that exists in hemlock stands. Some, like the black-throated green warbler (*Dendroica virens*) and the blue-headed vireo (*Vireo solitarius*), are only present in forests with hemlocks (hemlock obligates) (Quimby 1995).

Hemlocks are widely appreciated for their aesthetics. Their grandiosity and gracefulness captivated Pennsylvania into making it the State Tree in 1931 (Rendell et al. 2003). Hemlocks are commonly planted as a tree, shrub, or hedge in ornamental landscapes in the US and Europe. At least 274 cultivars of eastern hemlock are known to exist, making it one of the most cultured landscape tree species (Quimby 1995; Swartley 1984). The recreational appeal of hemlock forests is evident by the millions of people that visit National Parks in eastern US every year, the several Appalachian Trail clubs and a specific recreational area in Pisgah National Forest specially dedicated to the Carolina hemlock (ATC 2009; Quimby 1995; ROSI 2008).

The economic value of hemlocks as timber and pulp is considerably low. However, there has been an effort to quantify economically the services provided by forests, as these resources are being threatened and forest managers need this information to evaluate cost/benefits of treatments (Cleaves 2008; Costanza et al. 1997; Costanza 1998; Farber et al. 2002; Heal 2000; Loomis et al. 2000). In the groundbreaking work of

Costanza et al. (1997), ecosystem services performed by forests have been valued at \$969 per hectare per year. Considering the specific case of hemlock forests, that excludes services as food production and raw material, the value of each hectare of hemlock forest per year was valued in \$788. This value does not account for ecosystems services known to be provided by hemlock forests such as habitat/refugia for species of conservation interest. Several studies have also been conducted to determine how much people valued hemlock forests economically, that is, how much they would be willing to pay to protect them (Cleaves 2008; Holmes et al. 2008; Moore and Holmes 2008). Generally, these studies found that the value attributed to hemlock forests greatly exceeded control costs for exotic species in those forests. Additionally, it has been found that in an urban environment, good tree cover (between 40-60%) around a property has been associated with 0.5 to 6% higher property values (Morales 1980; Sander et al. 2010).

1.2 Hemlock pests in the eastern US

There are at least 24 species of insects, native and exotic, that feed on eastern hemlock, but few cause significant damage (Godman and Lancaster 1990). Of the native pests, the hemlock borer (*Melanophila fulvoguttata* Harris) and the eastern subspecies of hemlock looper (*Lambdina fiscellaria* Guenee) are considered the most important. The immature stages of the hemlock borer are phloem feeders, excavating galleries just beneath the bark of the tree. It is considered a secondary pest, as it can only successfully attack a host that is stressed either by wind-throw, drought, excessive stand openings, or attacks by other primary pests (Allen 2000; USDA-FS 2000). The hemlock looper has a

very wide host range, feeding on a range of both broad-leaved and needle-bearing hosts. As this pest usually does not consume the whole needle, the partially discolored foliage results in a general browning of heavily infested trees (Allen 2000; USDA-FS 1992). Native hemlock scale pests, like the hemlock scale (*Abgrallaspis ithacae* Ferris), are usually innocuous and only occasionally reach pestiferous levels.

The most damaging pests are non-native, partly because these species do not have the natural enemy complex that controlled their populations in their native range. Three introduced scale species from Japan, the elongate hemlock scale (*Fiorinia externa* Ferris), shortneedle conifer scale (*Nuculaspis tsugae* (Marlatt)), and Cryptomeria scale (*Aspidiotus cryptomeriae* Kuwana), frequently grow to damaging populations causing defoliation, branch dieback and eventually death, especially when combined with other stress factors. The elongated hemlock scale can attack 40 species of conifers, 14 of which are native to the US (Stimmel 2000; USDA-FS 2002). Despite the relevant impacts of these other exotic pests, the most threatening pest to eastern US hemlock forests health is the hemlock woolly adelgid, *Adelges tsugae* Annand. *Adelges tsugae* can potentially lead to the widespread decline or even elimination of eastern and Carolina hemlocks throughout part or maybe all of their range (Orwig et al. 2002).

1.3 *Adelges tsugae* life cycle

Adelges tsugae is included in the Adelgidae family within the Aphidoidea (Hemiptera), which includes some of the most destructive introduced species in North American forests. Members of this family can be easily distinguished from the

Aphididae (Aphidoidea: Hemiptera) by the absence of siphunculi and the retention of oviparity in all generations, and from both Aphididae and Phylloxeridae (Aphidoidea) by feeding only on certain genera of Pinacea (gymnosperms) instead of angiosperms (Havill and Footitt 2007).

Genetic analysis of *A. tsugae* through its present range showed that this pest seems to be native to all hemlock ranges with the exception of the eastern US. The native population could be differentiated into four clades—China, Taiwan, northern and southern Japan. It is unclear whether HWA is endemic to western North America or if it was introduced. The haplotypes found in western North America were distinct from the others, but did not form their own clade. The population in southern Japan has been established as the origin of the population introduced into the eastern US (Havill et al. 2006). In all of its range, with the exception of eastern US, *A. tsugae* is not considered a pest, usually occurring in low and innocuous densities. These populations are thought to be kept under control by the action of both host resistance and natural enemies (Havill and Montgomery 2008; McClure 1989; McClure and Cheah 1999; Tredici and Kitajima 2004). In the eastern US, *A. tsugae* was first discovered in the 1950's on *T. canadensis* in Richmond, VA (Souto et al. 1996). The pathway of introduction of *A. tsugae* into the eastern US is unknown, but it is thought to have come on nursery stock from Japan (Cheah et al. 2004a; Havill and Montgomery 2008). In the introduced range, *A. tsugae* found highly susceptible hosts, eastern and Carolina hemlocks, and was released from the control by its specific natural enemies (Orwig and Foster 1998).

In both native and introduced range, *A. tsugae* has a multigeneration, polymorphic life cycle. In the native range, *A. tsugae* is cyclically parthenogenic and holocyclic, that

is, it has both asexual (parthenogenic) and sexual reproduction, and alternates hosts. The primary host, where sexual reproduction takes place, is *Picea torano* (= *P. polita*) (Koch) Koehne, and the secondary host, where asexual reproduction takes place, is a *Tsuga* species (Montgomery et al. 2009a). Five generations with correspondent different morphs compose the *A. tsugae* life cycle; three take place on the primary host and two on the secondary host. The three forms on the primary host are called sexualis, fundatrix and gallicola, the last one is the winged generation that migrates to the secondary host. The two forms on the secondary host are called exulis and sexupara, the last one is the winged generation that migrates back to the primary host. The exulis can be further differentiated into sistens if they have a period of diapause during the first instar, or progrediens if they do not have that diapause period. As *A. tsugae* primary host, *P. torano*, is not present in North America, *A. tsugae* life cycle in this part of its range is parthenogenic and anholocyclic. In western North America sexupara are not produced, but in the eastern US, *A. tsugae* continues to produce winged sexuparae that leave the hemlocks but die without finding a suitable host for the sexual generation (Havill and Foottit 2007). After hatching, the first instar progrediens or sistens nymphs (crawlers) disperse for suitable feeding sites at the base of the needles (McClure 1989). Unlike other adelgids, that feed on cortical parenchyma cells and phloem solutes, *A. tsugae* feeds on storage cells, the xylem ray parenchyma cells (Young et al. 1995). When a crawler finds a suitable site, it introduces the stylet bundle into the plant tissue up to the xylem ray parenchyma cells just below the leaf abscission layer, and secretes saliva that hardens around the stylet bundle forming a salivary sheath. Once settled, the sistens nymphs undergo an aestival diapause while progrediens nymphs continue to develop. The

nymphs develop through four instars before becoming adults (McClure 1989). Winged adults and crawlers are the only mobile stages of HWA life cycle (Havill and Footitt 2007). The seasonality of each generation depends on the environmental conditions at each location that determine a faster or slower development. In the eastern US, progredientes can be present from March, April or May to July, sistentes can be present from May or June to January or March, and the sexuparae can be present from June to July when the tree conditions deteriorate (Gray and Salom 1996; Joseph et al. 2011c; McClure 1989).

The spread of *A. tsugae* in the eastern North America is influenced by geographical variation in climate and host abundance. The rate of spread has been estimated as 8 Km/yr in the North and 15 Km/yr in the South (Evans and Gregoire 2007). *Adelges tsugae* has already spread through more than half of eastern hemlock geographic range and continues to expand its distribution, but may be approaching the limit of its potential expansion (Morin et al. 2009). Several factors contribute to this rapid spread. Despite *A. tsugae* limited mobility, its small size and woolly nature, predisposes it for dispersal by wind, birds and mammals. Airborne eggs, crawlers, and sexuparae were caught on sticky traps as distant as 1,350 m from the center of an infested stand and the source of potential airborne forms was vertically uniform across the tree. Of 16 species of birds caught in hemlock forests, 13 species were found to carry HWA on their bodies as far as 2 Km away from the nearest hemlock tree. Although eggs are also carried by birds, crawlers were present in larger numbers in all the birds (McClure 1990). White-tailed deer, a species increasingly abundant in much of North America and in some places considered a pest as well, has also an important role on *A. tsugae* dispersal

(Garrott et al. 1993; McClure 1990). Close to 80% of the seedlings browsed by deer and growing at 750 m from an infested site became infested. In contrast, only 25% of the non-browsed seedlings became infested at the same distance. This illustrates the relative lower efficiency of wind when compared to deer, in the dispersal of *A. tsugae* to greater distances. Other important means of long-distance dispersal are human assisted, such as the transportation of trees for nursery stock, barked logs and bark products from infested sites. Egg masses and crawlers are firmly attached to the bark and can survive several days in logs (McClure 1990).

1.4 *Adelges tsugae* impact

Eastern and Carolina hemlocks are highly susceptible to *A. tsugae* (McClure 1992b; Montgomery et al. 2009a; Pontius et al. 2006). Trees often show injury after only one year of infestation. Symptoms consist of discoloration, desiccation, loss of the needles, and dieback of branches. As the infestation progresses, crown vigor decreases eventually leading to the death of the tree (McClure 1991a). *Adelges tsugae* can be fatal to hemlocks of all ages in 4-15 years (McClure 1991a; 1996; Orwig et al. 2002). In some areas, mortality due to *A. tsugae* infestation was as high as 95% (Bair 2002; Mayer et al. 2002; Orwig and Foster 1998). As a foundation species, the loss of eastern hemlock due to *A. tsugae* has led to immediate, short- and long-term changes in ecosystem structure and function (Ellison et al. 2005a). Initially, hemlock death results in changes in the carbon and nutrient inputs in both soil and streams, which in turn impacts both soil and stream fauna taking advantage of the increased resources (Eschtruth et al. 2006; Orwig et

al. 2008). In the biweekly intervals, dead trees create novel habitat types in streams (fallen logs) and in the forest ground (Ellison et al. 2005a). In the bimonthly experiments, eastern hemlock does not naturally re-establish following mortality due to *A. tsugae*. It is often replaced by a different ecosystem type altogether, dominated by hardwoods birches (*Betula* spp.), oaks (*Quercus* spp.) and maples (*Acer* spp.), and by *Rhododendron* in riparian areas or yellow poplar (*Liriodendron tulipifera*) when *Rhododendron* is absent (Evans et al. 2011; Small et al. 2005; Spaulding and Rieske 2010; Stadler et al. 2005; Sullivan and Ellison 2006), resulting in a change in terrestrial and aquatic ecosystem processes (Daley et al. 2007; Ford and Vose 2007; Siderhurst et al. 2010; Stadler et al. 2005). This change in ecosystem type leads to the loss of arthropod (Adkins and Rieske 2013; Dilling et al. 2007; Ellison et al. 2005b; Rohr et al. 2009; Sackett et al. 2011), bird (Allen et al. 2009; Stodola et al. 2013; Swartzentruber and Master 2005; Tingley et al. 2002), fish (Ross et al. 2003; Siderhurst et al. 2010), aquatic invertebrates (Snyder et al. 2002a; Willacker et al. 2009), amphibian (Brooks 2001), and plant (Eschtruth et al. 2006) species associated uniquely with hemlock forests.

Economic impact of *A. tsugae* for the eastern US has been calculated as 215.4 million dollars per year. This value accounts only for direct costs, such as: federal government expenditures (survey, research, regulation, management, and outreach) (4.3 million/yr), local government expenditures (tree removal, replacement, and treatment) (66 million/yr), household expenditures (tree removal, replacement, and treatment) (44 million/yr), residential property value losses (100 million/yr) and timber value losses to forest landowners (1.1 million/yr) (Aukema et al. 2011). It was also found that the

impact on residential property values is not restricted to the property where the infested trees are located, but also the surrounding areas (Holmes et al. 2006; Holmes et al. 2010).

1.5 *Adelges tsugae* control in the eastern US

1.5.5 Abiotic Factors

The amount of time on eastern hemlock tree can withstand *A. tsugae* attack can be affected by several environmental variables (Faulkenberry et al. 2009; Mayer et al. 2002; Orwig and Foster 1998; Orwig et al. 2002; Orwig et al. 2012; Pontius et al. 2006; Royle and Lathrop 2000; Sivaramakrishnan and Berlyn 2000; Young et al. 2000). Water stress has been consistently associated with a faster decline in tree health (Orwig and Foster 1998; Orwig et al. 2002; Pontius et al. 2006; Sivaramakrishnan and Berlyn 2000; Young et al. 2000). Latitude and longitude have also been found to be strongly correlated with *A. tsugae* infestation as it relates to the infestation front, that is, susceptibility of a hemlock stand to infestation depends on whether there are infested stands nearby (Faulkenberry et al. 2009; Mayer et al. 2002; Orwig et al. 2012). Other landscape characteristics such as elevation, slope, aspect, and terrain shape index, have also been found to be relevant but more inconsistently (often depends on the season) (Faulkenberry et al. 2009; Pontius et al. 2006; Royle and Lathrop 2000; Young et al. 2000).

Low winter temperature is the most significant mortality factor controlling *A. tsugae* population and limiting its spread in the eastern US (Evans and Gregoire 2007; Morin et al. 2009; Orwig et al. 2012; Parker et al. 1998; 1999). However, recent studies

have called attention to: 1) the potential effects of climate change, which would make previous unsuitable habitats, suitable, and may lead to the expansion of this pests' distribution (Paradis et al. 2008); and 2) the possibility that *A. tsugae* is adapting to the conditions in the introduced range, exhibiting a greater tolerance to colder temperatures (Butin et al. 2005; Skinner et al. 2003).

2.5.5 Host plant resistance and tolerance

While the eastern and Carolina hemlocks were found to be highly susceptible to *A. tsugae*, the Chinese hemlock (*T. chinensis* (Franch.) E. Pritz.) was found to be highly resistant to the pest (Montgomery et al. 2009a; Tredici and Kitajima 2004). Attempts have been made to create resistant hybrids that retain as much as possible the characteristics of the native species. Carolina and Chinese hemlocks crosses have been successful, with hybrids having intermediate resistance to *A. tsugae*. However, it was not possible to cross Chinese and eastern hemlocks (Montgomery et al. 2009a). Reports of hemlock individual trees and stands surviving, in areas otherwise devastated by *A. tsugae*, have raised doubts about the absence of innate resistance in eastern and Carolina hemlocks (Oten 2011; Radville 2012). In order to guide the search for innately resistant trees, potential characteristics that confer resistance have been studied (Ingwell et al. 2009; Lagalante et al. 2007; Oten 2011; Pontius et al. 2006). Terpenoid volatiles (Lagalante et al. 2007), foliar chemistry (Ingwell et al. 2009; Pontius et al. 2006), and physical (Oten et al. 2012) and chemical (Oten 2011) characteristics of the needle cushion have been associated with resistance. All these characteristics vary within

eastern and Carolina hemlock populations (Ingwell et al. 2009; Lagalante et al. 2007; Pontius et al. 2006), and the foliar chemistry has been associated with some level of resistance in these species (Pontius et al. 2006). Infusion of terpenoids onto infested clippings for control of *A. tsugae* has been performed, but with unfavorable results (Iosue 2008). Screening of surviving trees in Connecticut and New Jersey for resistance using grafting and artificial infestation was also performed (Radville 2012). The trees were found to not be innately resistant, but grafting was found to significantly reduce the *A. tsugae* density (Radville 2012).

Foliar concentrations of calcium, potassium, nitrogen, and phosphorous were found to be strongly correlated with *A. tsugae* densities. Higher nitrogen and potassium concentrations increased, while higher calcium and phosphorous concentrations decreased *A. tsugae* densities (Pontius et al. 2006). Nitrogen fertilization of hemlocks increased *A. tsugae* densities (Joseph et al. 2011a; Joseph et al. 2011b; McClure 1991b) and hindered the effect of chemical treatments (McClure 1992a). Nitrogen is a limiting nutrient for most plants and animals. Nitrogen fertilization increases tree health, which can potentially making it more capable of withstanding attacks, but also increases the nutritional quality and palatability of the tree for the pest (McClure 1991b; Pontius et al. 2006). However, in the case of *A. tsugae*, the increase in pest density has been found to severely outweigh the gains in tree health in the field. In cascade effect, the healthier *A. tsugae* from fertilized trees seem to also be more palatable for specific predators, leading to increased predation (Joseph et al. 2011a). Notwithstanding, fertilization of hemlocks with calcium and phosphorous can potentially be used to suppress *A. tsugae* densities (Pontius et al. 2006).

1.5.3 Cultural Control

In the initial phases of *A. tsugae* infestation, hemlock reproduction can be encouraged by regeneration cuts. In mature stands—average tree DBH 40 to 46 cm—a two- or three-cut shelterwood system (removal of residual canopy to promote seedling development) is recommended. In immature stands (average tree DBH < 38 cm), the removal of trees of lower vigor and thinning is more appropriate (Brooks 2004; Kelty 2000; Lancaster 1985).

1.5.4 Genetic resources conservation

In the event of the worst case scenario where the effective management of *A. tsugae* fails and eastern and Carolina are eliminated from most of their range, there is a program of genetic conservation with seed collections and ex-situ conservation (Jetton et al. 2008a). The first efforts have focused on Carolina hemlock due to its smaller population size and the fact that *A. tsugae* rapidly affected all of its geographic range (Jetton et al. 2008a). Seed collection throughout its range and the establishment of field *ex situ* conservation banks in Chile for Carolina hemlock were completed in 2006 (Jetton et al. 2008b). The genetic variability in the *ex situ* conservation banks was found to adequately reflect the genetic variability in natural stands of Carolina hemlock (Potter et al. 2010). The southern part of the geographic range of the eastern hemlock is more genetically diverse and is under greater threat from *A. tsugae* (Lemieux et al. 2011), as

such, seed collection started in this part of the range and was ongoing as of 2010 (Jetton et al. 2010). Seed collections on the central and northern part of the geographic range were planned to start in 2010. Ex site conservation banks for both Carolina and eastern hemlock were established in Brazil in 2010 (Jetton et al. 2010).

1.5.5 Chemical control

Foliar insecticides—insecticidal soap, horticultural oil, diazinon, dimethoate, ethion, fluvalinate, and malathion—were initially used and revealed good efficacy when care was taken to completely drench the branches for complete coverage (McClure 1987). Except for the insecticidal soap and oil, all the other insecticides have serious applicator risks, being classified as “most toxic” (PAN Bad Actor Chemical), and are moderately to highly toxic for beneficial insects. Another drawback to the use of these chemicals is the need for frequent, even annual, reapplication. Short-lived systemic insecticides—oxydemetonmethyl, dicrotophos, and acephate—followed and were found to provide excellent levels of control when injected or implanted through the bark of the tree (McClure 1992a). In the early 1990s, a new class of systemic insecticides, the neonicotinoids, became available. Several—imidacloprid, dinotefuran, acetamiprid, and thiamethoxam—have been found to successfully control *A. tsugae* populations (Cowles et al. 2006; Fidgen et al. 2002; Frank and Lebude 2011; Tattar et al. 1998; Werner and Cowles 2007). Imidacloprid was the first and is still the insecticide of this class most widely used against *A. tsugae*. Due to their systemic activity neonicotinoids can be applied by soil drench, soil injection, and several methods of trunk injection (Cowles et

al. 2006; Frank and Lebude 2011). Soil applications have been successful, but success with trunk injections has been inconsistent (Cowles et al. 2006; Doccola et al. 2007). Since xylem sap flow is essential for transporting and distributing the insecticide from the soil to the canopy of the tree, treatment should be applied prior to severe damage to the trees and several weeks allowed for the insecticide to be transported to where the adelgid feeds. Damaged trees have difficulty transporting and distributing the insecticide and the application takes longer to impact the *A. tsugae* population (Doccola et al. 2002). Treatment should be timed for spring or fall when water uptake by the trees is highest (Ford et al. 2007; Tattar et al. 1998). The timing of application for soil and trunk applications should also consider the time necessary—from 2 weeks to 3 months—for the insecticide to be transported up the tree and to affect the *A. tsugae* population (Fidgen et al. 2002; Frank and Lebude 2011; Tattar et al. 1998). Soil and trunk application of imidacloprid were found to take 1 to 3 months to control *A. tsugae*, while both dinotefuran and thiamethoxam were found to be faster (Fidgen et al. 2002; Frank and Lebude 2011). Residual activity of up to 3 years were observed for soil applications of imidacloprid, thiamethoxam, dinotefuran, and acetamiprid (Cowles et al. 2006; Dilling et al. 2010; Fidgen et al. 2002; Frank and Lebude 2011). Foliar applications provide faster control, but soil applications provide longer residual activity (Frank and Lebude 2011).

In a forest setting, chemical control is not viable on an area-wide basis, due to the costs involved and the environmental consequences of such applications (Cowles et al. 2006; Souto et al. 1996). Imidacloprid was found to significantly negatively affect soil microarthropod populations abundance (Knoepp et al. 2012); and canopy detritivore specific richness and abundance; phytophaga specific richness; and fungivore,

phytophaga, scavenger, and transient phytophaga abundance (Dilling et al. 2009). Conversely, secondary pest outbreaks of spruce spider and hemlock rust mites have also been associated with imidacloprid treatments for the control of *A. tsugae* (Raupp et al. 2004). Considering the close association of eastern hemlock with bodies of water, the possibility of insecticide leaching has to be considered as well. In Churchel et al. (2011), a study of four streams in the southern Appalachian region of Georgia and North Carolina where surrounding eastern hemlocks were treated with imidacloprid, found only a trace amount of imidacloprid in one water sample from one stream over a period of two years, and no effect was observed on the aquatic macroinvertebrates in that stream. While unlikely, leaching of imidacloprid to surrounding bodies of water is possible and especial care should be used when applying imidacloprid in areas with soil types that may not bind imidacloprid as tightly (e.g., low organic matter content) (Churchel et al. 2011). An optimization of the dosage for each tree and the adoption of tablet insecticide formulation should also minimize the risk of contaminating aquatic resources (Cowles 2009).

Despite the high costs of chemical control, the rapid decline of large older hemlocks due to *A. tsugae* infestation has led to the use of chemical control in forest areas to save these trees (Werner and Cowles 2007). Recently, the integration of chemical and biological control has also been proposed. The use of chemical control would save the larger trees and the smaller trees would sustain the increase of the biological control agents' population. When the larger trees no longer had chemical protection and were re-infested by *A. tsugae*, the biological control agents' population would have a larger population that would be able to keep the re-infestation under control (Salom et al. 2011). Although both lethal and sublethal effects have been observed on

the specific predators, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *Sasajiscymnus tsugae* (Sasaji and McClure) (Coleoptera: Coccinellidae), after feeding on *A. tsugae* on imidacloprid treated hemlock branches, when given the choice between insecticide treated and untreated branches, predators would not feed on treated branches (Eisenback et al. 2010). The use of chemical and biological control seems to be compatible, since it is likely that predators will only colonize treated trees when there is no insecticide activity.

1.5.6 Biological control

Adelges tsugae activity during the winter (McClure 1989), combined with its physiological defenses (feeding deterrents) (Jones et al. 2012), allow this pest to escape predation by most generalist predators. The limitations of chemical control in forest areas makes classical biological control a desirable option. Biological control can potentially protect the eastern hemlock stands with a cost effective, long term solution. The classical biological control program for the control of *A. tsugae* aims to re-establish the control of *A. tsugae* by its natural enemies complex seen in the native range in the introduced range (Legner and Bellows 1999; USDA-FS 2005).

1.5.6.1 Competition

A great population regulatory factor for *A. tsugae* in the eastern US is intraspecific competition. The survival and fecundity of *A. tsugae* is highest on new

growth. As the *A. tsugae* population increases, tree health deteriorates and trees are unable to produce new growth, and the *A. tsugae* population crashes in a negative density-dependent feedback mechanism. With the crash of the *A. tsugae* population, the tree is able to recover to produce some new growth, which in turn leads to a new increase in *A. tsugae* population. This cycle continues until the tree is no longer able to recover and dies (McClure 1991a).

As native pests rarely reach damaging populations, competition of these species with *A. tsugae* is unlikely. Both positive (Danoff-Burg and Bird 2002; McClure 2002; Preisser et al. 2008) and negative (Preisser and Elkinton 2008) interspecific interactions between the exotic pests, *A. tsugae* and *F. externa*, have been reported. The nature of the interaction (positive or negative) seems to be related to the timing of the infestation by *F. externa* in relation to *A. tsugae*. Initial infestation by *A. tsugae* seems to impact host tree defenses and facilitate secondary infestation by *F. externa*. The impact of both pests leads to a faster decline of the host tree (Danoff-Burg and Bird 2002; McClure 2002). When infestation by *A. tsugae* and *F. externa* was simultaneous, *A. tsugae* densities and damage to the host tree was lower than in infestations with just *A. tsugae* (Gómez et al. 2012; Preisser and Elkinton 2008).

1.5.6.2 Pathogens

Twenty fungal genera and 79 entomopathogenic fungi isolates were found in association with *A. tsugae* in the eastern US and southern China. Of all isolates, two strains of *Beauveria bassiana* Balasamo, one of *Lecanicillium lecanii* Zimmermann, one

of *Metarhizium anisopliae* Metchnikoff, demonstrated high efficacy against *A. tsugae* (Reid et al. 2010). *Beauveria bassiana* and *L. lecanii* were formulated with oil and whey carriers. The whey promotes the development of conidia in the absence of the host (Grassano 2008). These formulations were used in several small-scale forest trials and applied onto selected hemlock branches strongly infested with *A. tsugae* first instar nymphs (Costa et al. 2005; Gouli et al. 2008). Applications are only effective against stages that do not have the woolly protective cover (Costa et al. 2005). All field trials indicated significant reductions of HWA populations (Costa et al. 2005; Gouli et al. 2008). Two applications of fungal formulations in order to target the first instar nymphs in all the new growth, resulted in a 23% to 25% increase in *A. tsugae* mortality in relation to single applications (Gouli et al. 2008).

1.5.6.3 Predators

The first effort toward the implementation of biological control of HWA in eastern North America started in 1992. McClure (1995b) collected several predators from *A. tsugae* in Japan. From those predators, an oribatid mite, *Diapterobates humeralis* (Hermann), and a small coccinellid, *S. tsugae*, were imported for the biological control of *A. tsugae*. The mite was released in 1993 but did not survive the winter (McClure 1995a). *Sasajiscymnus tsugae* was imported, evaluated, mass reared and cleared for release in 1995 (Sasaji and McClure 1997). Field releases started in 1995 in Connecticut and more than 2 million *S. tsugae* have been released throughout the eastern US (Hakeem et al. 2010). These predators have also been made commercially available recently (Tree

SaversTM 2012). Post-release evaluations indicated successful overwintering, reproduction and dispersal of *S. tsugae*, but establishment of the predator, impact on *A. tsugae* populations, and recovery of hemlocks seem to be variable. Hakeem et al. (2010) monitored several *S. tsugae* releases in the southern Appalachian and was only able to recover this predator from locations where releases were made 5 to 7 years ago. This indicates that this species is actually establishing in several locations but, with the sampling method currently used, the predator population takes 5 to 7 years to reach a detectable level. *Sasajiscymnus tsugae* was found to be less effective in locations where the trees are subject to other stress factors besides *A. tsugae* infestation and with very high initial *A. tsugae* densities (Cheah et al. 2004a).

Additional foreign explorations for natural enemies were carried out from 1995 until 1997 in Yunnan, Sichuan and Shannxi provinces, in China. Three previously unknown lady beetles, *Scymnus camptodromus* Yu et Liu (Coleoptera: Coccinellidae), *Scymnus sinuanodulus* Yu et Yao (Coleoptera: Coccinellidae) and *Scymnus ningshanensis* Yu et Yao (Coleoptera: Coccinellidae), were found to be the most abundant predators in different locations (Montgomery and Keena 2011; Yu et al. 2000). The predators were imported, evaluated in quarantine and cleared for release (Butin et al. 2003; Butin et al. 2004; Lu and Montgomery 2001; Lu et al. 2002). However, due to difficulties in mass rearing the predators, *S. camptodromus* has not been released, and *S. ningshanensis* and *S. sinuanodulus* releases were delayed until 2004 (Cheah et al. 2004b; Cheah et al. 2004a).

In 1997, a predator native to western North America, *Laricobius nigrinus*, was found to consistently feed only on *A. tsugae* (Zilahi-Balogh et al. 2003). Beetles were

imported to Virginia for evaluation in a quarantine facility. The predator was cleared for release in 2000 and in 2003 releases started (Cheah et al. 2004a; Lamb et al. 2006). Since then, mass rearing has become more efficient and larger numbers are being released every year (Salom et al. 2008). *Laricobius nigrinus* is a specialist predator of *A. tsugae*. Its life cycle is synchronous with *A. tsugae*, and, though it might feed on other adelgids, it only develops on *A. tsugae* (Zilahi-Balogh et al. 2002; Zilahi-Balogh et al. 2003). Females oviposit within *A. tsugae* ovisacs from January to March. After hatching the larvae go through four instars and the mature larvae drops to the soil to pupate. Oviposition and subsequent larval development coincides with oviposition by the *A. tsugae* sistentes adults. The developmental time is inversely proportional to temperature, decreasing with increasing temperature. Eclosed adults remain in the soil in a state of aestival diapause that coincides with diapausing first instar *A. tsugae* sistentes. Adults resume activity in the fall coinciding with resumption of development by *A. tsugae* sistentes (Zilahi-Balogh 2001). Field studies using cage studies found that the adults feed on 3 to 6 *A. tsugae* sistentes nymphs per day (Lamb et al. 2006). Release methods and dispersal evaluations started in 2003. Of 22 release sites evaluated, 13 had established *L. nigrinus* populations. Establishment was correlated with minimum winter temperature and release size. Higher minimum winter temperature and larger release sizes promoted establishment (Mausel et al. 2010). *Laricobius nigrinus* was found to slowly disperse initially up the release tree and then away from the release tree at a rate of ≈ 39 m/yr. This information not only supports the use of this predator but also guides future monitoring as it indicates that sampling *L. nigrinus* only from the lower crown would likely underestimate its presence (Davis et al. 2012). Studies on predator interactions

between *L. nigrinus* and the previously released specialist, *S. tsugae*, and generalist, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), showed that individuals of one species in general avoid the individuals of the other species (Flowers et al. 2007), and feeding and reproduction were not affected by the presence of other species (Flowers et al. 2005; 2006).

A native eastern North America *Laricobius* species has also been found on *A. tsugae* infested hemlock—*Laricobius rubidus* Le Conte (Coleoptera: Derodontidae). This predator feeds mainly on pine bark adelgid, *Pineus strobi* Hartig, and prefers this prey when given the choice, but is able to feed and develop on *A. tsugae* when its preferred prey is not available (Zilahi-Balogh et al. 2005). Like *L. nigrinus*, *L. rubidus* females lay their eggs in woolly ovisacs, the larvae develop through 4 instars and the mature larvae drop to the soil to pupate. Although this species is not as synchronized with *A. tsugae* as *L. nigrinus*, it is also active from fall through mid-spring and there is a considerable overlap of feeding by both species (Mausel 2005; Mausel et al. 2008). Feeding and reproduction on *A. tsugae* of either *L. nigrinus* or *L. rubidus* were not affected by the presence of the other species (Story et al. 2012). *Laricobius nigrinus* and *L. rubidus* have been found to be interbreeding at sites where *L. nigrinus* was released in the eastern US (Havill et al. 2012). Ongoing studies are assessing whether the hybridization between these two species will enhance or hinder *A. tsugae* control (Fischer pers. comm.).

Further exploration in western North America, China and Japan continues to find new potential biological agents for evaluation. In western North America, *Scymnus coniferarum* (Crotch) (Coleoptera: Coccinellidae) was found in association with *A. tsugae*

in Washington State (Montgomery et al. 2009b; Montgomery et al. 2011a), and *Leucopis* spp. larvae and adults were found to be positively correlated to HWA densities on *T. heterophylla* in the states of Oregon and Washington (Grubin et al. 2011; Kohler et al. 2008). *Scymnus coniferarum* is a specific predator of adelgids. In the laboratory it was able to complete development from egg to adult on *A. tsugae* and *P. strobi* (Montgomery et al. 2011a). *Leucopis argenticollis* Zetterstedt and *Leucopis piniperda* Malloch larvae were found to feed and develop optimally on *A. tsugae*, but were also able to feed and develop on other adelgids (Grubin et al. 2011). Surveys in China in 2002 and 2003 discovered *Laricobius baoxingensis* Zilahi-Balogh and Jelínek and *Laricobius kangdingensis* Zilahi-Balogh and Jelínek (Gatton et al. 2009), and *Tetraphleps galchanoides* Ghauri (McAvoy et al. 2007), respectively, associated with *A. tsugae*. Neither *L. baoxingensis* or *L. kangdingensis* were successfully reared in the quarantine laboratory (Gatton 2005). Both *L. kangdingensis* and *T. galchanoides* were found to feed preferentially on *A. tsugae* (Gatton 2005; McAvoy et al. 2007). However, *T. galchanoides* was also found to feed on non-target adelgids, aphids, and even on *Laricobius* larvae (Montgomery et al. 2011a). In Japan, a new *Laricobius* species, *Laricobius osakensis* Montgomery and Shiyake (Derodontidae Coleoptera), was found in association with *A. tsugae* in the Osaka region in 2005 (Salom and Lamb 2008).

In 2007 the HWA Predator Release and Recovery Database was developed to standardize and centralize the information on the release, monitoring, and recovery of the predators introduced for the control of *A. tsugae* (Roberts et al. 2011).

1.6 *Laricobius osakensis* biology

Laricobius osakensis was discovered in May 2005 on *Tsuga sieboldii* Carrière in Osaka, Japan. In March 2006, A. Lamb and T. McAvoy went to Japan and brought three hundred adult beetles and several hundred larvae back to the Beneficial Insects Quarantine Laboratory at Virginia Tech. Several studies have since been carried out: development at two different temperatures, feeding and oviposition rates, and field studies in Japan (Lamb et al. 2011; Salom and Lamb 2008; Vieira et al. in press).

Several predators were found in association with *A. tsugae* in Japan, but *L. osakensis* in particular was found to be a key predator of *A. tsugae*. *Laricobius osakensis* adult females begin oviposition one to two weeks prior to *A. tsugae* oviposition (Vieira et al. in press), while *L. nigrinus* only starts oviposition when *A. tsugae* progredientes eggs are present (Zilahi-Balogh et al. 2003). However, it seems that once oviposition starts it does not stop regardless of the stage of *A. tsugae* offered or conditions. Field collected *L. osakensis* were already ovipositing and continued to do so in the laboratory regardless of the prey stage (2nd instar nymphs) (pers. obs.), and even at very low temperatures (0° C). At -7° C, oviposition stopped, but the predators survived and still showed signs of feeding. Emergence of *L. osakensis* occurs one month later (end of October) than *L. nigrinus*. It was discovered that larger larvae (3rd and 4th instars) were able to feed and develop on *A. tsugae* nymphs, something rarely observed in *L. nigrinus*. *Laricobius osakensis* also showed higher oviposition rate and faster larval development than *L. nigrinus* (Table 1.1). Pupae showed similar survival both at 12 and 15°C, 65 and 64%

respectively, and after 30 days most individuals at 15°C had molted (Salom and Lamb 2008).

Laricobius osakensis feeding and reproduction on *A. tsugae* were not affected by the presence of either *L. nigrinus* or *L. rubidus* (Story et al. 2012). Preliminary studies on the hybridization between *L. osakensis* and *L. nigrinus* indicate that they mate, but the eggs produced are not viable (Fischer et al. 2011).

1.7 Research rationale

Hemlock woolly adelgid continues to spread and kill eastern and Carolina hemlocks through the eastern United States (USDA-FS 2012). Several natural enemies have been released as part of a natural enemy complex for the control of *A. tsugae*. Yet, *A. tsugae* populations continue to reach densities that are lethal to eastern and Carolina hemlocks (Cheah et al. 2004a). As biological control continues to be the preferred option for the control of *A. tsugae* in forest areas, the search for additional predators to supplement the natural enemies complex already released into the eastern US continues (Onken and Reardon 2011a).

Laricobius osakensis has the potential to be an important contributor to the natural enemy complex for the control of *A. tsugae*. To ensure the selection of the best natural enemies and their safe introduction, a series of pre-release evaluations have to be conducted (Legner and Bellows 1999). Pre-release evaluations of *L. osakensis* will focus on 1) its host specificity; 2) feeding and oviposition potential compared with the previously released *L. nigrinus*; 3) behaviour and potential interactions with *L. nigrinus*;

and 4) survivorship, feeding and reproduction within sleeve cages in a field setting. Host range studies provide a tool for evaluating the environmental risk involved in the introduction of a given natural enemy. Recent studies had shown non-target effects of introduced biological control agents for arthropods. As a consequence, host range studies for these biological control agents are now recognized as a very important mandatory step (Butin et al. 2002; FAO and IPPC 2006; McEvoy and Coombs 2000; NAPPO 2006). Comparing feeding and oviposition of a potential natural enemy with a related previously released species provides evidence on whether the new natural enemy has an added value in relation to the natural enemies already released. Predator behavior and its interaction with the environment provided valuable information that can be used for its manipulation in rearing, releases and monitoring (Etzel and Legner 1999). With several natural enemies previously released, it is necessary to guarantee that they will not hinder each other's impact on the pest population (Mills 2006). Cage studies have been frequently used to evaluate natural enemies in the field. These studies provide insight into the natural enemy's potential to establish and impact the pests' population (Luck et al. 1999). Results from these pre-release studies can provide evidence of the low risk posed by the introduction of *L. osakensis*, and of its potential as a new addition of the natural enemies. The success of the predator in field trials would also justify future investment on this predator.

1.8 Objectives

1. Determine host range for *Laricobius osakensis* Montgomery and Shiyake

2. Determine and compare the functional and numerical response to *A. tsugae* density of:
 - a. Female and male *L. osakensis* and *L. nigrinus* adults
 - b. Larvae of *L. nigrinus* and *L. osakensis* (just functional response)
3. Determine *L. osakensis* survivorship, reproduction, development and feeding of both adults and larvae on *A. tsugae* populations in sleeve cages in the field throughout the winter and spring.
4. Determine the behavior of *L. osakensis* and *L. nigrinus* in individual and paired assays.
 - a. Determine the behavior pattern of female and male *L. osakensis* and *L. nigrinus* adult behavioral pattern during day and night, in spring and winter conditions.
 - b. Determine how the predators interact with the prey, the environment, and other predators.
 - c. Determine if the interactions between predators have potential indirect negative effects on their effectiveness as biological control agents.

Table 1.1 Mean development time (days \pm S. D.) of *Laricobius osakensis* eggs and larvae compared with the development time of the same stages for *Laricobius nigrinus*. Table taken from Salom and Lamb (2008).

		Developmental Rate (Days \pm S.D.)	
Temperature	Stage	<i>L. osakensis</i>	<i>L. nigrinus</i>
9°C	Egg	17.31 \pm 0.63	16.3 \pm 0.86
12°C	Egg	12.2 \pm 0.92	11.5 \pm 1.05
9°C	Larvae	24.14 \pm 5.33	32.8 \pm 2.92
12°C	Larvae	15.45 \pm 3.55	25.1 \pm 2.77

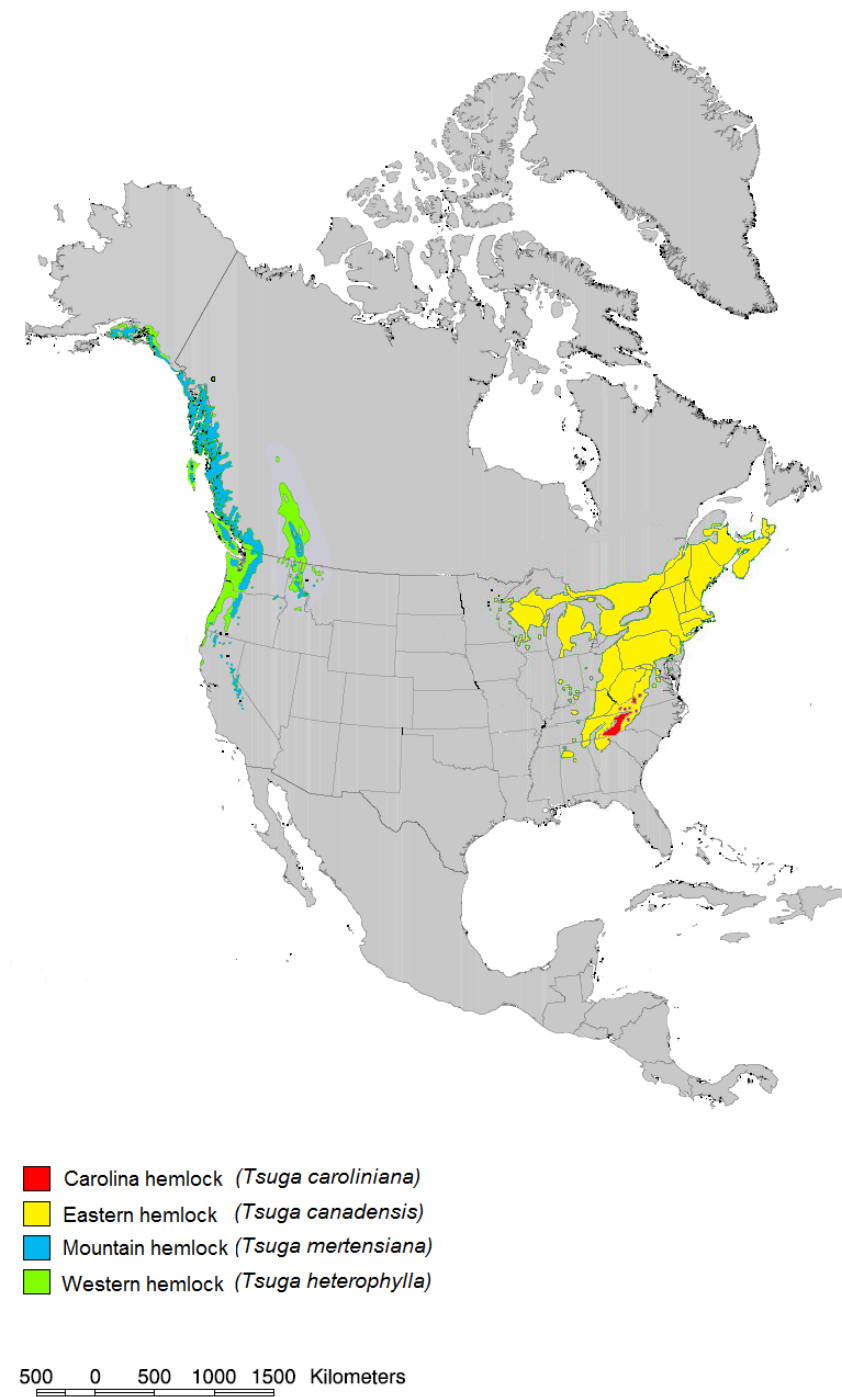


Figure 1.1 Geographic distribution of the four North America hemlock species – *Tsuga caroliniana* (red), *T. canadensis* (yellow), *T. mertensiana* (blue) and *T. heterophylla* (green). Redrawn from maps in USGS (1999) .

**Chapter 2 Host Range of *Laricobius osakensis* (Coleoptera: Derodontidae), a New
Biological Control Agent of Hemlock Woolly Adelgid (Hemiptera: Adelgidae)**

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Host Range of *Laricobius osakensis* (Coleoptera: Derodontidae), a New Biological Control Agent of Hemlock Woolly Adelgid (Hemiptera: Adelgidae)

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ABSTRACT Hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an invasive pest of eastern hemlock (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*T. caroliniana* Englem.) in eastern United States. Host-range tests for *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae), imported from Japan, were conducted under quarantine in Blacksburg, VA, to determine the suitability of this predator as a biological control agent of *A. tsugae*. Host-range testing for *L. osakensis*, involved no-choice and paired choice feeding, oviposition, and development tests with *A. tsugae*, three other adelgids, and three nonadelgid species. *L. osakensis* fed and laid more eggs on *A. tsugae* over all the other host species. The difference was greater in paired-choice tests. *L. osakensis* completed development only on *A. tsugae*. The overall results of the host range study indicate that *L. osakensis* is a specific predator of *A. tsugae*, supporting its potential as a biological control agent, and is not a threat to nontarget species populations.

KEY WORDS Host range, biological control, *Laricobius osakensis*, *Adelges tsugae*, hemlock woolly adelgid

Hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) (Annand 1924) is the single greatest threat to hemlock forests in the eastern United States (Orwig et al. 2002). This insect is native to all hemlock ranges except in those found in eastern North America (Havill and Footitt 2007). In its native range it is not usually considered a pest as it very rarely reaches outbreak numbers (McClure 1989, Montgomery and Lyon 1995).

In North America, *A. tsugae* was first reported in the Pacific Northwest on *Tsuga heterophylla* (Raf.) Sargent and *T. mertensiana* (Bong.) Carrière, where both species are rarely injured (Annand 1924). A recent genetic analysis of *A. tsugae* across all its distribution proved that the population present in western North America is distinct, yet does not form its own clade (Havill et al. 2006). The fact that the population in western North America is under control both by the action of natural enemies and host resistance seems to indicate that *A. tsugae* might be native to this region (Havill and Footitt 2007). The same genetic analysis provided evidence that the clade introduced into the eastern U.S. came from southern Japan (Havill et al. 2006). In the eastern U.S., this invasive species was first detected on eastern hemlock, *T. canadensis* (L.) Carrière, in Richmond, VA, in the early 1950s (Souto et al. 1996).

Despite the relative low economic value of hemlocks as a timber species in the forest setting, there is a general recognition of their great ecological value (Snyder et al. 2002, Ross et al. 2003, Ward et al. 2004). Carolina hemlock (*Tsuga caroliniana* Englem.) is a rare endemic species to the Appalachian Mountains (Farjon 1990). The loss of this species in this area means the loss of the species worldwide (Ward et al. 2004). Hemlocks are also frequently dominant in several forest settings in the eastern U.S. greatly affecting the characteristics of the habitat where they are present (Tyrrell and Crow 1994, Ross et al. 2003, Morkeski 2007). Several species of birds, fish, invertebrates, amphibians, reptiles, mammals, and other plants depend greatly on the environment that exists in hemlock stands. Some species, like the black-throated green warbler (*Dendroica virens*) and the blue-headed vireo (*Vireo solitarius*), are only present in hemlock forests (hemlock obligates) (Degraaf and Chadwick 1987, Quimby 1995, Ross et al. 2004).

Natural control of *A. tsugae* in its native areas seems to be because of a combination of both tree resistance and natural enemies (McClure and Cheah 1999, Tredici and Kitajima 2004, Havill et al. 2008). Unlike other hemlock species, *T. canadensis* and *T. caroliniana* are highly susceptible to *A. tsugae* (Orwig and Foster 1998, Mayer et al. 2002). Infested trees show poor crown, reduced terminal branch growth and needle loss. This pest has been reported to be fatal to hemlocks of all ages in as little time as 4 yr (McClure 1991).

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Since its introduction, *A. tsugae* has already spread throughout 50% of 1.3 million ha of *T. canadensis* ecosystem and it seems to only be slowed by low temperatures (Parker et al. 1998).

In addition to the lack of resistance there are no known natural enemies of *A. tsugae* in the eastern U.S. (Montgomery and Lyon 1995, Wallace and Hain 2000). The only natural regulation of *A. tsugae* populations in the eastern U.S. appear to be weather (Parker et al. 1998) and negative density-dependent feedback mechanisms (McClure 1991).

In 1995, *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae), found in association with *A. tsugae* in Japan, was the first natural enemy released for the management of *A. tsugae* in eastern U.S. (Sasaji and McClure 1997, Cheah and McClure 1998). Other natural enemies that have been found across the natural range of *A. tsugae* have been released. These include one coccinellid, *Scymnus sinuatus* Yu et Yao (Cheah et al. 2004), and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), from western North America (Lamb et al. 2006). Despite all this effort, satisfactory control of *A. tsugae* has not yet been achieved.

Foreign exploration continues to find new natural enemies to augment the current assemblage of natural enemies being released (McAvoy et al. 2007, Kohler et al. 2008). *Laricobius osakensis* Montgomery and Shiyake (Montgomery et al. 2011) was discovered in May 2005 in association with *A. tsugae* on *T. sieboldii* saplings in Japan. In March 2006, 300 adult beetles and several hundred larvae were collected and constituted the parental line for the colony that has been reared in the Beneficial Insects Quarantine Laboratory at Virginia Tech.

Both adult and larvae of the genus *Laricobius* are predacious on woolly adelgids (Hemiptera: Adelgidae) (Lawrence and Hlavac 1979, Lamb et al. 2008). *L. nigrinus* was found to be a specialist predator of *A. tsugae* (Zilahi-Balogh et al. 2002). Its life cycle is synchronous with *A. tsugae* and only develops on this species, although it might feed on other adelgid species (Zilahi-Balogh et al. 2003). Postrelease evaluation of impact and establishment for this species has been encouraging (Mausel et al. 2008, Mausel et al. 2010). It is expected that *L. osakensis* will follow the same strong association with *A. tsugae*.

Preliminary field and laboratory studies for *L. osakensis* indicate promising attributes as biological agent of *A. tsugae*. Ongoing field studies in Japan indicate that this species is a key species in the control of *A. tsugae* populations (Lamb et al. 2008). Based on laboratory studies, this species has a lower temperature threshold and higher feeding, oviposition and larval development rate than *L. nigrinus*. It also begins oviposition sooner, 2 wk after emergence, while *L. nigrinus* only begins oviposition 2–3 mo after emergence. However, emergence of *L. osakensis* in the laboratory occurs in the fall, ~1 mo later than *L. nigrinus* (A.B. Lamb, unpublished data).

The primary objective of this study was to determine the host specificity of *L. osakensis*. A suitable

biological control agent should be specific to the target pest, *A. tsugae*, thus minimizing any nontarget or negative ecological effects (van Lenteren et al. 2006).

Materials and Methods

Prey acceptance and suitability tests were conducted in three separate periods between 2006 and 2009. No-choice adult feeding and oviposition tests were conducted in 2006 for *A. tsugae* and two test species, *Adelges piceae* (Ratzeburg) (Hemiptera: Adelgidae) and *Pineus strobi* (Hartig) (Hemiptera: Adelgidae). In 2008, adult feeding, oviposition, and larval development were evaluated in paired-choice tests using *A. tsugae* with one of five test species: *A. piceae*, *P. strobi*, *Fiorinia externa* Ferris (Hemiptera: Diaspididae), *Chionaspis pinifoliae* (Fitch) (Hemiptera: Diaspididae), and *Prociphilus tessellatus* (Fitch) (Hemiptera: Aphididae). In 2009, additional no-choice, paired-choice and development tests were performed. No-choice feeding and oviposition were evaluated using *A. tsugae* and four other test species: *A. piceae*, *Adelges abietis* (L.) (Hemiptera: Adelgidae), *F. externa*, and *P. tessellatus*. Paired-choice oviposition was evaluated using *A. tsugae* paired with one of three test species, *A. piceae*, *A. abietis*, or *F. externa*. Development tests were conducted using *A. tsugae* and five test species, *A. tsugae*, *A. piceae*, *A. abietis*, *P. strobi*, *F. externa*, and *P. tessellatus*.

Test beetles were collected using a beating tray from *A. tsugae*-infested *T. sieboldii* in Kobe, Japan (N34°44.445' E 135° 10.566') in 2006 and 2008. They were shipped for tests in the quarantine facility at Virginia Polytechnic Institute and State University, Blacksburg, VA.

Tests in 2009 used F₁ generation females produced by *L. osakensis* adults collected from *A. tsugae*-infested *T. sieboldii* in Kobe and Takatsuki, Japan (N34°57.565' E 135°36.681'). They were maintained in environmental chambers at 6°C, 12:12 (L:D) h, and 70–90% RH on field collected *A. tsugae* infested eastern hemlock twig cuttings. The resulting progeny (F₁ generation) that started emerging from aestivation as adults in November 2008 were used in this study.

Test Prey Species. Five species of alternate prey were selected for the host range tests based on taxonomic or ecological similarity to *A. tsugae*, and ecological importance. All test prey species belong to the order Hemiptera from five genera in three families (McClure 1989, Farjon 1990, Baker 1994, Kolk and Starzyk 1996, Desrochers et al. 2002, U.S. Department of Agriculture [USDA] 2002). They are listed in Table 1 with their associated host plants, place of origin, the times at which each stage is present in the field and the reason for being selected for the host range tests. Although *P. tessellatus* is generally considered a pest, it has ecological importance as prey for *Feniseca tarquinius* (Fabricius) (Lepidoptera: Lycaenidae), the only strictly carnivorous caterpillar in the U.S. (Scott 1997).

Test prey species collected from the field were on their secondary host plant at the time of the test. In 2006, both alternate test prey were collected in Vir-

Table 1. List of prey species used in host range tests for *L. osakensis* with prey origin and family, their plant hosts, times at which each stage (egg, nymph, wingless, and winged adult) is present, dormant (D) stage and reason, for selection in test

Prey	Plant host by genus	Origin	Eggs	Nymphs	Wingless adults	Winged adults	Selection rationale
Adelgidae							
Hemlock woolly adelgid	Primary: <i>Picea</i> (not present in U.S.)	Japan	Mar.–Apr.	Apr.–May	Feb.–June	June	Target
<i>Adelges tsugae</i> (Annand) ^a	Secondary: <i>Tsuga</i>		June–July	July–Nov. (D)			
Balsam woolly adelgid	Primary: <i>Picea</i>	Europe	June–July	Nov.–Apr. (D)	Aug.–Oct.	—	Taxonomic similarity
<i>Adelges piceae</i> (Ratzeburg) ^b	Secondary: <i>Abies</i>		Sept.–Oct.	June–Aug.	May–July		
Eastern spruce gall adelgid	Primary: <i>Picea</i>	Europe	May–June	Dec.–Apr. (D)	May	Aug.–Sept.	Taxonomic similarity
<i>Adelges abietis</i> (L.) ^c	Secondary: none	Eastern North America	Aug.–Sept.	June–Nov.	Aug.–Sept.		
Pine bark adelgid	Primary: <i>Picea</i>		Apr.–May	Dec.–Apr. (D)	April	May–Nov.	Taxonomic similarity
<i>Pinus strobi</i> (Hartig) ^d	Secondary: <i>Pinus</i>		June–Nov.	May–Nov.	May–Nov.		
Aphididae							
Woolly alder aphid	Primary: <i>Acer</i>	North America	Oct.–Mar. (D)	May–Sept.	May–Sept.	July Sept.	Ecological importance
<i>Prociphilus tessellates</i> (Fitch) ^e	Secondary: <i>Alnus</i>						
Diaspididae							
Pine needle scale	<i>Pinus</i> , <i>Picea</i> , <i>Tsuga</i> , <i>Chionaspis pinifoliae</i>	North America	Oct.–Mar. (D)	Apr.–June	July	—	Ecological similarity
(Fitch) ^f	<i>Abies</i>						
Elongated hemlock scale	<i>Tsuga</i> , <i>Picea</i> , <i>Abies</i>	Japan	Sept.–Apr. (D)	July–Sept.	Aug.–Sept.	—	Ecological similarity
<i>Florinia externa</i> Ferris ^g				May–Sept.	May–Sept. (D)		

^a McClure (1989).
^b USDA (1989).
^c Desrochers et al. (2002).
^d Martineau (1984).
^e Baker (1994).
^f Luck and Dahlsten (1974).
^g McClure (1978).

ginia: *A. picea* from Fraser fir, *Abies fraseri* (Pursh) in a mixed stand in Blacksburg and *P. strobi* from a *Pinus strobus* (L.) stand at Mountain Lake, Giles Co., VA. In 2008, all alternate test prey were collected in Virginia with the exception of *F. externa*, which was collected from a *T. canadensis* stand in Snyder Co., PA. *A. piceae* and *C. pinifoliae* were collected from *A. fraseri* and *T. canadensis*, respectively, from a mixed stand in Blacksburg, VA. *P. strobi* was collected from a *P. strobus* stand in Mountain Lake and *P. tessellatus* was collected from *Alnus serrulata* (Ait.) Willd in Big Stony Creek, Giles Co., VA. In 2009, *P. tessellatus* was collected from the same location as in 2008. *A. abietis* was collected from *Picea abies* (L.) Karst in Mountain Lake. *A. piceae* was collected from *A. fraseri* in an abandoned Christmas tree plantation in Laurel Springs, Ashe Co., NC. *F. externa* was collected from an ornamental *T. canadensis* tree in Boone, Watauga Co., NC. *P. strobi* was collected from *P. strobus* in the Cherokee National Forest, Monroe County, TN.

All stages (egg, nymphs, or adults) were offered in the feeding and oviposition tests according to the stage collected from the field at the time of the test. *L. osakensis* adults can feed on nymphs and adults alone, but will feed on eggs if they are present. For the development tests, with the exception of *P. tessellates*, only prey with eggs were used as *Laricobius* larvae are known to only feed on eggs in their development. However, *L. osakensis* later instars may be able to feed on nymphs as well. *P. tessellates* could only be found

on *A. serrulata*, the secondary host, where it reproduces asexually, giving live birth.

A. tsugae differs from all the other prey tested (Table 1) in that it undergoes aestival diapause in the summer and develops throughout the winter. In contrast, the other prey go through hibernal diapause in the winter and develop from spring through fall. As *L. osakensis* is synchronized with *A. tsugae*, it also undergoes aestival diapause and develops throughout the winter, when the alternate prey are dormant (A.B. Lamb, unpublished data).

To minimize damage to test prey in the families Adelgidae and Diaspididae, which remain attached to the host plants once crawlers settle, all test prey were left intact on their host. The test prey used occur naturally in a forest setting on conifers in Virginia and would likely be encountered by *L. osakensis* when it is released into the environment.

Prey Acceptance. Adult Feeding Tests. Prey acceptance by adult *L. osakensis* was examined through both no-choice and paired-choice feeding experiments using adults or eggs of each test prey.

No-choice feeding tests using adult prey were *A. tsugae*, *A. piceae*, *A. abietis*, *P. tessellates*, and *F. externa* in 2009 and *A. tsugae*, *A. piceae*, and *P. strobi* eggs were used in 2006. Paired-choice feeding tests in 2008 were *A. tsugae* with either *A. piceae*, *P. tessellates*, *F. externa*, *P. strobe*, or *C. pinifoliae*. Each test was for 5, 3, and 7 d, respectively, with 8–10 replications. One female *L. osakensis* was used for each replicate, 24 *L. osakensis*

females were used in the tests in 2006 and 50 *L. osakensis* females were used in 2008 and 2009.

L. osakensis female adults, starved for 24 h to control satiation, were randomly assigned to 100 × 25 mm² polystyrene petri dishes (Nalgene Lab-Tek) containing one test prey (no-choice test) or hemlock woolly adelgid and an alternate test prey (treatments) on sections of the host plant (paired-choice test). Two layers of 90 mm diameter filter paper (GE Healthcare Whatman) were placed at the base of the petri dish.

When using adult prey, a sample of 10 individuals of each test prey, including hemlock woolly adelgid, was selected and measured. The volume for 20 *A. tsugae* adults and the number of a specific test prey with the same volume was calculated. Excess individuals were removed from the host plant with fine forceps.

When using test prey eggs, the eggs were counted before exposure to *L. osakensis*. For most test prey the eggs are loosely surrounded by waxy wool filaments and can be easily counted without compromising the integrity of the egg mass. In contrast, *A. tsugae* lays its eggs within tight woolly ovisacs making it difficult to count the eggs without destroying the ovisac. The equation $y = 19.41x - 27.95$, relating the number of eggs (y) in an ovisac and ovisac area (millimeters square) (x), was used to determine the number of eggs offered (Zilahi-Balogh et al. 2002). Before exposure to *L. osakensis*, the area of the *A. tsugae* ovisacs offered was determined using a dissecting scope with a calibrated ocular scale, and the number of eggs within estimated.

On completion of the test, *L. osakensis* adults were removed and the remaining prey adults or eggs were counted. Tests were conducted at 12°C in 2006 and 9°C in 2009, 12:12 (L:D) h, and 75–87% RH in an environmental chamber in a completely randomized experimental design.

Oviposition Tests. No-choice (single-prey) and paired-choice tests were conducted to evaluate acceptance and preference of test prey by *L. osakensis* for oviposition. No-choice treatments were: *A. tsugae*, *A. piceae*, and *P. strobi* in 2006, and *A. tsugae*, *A. piceae*, *A. abietis*, *P. tessellatus*, and *F. externa* in 2009. Paired-choice tests were *A. tsugae* with *P. strobi*, *C. pinifoliae*, or *P. tessellatus* in 2008, and with *A. piceae*, *A. abietis*, or *F. externa* in 2009.

Tests were conducted in 100 × 25 mm² (Nalgene Lab-Tek) plastic petri dishes lined with two layers of 90 mm diameter filter paper (GE Healthcare Whatman) at the base. *L. osakensis* females were randomly assigned to petri dishes that contained either a single test prey with their associate plant host (no-choice test) or *A. tsugae* plus another test species (paired-choice test). In the paired-choice tests, *A. tsugae* with test prey were placed 4–5 cm apart and the female beetle placed in the middle. Each treatment had 8–11 replications. One female *L. osakensis* was used for each replicate, 24 *L. osakensis* females were used in the tests in 2006, 44 were used in 2008 and 50 were used in 2009. The duration of each test was 5 d in 2006, 7 d in 2008 and 3 d in 2009 and counts were made on the number of *L. osakensis* eggs deposited on each test prey. Tests were conducted at 12°C in both 2006 and 2008 and at

9°C in 2009, 12:12 (L:D) h, and 75–87% RH in an environmental chamber using a completely randomized experimental design.

Prey Suitability. Survivorship of each stage of *L. osakensis*, from the egg to adult, was assessed on prey species (Table 1). Two approaches were used, monitoring individuals and in groups.

In 2009, procedures for development in groups followed the rearing procedure in place for *L. osakensis* and modified from Lamb et al. (2005). This was done to minimize disturbance and optimize development on all test prey species. When the prey were on branches or needles, clusters of infested twigs were arranged in rows in a rectangular piece of floral foam (≈15 × 10 × 2.5 cm) soaked with water and wrapped in parafilm. When the prey was on the bark, the undersides of the bark sections were waxed to limit the loss of moisture. Forty or 80 *L. osakensis* eggs, depending on availability, were transferred individually with a fine brush onto test prey. The clusters were placed into individual metallic funnels (25.4 cm diameter/top, 7.3 cm diameter/bottom, 20.3 cm height) with a metal grid and a plastic cylinder with ventilation on top and a glass jar at the bottom to collect the prepupal larvae that drop. In nature, mature larvae drop from the foliage to settle into the soil and pupate (Lamb et al. 2007). Less frequently, immature larvae accidentally dropped as well. The prepupal larvae stage can be distinguished from immature larvae as they curl into tight spheres when disturbed and remain like that for >10 s. Immature larvae cannot curl as tightly (there is space within the curl), are much more active (more movement), and return to an extended position in <10 s. The number and stage of larvae that dropped were recorded. Immature larvae were measured to determine larval stage and returned to the foliage in the funnel to complete development. Prepupal larvae were placed into a ventilated container filled up to 4 cm with a soil mix of one part sand, two parts peat moss, two parts sphagnum, and one part of water. These containers were placed in an environmental chamber at 15°C and 75–87% RH for pupation. Water was added weekly to maintain moisture and the temperature was manipulated during aestivation to synchronize adult emergence with *A. tsugae* breaking aestival diapause in the Fall (Lamb et al. 2007). The number of adults that emerged from each treatment was recorded.

Individual development was monitored in 2008 to determine the maximum development stage reached. This was done in a much more confined space. In 2008, 10 *L. osakensis* eggs were followed for each test prey. Each egg was transferred with a fine brush onto test prey in a 100 × 25 mm² polystyrene petri dish (Nalgene Lab-Tek) lined with two layers of 90 mm diameter filter paper (GE Healthcare Whatman). In 2009, similar assays were conducted, but instead of one egg per assay, six eggs were placed in each petri dish with four replications for each prey. Eggs of *L. osakensis* were examined daily for hatch. Larval stages were examined daily or every other day for survivorship at each stage. Fresh prey was added at each check. The

Table 2. Mean vol. of adults (mm³) or no. of eggs (±SE) of prey consumed per day, per *L. osakensis* female, in no-choice tests in 2006 and 2009 (—) indicate no data were collected

Test prey	n	Mean vol. of adults	n	Mean no. of eggs ± SE
		(mm ³) ± SE consumed per day/female in 2006 ^{a,b}		
<i>A. tsugae</i>	8	17.65 ± 2.11a	10	0.77 ± 0.06a
<i>P. strobi</i>	8	8.37 ± 0.94b	—	—
<i>A. piceae</i>	8	1.40 ± 0.34c	10	0.17 ± 0.01bc
<i>A. abietis</i>	—	—	10	0.31 ± 0.07b
<i>F. externa</i>	—	—	10	0.00 ± 0.00c
<i>P. tessellatus</i>	—	—	10	0.07 ± 0.01c

^a Means followed by the same letter for each year are not significantly different at *p* = 0.05, Tukey-Kramer honestly significant difference test.
^b 3-d test period.
^c 5-d test period.

maximum larval stage that *L. osakensis* reached in each assay was recorded.

Development data for both individual and group development tests were summarized to determine the overall maximum possible development of *L. osakensis* in each test prey. Group development tests involved much less disturbance and can provide a better indication of prey suitability. However, the limitation in disturbance also limits the information that can be obtained. Combining both assays allow for a complete set of data step by step (individual assay) and the reliability of the information provided by group assays (immature or prepupal larvae that dropped) when available.

Data Analyses. Box plots were used for detection of outliers. Extreme outliers were determined as being out of range of both box (25 and 75th quantiles) and whiskers (standard deviation) in the box plots. Three outliers were detected and removed when necessary before any statistical treatment. All data were tested for normality using goodness-of-fit test and for heterogeneity of variance through plots of the residuals.

Feeding no-choice tests were analyzed using a one-way analysis of variance (ANOVA). For the no-choice oviposition tests, as count data modeled by the Poisson distribution, the GLIMMIX procedure in SAS (SAS Institute Inc. 2008a) was used for the overall ANOVA. The Tukey-Kramer honestly significant difference test was used to determine significant differences among treatments in both tests.

Table 3. Mean no. of eggs (±SE) of *A. tsugae* and alternate test prey consumed per day, per *L. osakensis* female, in paired-choice tests for 7 d

Alternate prey	n	Mean no. of eggs consumed per day/female ± SE			<i>t</i>	df	<i>P</i> ^a
		<i>A. tsugae</i>	Alternate prey	Difference			
2008							
<i>A. piceae</i>	10	0.86 ± 0.13	0.24 ± 0.03	0.61 ± 0.13	4.56	9	0.0014
<i>P. strobi</i>	10	0.53 ± 0.05	0.29 ± 0.07	0.24 ± 0.10	2.24	9	0.0347
<i>P. tessellatus</i>	10	1.13 ± 0.17	0.19 ± 0.06	0.94 ± 0.21	4.42	9	0.0017
<i>F. externa</i>	10	0.51 ± 0.04	0.00 ± 0.00	0.51 ± 0.04	13.50	9	<0.0001
<i>C. pinifoliae</i>	10	0.86 ± 0.10	0.00 ± 0.00	0.86 ± 0.10	8.78	9	<0.0001

^a Significance at *P* = 0.05, Tukey-Kramer honestly significant difference test.

Table 4. Mean no. of eggs (±SE) laid per day, per *L. osakensis* female, in no-choice tests conducted in Mar. of both 2006 and 2009

Test prey	n	Mean no. of eggs laid per day/female ± SE ^a
2006 ^b		
<i>A. tsugae</i>	8	3.70 ± 0.67a
<i>A. piceae</i>	8	0.10 ± 0.05b
<i>P. strobi</i>	8	0.70 ± 0.11b
2009 ^c		
<i>A. tsugae</i>	10	1.10 ± 0.16a
<i>A. piceae</i>	10	0.30 ± 0.15b
<i>A. abietis</i>	10	0.20 ± 0.08b
<i>F. externa</i>	10	0.03 ± 0.04b
<i>P. tessellatus</i>	10	0.20 ± 0.12b

^a Means followed by the same letter for each year are not significantly different at *P* = 0.05, Tukey-Kramer honestly significant difference test.
^b 3-d test (12°C).
^c 5-d test (9°C).

In feeding and oviposition paired-choice tests, paired *t*-tests were used to determine prey preference. Larval development was summarized by the stages that *L. osakensis* reached for each test prey. The percentage of adults obtained on each host was modeled by a binomial response (success/fail to develop from egg to adult on the host). All statistical tests were carried out using JMP (SAS Institute Inc. 2007) and SAS (SAS Institute Inc. 2008b), at *P* = 0.05 significance level.

Results

Prey Acceptance. Adult Feeding Tests. *L. osakensis* adults fed on all test prey with the exception of *F. externa* and *C. pinifoliae*. In no-choice tests, during both test periods, significantly more adults or eggs of *A. tsugae* were eaten than adults or eggs of all other test prey species (*F* = 36.55; *df* = 23; *P* < 0.0001–2006 and *F* = 50.94; *df* = 49; *P* < 0.0001–2009) (Table 2). Significantly more *P. strobi* eggs were consumed than *A. piceae* eggs (*q* = 2.52; *df* = 7; *P* = 0.004), and a significantly greater volume of *A. abietis* adults were consumed than both *P. tessellates* (*q* = 2.84; *df* = 9; *P* = 0.003) and *F. externa* (*q* = 2.84; *df* = 9; *P* < 0.0001). In the paired-choice test of egg prey, *A. tsugae* was preferred over all four test species. *L. osakensis* did not feed on any *F. externa* or *C. pinifoliae* eggs (Table 3).

Table 5. Mean no. of eggs (\pm SE) laid per *L. osakensis* female on *A. tsugae* and on alternate test prey in paired-choice tests conducted in 2008 and 2009, with the duration of 7 and 3 d, respectively

Alternate prey	n	Mean no. of eggs laid per day/female \pm SE			t	df	P ^a
		<i>Adelges tsugae</i>	Alternate prey	Difference			
2008							
<i>A. piceae</i>	10	2.17 \pm 0.24	0.00 \pm 0.00	2.17 \pm 0.24	9.17	9	<0.0001
<i>P. strobi</i>	10	1.73 \pm 0.18	0.00 \pm 0.00	1.73 \pm 0.18	9.67	9	<0.0001
<i>P. tessellatus</i>	10	2.03 \pm 0.26	0.00 \pm 0.00	2.03 \pm 0.26	8.68	9	<0.0001
<i>F. externa</i>	7	1.73 \pm 0.21	0.01 \pm 0.01	1.71 \pm 0.21	8.26	6	<0.0001
<i>C. pinifoliae</i>	7	0.89 \pm 0.23	0.00 \pm 0.00	0.89 \pm 0.23	3.78	6	0.0092
2009							
<i>A. piceae</i>	10	1.10 \pm 0.59	0.00 \pm 0.00	1.10 \pm 0.59	5.88	9	0.0002
<i>A. abietis</i>	10	0.70 \pm 0.21	0.07 \pm 0.07	0.63 \pm 0.24	2.58	9	0.0298
<i>F. externa</i>	10	0.90 \pm 0.27	0.00 \pm 0.00	0.90 \pm 0.27	3.31	9	0.0091

^a Significance at $P = 0.05$, Tukey Kramer honestly significant difference test.

Oviposition Tests. In no-choice tests, during both test periods, *L. osakensis* females laid significantly more eggs in *A. tsugae* than in any other test prey ($F = 54.95$; $df = 21$; $P < 0.0001$ –2006 and $F = 9.37$; $df = 45$; $P < 0.0001$ –2009) (Table 4). Females laid at least one egg in all alternate test prey. In the paired-choice tests, *L. osakensis* laid eggs almost exclusively on *A. tsugae* in both 2008 and 2009 (Table 5), with the only exception being on *A. abietis*.

Prey Suitability. *L. osakensis* could only complete development to the adult stage on a diet of *A. tsugae* (Table 6). *A. piceae*, *A. abietis*, and *P. strobi* supported larval development to the fourth instar, as observed in group assays, but did not support further development. Larvae provided with *P. tessellatus*, *F. externa*, and *C. pinifoliae* died in the first instar.

Discussion

Test results on oviposition, feeding, and larval development indicate that *L. osakensis* is highly specific to *A. tsugae*. Although it can feed on other adelgid species, there is a distinct preference for *A. tsugae* in both feeding and oviposition tests, and it is only able to complete development on *A. tsugae*. Because *L. osakensis* failed to complete development on all other tests species, they are not considered suitable hosts. The preference is especially accentuated in the paired-choice oviposition tests. *L. osakensis* laid eggs on all test prey species, even on the petri dishes, when presented with no option. However, when given the

choice between *A. tsugae* and the other prey, it laid eggs almost exclusively on *A. tsugae*.

The amount of feeding is greatly reduced when only the alternate prey is provided. In addition, *L. osakensis* phenology under natural conditions would limit feeding on these alternate prey. Being active in winter, *L. osakensis* is synchronized with *A. tsugae* activity, while the alternate prey species are dormant. Thus, the window of time at which *L. osakensis* would have the opportunity to encounter these other species is very limited. This limited ability to feed on other prey species may make it difficult for *L. osakensis* to survive fluctuating *A. tsugae* populations. However, its threat to nontarget species is minimal at most. As *A. tsugae* will be always preferred if present, any feeding on alternate prey would be limited and development is very unlikely.

L. osakensis was maintained on *A. tsugae* before tests. This might have introduced bias toward *A. tsugae* in the feeding and oviposition tests, but not in the development tests, as the eggs were the stage transferred to the alternate prey. In this case, only *A. tsugae* supported *L. osakensis* development to the adult stage.

The information gathered (Table 6) clearly justifies the use of both individual and group approaches. The group assays wouldn't be able to provide information for prey in which *L. osakensis* larvae could not develop past the third instar, as it was the case for *P. tessellatus*, *F. externa* and *C. pinifoliae*. Similarly, the individual assays would not be able to provide reliable information on the maximum larval instar reached on each

Table 6. *L. osakensis* development on hemlock wooly adelgid and on the alternate test prey

Stage	<i>A. tsugae</i>	<i>A. piceae</i>	<i>A. abietis</i>	<i>P. strobi</i>	<i>P. tessellatus</i>	<i>F. externa</i>	<i>C. pinifoliae</i>
Egg	80	80	80	40	40	40	10
Instar 1	✓	✓	✓	✓	✓	✓	✓
Instar 2	✓	✓	✓	✓	—	—	—
Instar 3	✓	✓	✓	✓	—	—	—
Instar 4	✓	✗	✗	✗	—	—	—
Pre-pupae	✗ 71	—	—	—	—	—	—
Pupae	—	—	—	—	—	—	—
Adult	✗ 19	✗ 0	✗ 0	✗ 0	✗ 0	✗ 0	✗ 0

Individuals reaching the developmental stage feeding on test prey observed in individual assays are indicated with a ✓. Individuals reaching the developmental stage feeding on test prey observed in group assays are indicated with a ✗.

prey. This was the case for all the other prey. Using only individual assays one would conclude that *L. osakensis* couldn't develop on *A. tsugae*, which we knew was inaccurate because this predator has been reared in Virginia Tech on this host for years. It was also possible to determine that *L. osakensis* developed up to the fourth larval instar using *A. piceae*, *A. abietis*, and *P. strobi* instead of the third larval instar as observed in individual assays. The fourth larval instar was detectable on the group assays because, as previously discussed in the methodology, some immature larvae did drop to the jars.

L. osakensis follows the pattern previously described for other *Laricobius* species showing a feeding preference for adelgids. This is not surprising as previous records describe members of the genus *Laricobius* as adelgid specialists (Lawrence and Hlavac 1979). Based on our overall results, *L. osakensis* appears to be highly host specific on *A. tsugae*, preferring it over all the other prey species tested. This is a favorable attribute of a biological control agent. The possibility of nontarget effects of releasing this predator into the environment seems to be negligible and the potential benefit that could come with controlling *A. tsugae* largely surpasses the minimal risk.

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References Cited

- Annand, P. N. 1924. A new species of *Adelges* (Hemiptera: Phylloxeridae). Pan-Pacific Entomol. 1: 79–82.
- Baker, J. R. 1994. Woolly alder aphid ornamentals and turf. Department of Entomology insect note, ENT/ort-06 North Carolina University, North Carolina Cooperative Extension Service, NC.
- Cheah, C.A.S.-J., and M. S. McClure. 1998. Life history and development of *Pseudoscyrmnus tsugae* (Coleoptera: Coccinellidae), a new predator of the hemlock woolly adelgid (Homoptera: Adelgidae). Environ. Entomol. 27: 1531–1536.
- Cheah, C.A.S.-J., K. S. Shields, P. M. Moore, J. E. Ogronick, and G. L. Bradford. 2004. Reproductive strategies of *Scymnus* ladybeetle predators of HWA, p. 14. In K. W. Gottschalk (ed.), Proceedings, XV U.S. Department of Agriculture interagency research forum on gypsy moth and other invasive species. Annapolis, MD, U.S. Department of Agriculture Forest Service–Northeastern Research Station, General Technical Report NE-332.
- Degraaf, R. M., and N. L. Chadwick. 1987. Forest type, timber size class, and New England breeding birds. J. Wildl. Manage. 51: 212–217.
- Desrochers, P., C. Hébert, N. Laflamme, P. O'Donnell, and C. Turbis. 2002. Insects and diseases of Canada's forests. Natural Resources Canada. Canadian Forestry Service, Sainte-Foy, Quebec.
- Farjon, A. 1990. Pinaceae, drawings and descriptions of the Genera: *Abies*, *Cedrus*, *Pseudolarix*, *Keteleeria*, *Nothotsuga*, *Tsuga*, *Cathaya*, *Pseudotsuga*, *Larix* and *Picea*. Koeltz Scientific Books, Königstein, Federal Republic of Germany.
- Havill, N. P., and R. G. Foottit. 2007. Biology and evolution of Adelgidae. Annu. Rev. Entomol. 52: 325–349.
- Havill, N. P., M. E. Montgomery, G. Yu, S. Shiyake, and A. Caccione. 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Ann. Entomol. Soc. Am. 99: 195–203.
- Havill, N. P., C. S. Campbell, T. F. Vining, B. LePage, R. J. Bayer, and M. J. Donoghue. 2008. Phylogeny and biogeography of *Tsuga* (Pinaceae) inferred from nuclear ribosomal ITS and chloroplast DNA sequence data. Syst. Bot. 33: 478–789.
- Kohler, G. R., V. L. Stiefel, K. F. Wallin, and D. W. Ross. 2008. Predators associated with the hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific Northwest. Environ. Entomol. 37: 494–504.
- Kolk, A., and J. R. Starzyk. 1996. Larch woolly adelgid (*Adelges laricis* Vallot) and *Sacchiphantes abietis* (L.), p. 705. In Kolk A. and J. R. Starzyk (eds.), The Atlas of Forest Insect Pests (Atlas skodliwych owadów lesnych). Multico Publishing Company, Warsaw, Poland.
- Lamb, A. B., S. M. Salom, and L. T. Kok. 2005. Guidelines for rearing *Laricobius nigrinus* Fender, pp. 309–317. In B. Onken and R. Reardon (eds.), Third Symposium on Hemlock Woolly Adelgid in the Eastern United States, 1–3 February 2005. Morgantown, WV, U.S. Department of Agriculture Forest Service, FHTET 2005-01.
- Lamb, A. B., S. M. Salom, and L. T. Kok. 2007. Factors influencing aestivation in *Laricobius nigrinus* (Coleoptera: Derodontidae), a predator of *Adelges tsugae* (Hemiptera: Adelgidae). Can. Entomol. 139: 576–586.
- Lamb, A. B., S. M. Salom, L. T. Kok, and D. L. Mausel. 2006. Confined field release of *Laricobius nigrinus* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in Virginia. Can. J. Forest Res. 36: 369–375.
- Lamb, A. B., S. Shiyake, S. M. Salom, M. E. Montgomery, and L. T. Kok. 2008. Evaluation of the Japanese *Laricobius* sp. n. and other natural enemies of hemlock woolly adelgid in Japan, pp. 29–36. In B. Onken and R. Reardon (eds.), Fourth Symposium on Hemlock Woolly Adelgid in the Eastern United States, 12–14 February 2008. Morgantown, WV, U.S. Department of Agriculture Forest Service, FHTET 2008-01.
- Lawrence, J. F., and T. F. Hlavac. 1979. Review of the Derodontidae (Coleoptera: Polyphaga) with new species from North America and Chile. Coleopt. Bull. 33: 369–414.
- Luck, R. F., and D. L. Dahlsten. 1974. Bionomics of the pine needle scale, *Chionaspis pinifoliae*, and its natural enemies at South Lake Tahoe, Calif. Ann. Entomol. Soc. Am. 67: 309–316.
- Martineau, R. 1984. Insects harmful to forest trees. Multi-science Publications Ltd., Canadian Forestry Service, Environment Canada and the Canadian Government Publishing Centre, Supply and Services, Ottawa, Canada.
- Mausel, D. L., S. M. Salom, L. T. Kok, and J. G. Fidge. 2008. Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera: Derodontidae) on hemlock woolly adelgid in Virginia. Environ. Entomol. 37: 1498–1507.

- Mausel, D. L., S. M. Salom, L. T. Kok, and G. A. Davis. 2010. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the Eastern United States. *Environ. Entomol.* 39: 440–448.
- Mayer, M., R. Chianese, T. Scudder, J. White, K. Vongpaseth, and R. Ward. 2002. Thirteen years of monitoring the hemlock woolly adelgid in New Jersey forests, pp. 50–60. In B. Onken, R. Reardon, and J. Lashomb (eds.), *Proceedings: Hemlock Woolly Adelgid in the Eastern United States Symposium*, 5–7 February 2002. New Jersey Agricultural Experiment Station, Rutgers–The State University of New Jersey and U.S. Department of Agriculture, East Brunswick, NJ.
- McAvoy, T. J., G.M.G. Zilahi-Balogh, S. M. Salom, L. T. Kok, and Z. Guoliang. 2007. Development and feeding of *Tetraneura galchanoides*, a predator of the hemlock woolly adelgid. *BioControl* 52: 339–350.
- McClure, M. S. 1978. Seasonal development of *Fiorinia externa*, *Tsugaspidiotus tsugae* (Homoptera: Diaspididae), and their parasite, *Aspidiotiphagus citrinus* (Hymenoptera: Aphelinidae): importance of parasite-host synchronism to the population dynamics of two scale pests of hemlock. *Environ. Entomol.* 7: 863–870.
- McClure, M. S. 1989. Evidence of a polymorphic life cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). *Ann. Entomol. Soc. Am.* 82: 50–54.
- McClure, M. S. 1991. Density-dependent feedback and population cycles in *Adelges tsugae* (Homoptera: Adelgidae) on *Tsuga canadensis*. *Environ. Entomol.* 20: 258–264.
- McClure, M. S., and C.A.S.J. Cheah. 1999. Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. *Biol. Invas.* 1: 247–254.
- Montgomery, M. E., and S. M. Lyon. 1995. Natural enemies of adelgids in North America: their prospect for biological control of *Adelges tsugae* (Homoptera: Adelgidae), pp. 89–102. In S. M. Salom, T. C. Tigner, and R. C. Reardon (eds.), *Proceedings of the First Hemlock Woolly Adelgid Review*. Charlottesville, VA, U.S. Department of Agriculture Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV.
- Montgomery, M. E., S. Shiyake, N. P. Havill, and R.A.B. Leschen. 2011. A New species of *Laricobius* (Coleoptera: Derodontidae) from Japan with phylogeny and a key for native and introduced congeners in North America. *Ann. Entomol. Soc. Am.* (in press).
- Morkeski, K. 2007. In-stream hemlock twig breakdown and effects of reach-scale twig additions on Appalachian headwater streams. M.S. thesis, Master of Science, Virginia Polytechnic Institute and State University Blacksburg, Virginia.
- Orwig, D. A., and D. R. Foster. 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. *J. Torrey Botanical Soc.* 125: 60–73.
- Orwig, D. A., D. R. Foster, and D. L. Mausel. 2002. Landscape patterns of hemlock decline in New England due to the introduced hemlock woolly adelgid. *J. Biogeogr.* 29: 1475–1487.
- Parker, B. L., M. Skinner, S. Gouli, T. Ashikaga, and H. B. Teillon. 1998. Survival of hemlock woolly adelgid (Homoptera: Adelgidae) at low temperatures. *Forest Sci.* 44: 414–420.
- Quimby, J. W. 1995. Value and importance of hemlock ecosystems in the United States. In S. M. Salom, T. L. Tignor, and R. C. Reardon (eds.), *Proceedings of the First Hemlock Woolly Adelgid Review*, Charlottesville, VA. U.S. Department of Agriculture Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV.
- Ross, R. M., L. A. Redell, R. M. Bennett, and J. A. Young. 2004. Mesohabitat use of threatened hemlock forests by breeding birds of the Delaware river basin in northeastern United States. *Natural Areas J.* 24: 307–315.
- Ross, R. M., R. M. Bennett, C. D. Snyder, J. A. Young, D. R. Smith, and D. P. Lemarie. 2003. Influence of eastern hemlock (*Tsuga canadensis* L.) on fish community structure and function in headwater streams of the Delaware River basin. *Ecol. Freshwater Fish* 12: 60–65.
- SAS Institute Inc. 2007. JMP user's guide. SAS Institute, Cary, NC.
- SAS Institute Inc. 2008a. The GLIMMIX procedure, pp. 2078–2419, SAS/STAT 9.2 user's guide. SAS Institute, Cary, NC.
- SAS Institute Inc. 2008b. SAS/STAT 9.2 user's guide, SAS Institute, Cary, NC.
- Sasaji, H., and M. S. McClure. 1997. Description and distribution of *Pseudocymnus tsugae* sp. Nov. (Coleoptera: Coccinellidae): an important predator of hemlock woolly adelgid in Japan. *Ann. Entomol. Soc. Am.* 90: 563–568.
- Scott, J. A. 1997. The butterflies of North America: a natural history and field guide. Stanford University Press, Stanford, CA.
- Snyder, C. D., J. A. Young, D. P. Lemarie, and D. R. Smith. 2002. Influence of eastern hemlock (*Tsuga canadensis*) forests on aquatic invertebrate assemblages in headwater streams. *Can. J. Fish. Aquat. Sci.* 59: 262.
- Souto, D. R., T. Luther, and B. Chianese. 1996. Past and current status of HWA in eastern and Carolina hemlock stands, pp. 9–15. In S. M. Salom, T. C. Tigner, and R. C. Reardon (eds.), *First Hemlock Woolly Adelgid Review*, Charlottesville, VA, U.S. Department of Agriculture Forest Service, Morgantown, WV.
- Tredici, P. D., and A. Kitajima. 2004. Introduction and cultivation of Chinese hemlock (*Tsuga chinensis*) and its resistance to hemlock woolly adelgid (*Adelges tsugae*). *J. Arboric.* 30: 282–287.
- Tyrrell, L. E., and T. R. Crow. 1994. Structural characteristics of old-growth hemlock-hardwood forests in relation to age. *Ecology* 75: 370–386.
- (USDA) U.S. Department of Agriculture. 1989. *Adelges piceae* (Ratzeburg), p. 98. Insects and diseases of trees in the south. USDA Forest Service-Forest Health Protection, Southern Region R8-PR16.
- (USDA) U.S. Department of Agriculture. 2002. Elongated hemlock scale (Pest Alert) NA-PR-01-02. (<http://www.na.fs.fed.us/spfo/pubs/pestal/ehscale/ehscale.pdf>).
- van Lenteren, J. C., J. Bale, F. Bigler, H.M.T. Hokkanen, and A.J.M. Loomans. 2006. Assessing risks of releasing exotic biological control agents of arthropod pests. *Annu. Rev. Entomol.* 51: 609–634.
- Wallace, M. S., and F. P. Hain. 2000. Field surveys and evaluation of native and established predators of the hemlock woolly adelgid (Homoptera: Adelgidae) in the southeastern United States. *Environ. Entomol.* 29: 638–644.
- Ward, J. S., M. E. Montgomery, C. A. Cheah, B. P. Onken, and R. S. Cowles. 2004. Eastern hemlock forests: guidelines to minimize the impacts of hemlock woolly adelgid. U.S. Department of Agriculture Forest Service, Northeastern Area, State & Private Forestry, Morgantown, WV.
- Zilahi-Balogh, G.M.G., L. T. Kok, and S. M. Salom. 2002. Host specificity of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a potential biological control agent of the hemlock woolly adelgid, *Adelges tsugae*

- Annand (Homoptera: Adelgidae). Biol. Control 24: 192–198.
- Zilahi-Balogh, G.M.G., L. M. Humble, A. B. Lamb, S. M. Salom, and L. T. Kok. 2003. Seasonal abundance and synchrony between *Laricobius nigrinus* (Coleoptera: Derodontidae) and its prey, the hemlock woolly adelgid (Homoptera: Adelgidae). Can. Entomol. 135: 103–115.
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**Chapter 3 Functional and Numerical Response of *Laricobius* spp. Predators
(Coleoptera: Derodontidae) on Hemlock Woolly Adelgid, *Adelges tsugae*
(Hemiptera: Adelgidae).**

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Functional and numerical response of *Laricobius* spp. predators (Coleoptera: Derodontidae) on hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae)

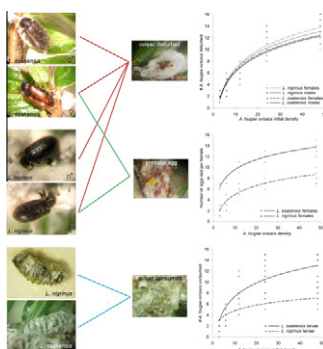
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HIGHLIGHTS

- *Laricobius osakensis* and *Laricobius nigrinus* are two specialized predators of *Adelges tsugae*.
- Functional and numerical response of adults and larvae of both species was assessed.
- Adults functional response was similar for both species.
- Numerical response was higher for *L. osakensis* across densities.
- *L. osakensis* larvae had a higher functional response than *L. nigrinus*.

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Biological control

ABSTRACT

Laboratory studies were carried out to determine the functional and numerical responses of the adults and larvae of *Laricobius osakensis* Montgomery and Shiyake and *Laricobius nigrinus* Fender to different densities of its prey, *Adelges tsugae* Annand. Males, females and larvae of both species showed a type II functional response. Overall attack rates and handling times for *L. osakensis* males and females were comparable. For *L. nigrinus* males and females, attack rates were similar but handling times differed significantly. Females of both species had the same attack rates and handling times, but *L. nigrinus* males showed a higher attack rate and longer handling time than *L. osakensis* males. Males killed significantly more *A. tsugae* adults than did females for both species, but there were no significant differences between species. The number of eggs laid by the predators (numerical response) as a function of *A. tsugae* density (d) was modeled by the equations: $n_e = 2.6691 \ln(d) + 3.4135$ and $n_e = 2.3863 \ln(d) - 0.5356$ for *L. osakensis* and *L. nigrinus*, respectively. *L. osakensis* laid significantly more eggs than *L. nigrinus* for all prey densities. While *L. osakensis* preferred to lay the eggs under the *A. tsugae* adults, most *L. nigrinus* eggs were in the wool. Attack rates were similar, but *L. osakensis* larvae handling time was significantly less than that of *L. nigrinus* larvae. The higher numerical response, combined with the higher functional response of *L. osakensis* larvae indicates that this species can potentially be more effective than *L. nigrinus*.

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1. Introduction

Hemlock forests in the eastern US are being threatened by the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera:

Adelgidae) (Kizlinski et al., 2002; Orwig and Foster, 1998; Orwig et al., 2002). This exotic pest from Japan can colonize all *Tsuga* species, but is only fatal for the two species native to the eastern US, the eastern (*Tsuga canadensis* (L.) Carrière) and Carolina (*Tsuga caroliniana* Englemann) hemlocks (Havill et al., 2006; Montgomery et al., 2009). Resistance shown by other *Tsuga* species in the native range of the pest is believed to be due to the action of both natural

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enemies and host resistance, however, both conditions are not present in the eastern US (Havill and Montgomery, 2008; McClure and Cheah, 1999).

The expanding geographic distribution of *A. tsugae* in the eastern US poses serious challenges for resource managers (Anonymous, 2010; Cowles, 2009; Evans and Gregoire, 2007). In forests, the costs and environmental/health concerns make chemical control for this pest impractical (Cowles et al., 2006). Biological control, on the other hand, can potentially provide self-sustaining control, where agents can disperse to adjacent areas, ideally reducing the amount of interventions necessary (USDAFS, 2005).

The approach for biological control of *A. tsugae* has focused on building a predator community in the eastern US that collectively would be able to keep the pest population below damaging levels. So far three predators – *Sasajiscymnus tsugae* Sasaji (Coleoptera: Coccinellidae), *Scymnus sinuanodulus* Yu & Yao (Coleoptera: Coccinellidae), and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) – have been introduced in the eastern US for the biological control of *A. tsugae* (McDonald et al., 2008). Following some encouraging results of the releases of *L. nigrinus*, a specialized predator of *A. tsugae* from western North America, *Laricobius osakensis* Montgomery & Shiyake, a congeneric predator from Japan, has been studied and recently approved to be added to the current predator community (Mausel et al., 2010; Salom et al., 2008). *L. osakensis* was found to be a key predator from the native range of the *A. tsugae* present in the eastern US, and like *L. nigrinus* was also found to be a highly specialized predator of *A. tsugae* (Lamb et al., 2008; Vieira et al., 2011).

Previous studies indicated that *L. osakensis* and *L. nigrinus* should not interact antagonistically even at lower prey densities (Story, 2010). Knowledge of predator performance, including functional and numerical response, could further elucidate potential interactions. Functional response is the change in feeding rate of the predator with changes in prey density, and numerical response is the change in the predator population (reproduction and migration) as a response to changes in prey density (Solomon, 1949). Three types of functional response have been identified by Holling (1959), a linear increase (type I), an increase slowing up to a plateau (type II), and a sigmoid increase (type III). Insect predators frequently exhibit a type II response (Munyanza and Obrycki, 1997; Santos, 1975).

Predation of *A. tsugae* by adult *L. nigrinus* has been estimated in field cages (Lamb et al., 2006). However, no study has determined the functional response of both adults and larvae for either species. In order to have a comprehensive assessment of the potential impact of each species the whole life cycle should be considered. This study was undertaken to describe and compare the functional response of both adults and larvae of *L. nigrinus* and *L. osakensis* to *A. tsugae* density.

2. Materials and methods

2.1. Predators and prey source

2.1.1. Predator adults

L. osakensis adults were field collected in November and December 2009, from *Tsuga diversifolia* in Shiga Kogen, Shirane and Nikko localities in Japan. *L. nigrinus* adults were collected in February 2009 from *Tsuga heterophylla* in urban areas in Seattle, Tacoma and Olympia, WA. Both predators were maintained on field collected *A. tsugae*-infested eastern hemlock twig cuttings in environmental chambers at 9 °C, 12:12 (L:D) and 65–75% RH (Lamb et al., 2005).

2.1.2. Predator larvae

Predator larvae (3rd instar) used in the experiments were the progeny of wild *L. osakensis* adults collected in October 2010, from

T. diversifolia in Shiga Kogen, Japan; and *L. nigrinus* adults collected in March 2011 in urban areas in Seattle, WA. Both species were maintained on field collected *A. tsugae*-infested eastern hemlock twig cuttings in environmental chambers at 9 °C, 12:12 (L:D) and 65–75% RH.

To synchronize development, *L. osakensis* eggs and larvae were held at 11 °C, and *L. nigrinus* at 13 °C.

2.1.3. Prey

A. tsugae ovisacs offered to both adults and larvae of *L. osakensis* and *L. nigrinus* during both experiments were from a single branch of an eastern hemlock tree in Polk County, TN. The use of one branch in each trial minimized prey quality variability that can also affect predator functional and numerical response (Messina and Hanks, 1998).

2.2. Sex differentiation of adult predators

A total of 49 female and 97 male *L. osakensis*, and 89 female and 90 male *L. nigrinus* were identified by oviposition trials. During the trials, 31 *L. osakensis* and 6 *L. nigrinus* died. Oviposition trials were conducted in 55 × 20.3 mm² polystyrene Petri dishes lined with two layers of 42.5 mm diam. lightly moistened with distilled water, and *A. tsugae*-infested twigs. One specimen was assigned to each Petri dish, and placed in an environmental chamber at 9 °C, 12:12 (L:D) and 75–87% RH. After 3 days, *A. tsugae* ovisacs were searched for predator eggs. If eggs were found, the specimen was identified as a female, and in the absence of eggs, the specimen was identified as a male. Though less accurate for identifying males than by observing pupae, this method causes much lower mortality, and was appropriate since the experiments required ovipositing females.

2.3. Adult predators functional and numerical response

The functional and numerical response study was conducted from April 22 to 25, 2010, using *A. tsugae* adults with eggs from the sistentes generation as prey. The range of densities chosen was based on previous observations on the amount of prey consumed and used for oviposition by *L. osakensis* (Vieira et al., 2011) and *L. nigrinus* (Zilahi-Balogh et al., 2002). To provide the appropriate density, 40–50 mm twigs were cut from a single *T. canadensis* branch with 1/3 of each prey density. The three twigs generating the standard densities (3, 6, 12, 24 and 48 ovisacs) were placed in a floral foam cube (20 mm side) soaked in water and wrapped with parafilm in each trial. Trials were carried out in 110 mm diam. × 115 mm cylindrical containers with two 25 mm diam. ventilation holes on the sides covered with mesh, and the floral foam cube placed in the center.

Since adult specimens were field collected, the exact age is unknown. However, considering the information on the seasonal abundance of both species in their native environment, adult specimens of both species were likely 6–7 months old (Lamb et al., 2007; Zilahi-Balogh et al., 2002). Females used were mated.

Ten replicates were prepared for each density for *L. osakensis* males and *L. nigrinus* males and females. For *L. osakensis* females, only six replicates were prepared for each density due to the limited number of specimens available. One adult predator was placed in each container and maintained in an environmental chamber at 9 °C, 12:12 (L:D) and 75–87% RH for 72 h.

Twigs were examined under 20× magnification to count the number of ovisacs disturbed (all trials – functional response) and the number of eggs laid (only females – numerical response).

2.4. Functional response of predator larvae

The larval functional response study was conducted from April 16 to 23, 2011, using *A. tsugae* adults with eggs from the sistentes generation. The number of eggs within the *A. tsugae* ovisacs prior to the experiments was variable (76–207 eggs) with a mean \pm SE of 152.10 ± 15.43 .

To provide the appropriate density, twigs were cut from a single *T. canadensis* branch, adding up to the pre-determined prey densities (3, 6, 12, 24 and 48 ovisacs). The range of densities chosen was based on previous observations on the amount of prey consumed by 3rd instar *L. osakensis* and *L. nigrinus* larvae, and adjusting for prey stage (Story, 2010). Twigs generating these prey densities were placed in a $100 \times 25 \text{ mm}^2$ polystyrene Petri dish (Nunc Lab-Tek) lined with a 90 mm diam. filter paper (GE Healthcare Whatman).

Third instar *L. osakensis* and *L. nigrinus* (about 1 week from reaching the mature 4th instar) were randomly assigned to each Petri dish with the standard densities. Each density was replicated 10 times for each species. One single larva was used in each trial. The duration of the test was 7 days, after which the number of ovisacs consumed was recorded. Tests were conducted at 9 °C, 12:12 (L:D), and 75–87% RH in an environmental chamber using a completely randomized experimental design.

It is difficult to clearly differentiate an egg consumed by the predator larva from an egg that hatched, as in both cases only the chorion remains. However, evidence of larval feeding in ovisacs is distinctive – in undisturbed ovisacs some eggs do not hatch, while in an ovisac attacked by larvae, all eggs are consumed (personal observation). Consequently, only those ovisacs where all eggs were consumed were counted as eaten.

2.5. Adult predators behavioral information

When predators were removed from the trials, their location in the trial – hidden in the foliage or on the container walls – was recorded. In addition to the number of ovisacs disturbed, we also determined if the *A. tsugae* adult was alive after being disturbed and recorded the number of *A. tsugae* adults killed by the predator. *A. tsugae* adults that die naturally just dry out, becoming completely solid or the insides have a paste-like consistency. Those killed were generally punctured by the predator and liquid hemolymph was visible around or coming out of the wound. The location where the females laid the eggs and the number of eggs in each individual ovisac was also recorded.

2.6. Data analysis

The functional response of *L. osakensis* and *L. nigrinus* males, females and larvae on *A. tsugae* ovisacs was determined by the two-step approach suggested by Juliano (2001). The first step is the identification of the most appropriate type of functional response for the data, and the second step is the estimation of parameters such as attack rate and handling time by the use of a mechanistic model for that specific type of functional response, accounting for prey depletion as the trial progresses.

For the first step, the polynomial model,

$$N_e/N_0 = (\exp(P_0 + P_1N_0 + P_2N_0^2 + P_3N_0^3))/(1 + \exp(P_0 + P_1N_0 + P_2N_0^2 + P_3N_0^3)),$$

was fitted to the data using logistic regression and maximum likelihood (PROC CATMOD, SAS). N_0 is the initial number of prey, N_e is the number of prey attacked, and P_0 , P_1 , P_2 , and P_3 are the constant, linear, quadratic, and cubic parameters to be estimated. The most appropriate type of functional response for the data was then

determined by examining the slope of the relationship N_e/N_0 vs N_0 . For a type II functional response P_1 is negative because N_e/N_0 decreases initially as N_0 increases, while for a type III functional response the P_1 is positive and P_2 is negative, because N_e/N_0 increases and then decreases as N_0 increases.

For the second step of the analysis, nonlinear least-squares regression was used to estimate parameters that fit the data to the mechanistic model, accounting for prey depletion for the type of functional response determined in the first step. The model incorporating prey depletion for type II functional response is

$$N_e = N_0(1 - \exp[a(T_h N_e - T)]),$$

and for type III functional response is

$$N_e = N_0[1 - \exp[(d + bN_0)(T_h N_e - T)/(1 + cN_0)]],$$

where N_0 is the initial density, N_e is the number of prey attacked, T_h is the handling time per prey, T is the total time available, a , b , c , and d are constants related to the attack rate.

Comparisons of the functional response between males, females, and larvae of the two species were based on nonlinear least-squares with indicator variables. Significant differences of the attack rates (a) and handling times (T_h) between species were determined using the t -test.

The numerical response of the two species was modeled by nonlinear regression as a function of prey density offered, and compared using the GLIMMIX procedure following a Poisson distribution, blocked by density (SAS Institute Inc., 2008a). The number of *A. tsugae* killed by the adults was also compared using the GLIMMIX procedure in SAS following a Poisson distribution, blocked by density. The comparison of the location of each sex for both species and the oviposition site preferences for each species was performed using a contingency analysis. Statistical treatments were performed in SAS (SAS Institute Inc., 2008b) and JMP (SAS Institute Inc., 2009).

3. Results

3.1. Functional response of adult predators

We observed that *L. osakensis* females and males in some cases attacked HWA ovisacs, consumed eggs, but did not kill the adult. The consumption of both adults and eggs will have an impact on the prey population, therefore the response to density will be evaluated by the number of attacked HWA ovisacs regardless of the adult being killed or not.

The shape of the fitted curves (Fig. 1) indicated that the attack of *A. tsugae* ovisacs by *L. osakensis* and *L. nigrinus* males and females was best described by a type II functional response curve. The significant negative linear parameters (Table 1) further confirmed this pattern.

The relationship between the *A. tsugae* ovisac density (N_0) and the number of *A. tsugae* ovisacs attacked (N_e) by *L. osakensis* males (1), *L. osakensis* females (2), *L. nigrinus* males (3), or *L. nigrinus* females (4), accounting for prey depletion during the study, is illustrated in Fig. 2.

The attack rates for *L. osakensis* males, *L. osakensis* females, *L. nigrinus* males, and *L. nigrinus* females were 0.013, 0.015, 0.021 and 0.018, and the handling times were 3.55, 3.77, 4.33, and 3.63 h, respectively (Table 2). *L. nigrinus* males showed a significantly higher attack rate and longer handling time than *L. osakensis* males, and a significantly longer handling time than *L. nigrinus* females.

3.2. Numerical response of adult predators

For both species, the number of eggs laid by individual females increased as *A. tsugae* ovisac density increased, stabilizing at the

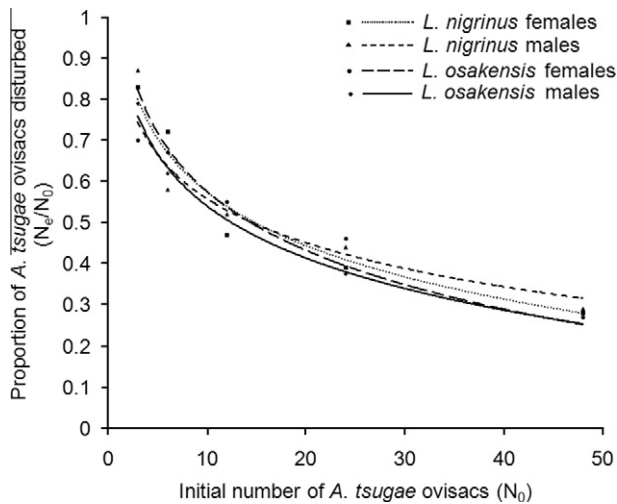


Fig. 1. Relationship between proportion of *A. tsugae* ovisacs disturbed and initial ovisac density for *L. osakensis* males (solid line), *L. osakensis* females (long dashes line), *L. nigrinus* males (short dashes line), and *L. nigrinus* females (dotted line).

Table 1

Maximum likelihood analysis of the response of *L. osakensis* and *L. nigrinus* females and males to varying densities of *A. tsugae* ovisacs. Significant negative linear parameter ($P < 0.05$) highlighted. Lo – *L. osakensis*, Ln – *L. nigrinus*, F – females, M – males.

Species	Sex	Source	Parameter	Estimate	SE	χ^2	P
Lo	F	Intercept	Constant (P_0)	1.9922	0.6163	10.45	0.0012
		N_0	Linear (P_1)	–0.2773	0.1146	5.86	0.0155
		N_0^2	Quadratic (P_2)	0.0109	0.00551	3.90	0.0484
		N_0^3	Cubic (P_3)	–0.00013	0.00007	3.48	0.0620
Lo	M	Intercept	Constant (P_0)	1.9785	0.6067	10.63	0.0011
		N_0	Linear (P_1)	–0.2872	0.1125	6.51	0.0107
		N_0^2	Quadratic (P_2)	0.0106	0.00543	3.80	0.0511
		N_0^3	Cubic (P_3)	–0.00012	0.00007	3.06	0.0802
Ln	F	Intercept	Constant (P_0)	1.9922	0.6163	10.45	0.0012
		N_0	Linear (P_1)	–0.2773	0.1146	5.86	0.0155
		N_0^2	Quadratic (P_2)	0.0109	0.00551	3.90	0.0484
		N_0^3	Cubic (P_3)	–0.00013	0.00007	3.48	0.062
Ln	M	Intercept	Constant (P_0)	1.2365	0.5977	4.28	0.0386
		N_0	Linear (P_1)	–0.1203	0.1137	1.12	0.2903
		N_0^2	Quadratic (P_2)	0.00361	0.00552	0.43	0.5134
		N_0^3	Cubic (P_3)	–0.00004	0.00007	0.37	0.5441

higher densities (24 and 48) (Fig. 3). The number of eggs (n_e) laid by *L. osakensis* or *L. nigrinus* females as a function of *A. tsugae* density (d) was modeled by the equations: $n_e = 2.6691 \ln(d) + 3.4135$ ($R^2 = 0.90$) and $n_e = 2.3863 \ln(d) - 0.5356$ ($R^2 = 0.88$), respectively.

L. osakensis females laid an average of 5.7, 7.8, 10.8, 11.2, and 12.8 eggs and *L. nigrinus* females laid an average of 1.8, 2.8, 5.8, 7.3, and 8.3 eggs, at *A. tsugae* ovisac densities of 3, 6, 12, 24 and 48, respectively. Overall, *L. osakensis* females laid significantly more eggs than *L. nigrinus* females ($F_{1,64} = 53.84$, $P < 0.0001$).

3.3. Functional response of predator larvae

The shape of the fitted curves (Fig. 4) and the significant negative linear parameters (Table 3) indicated that the attack of *A. tsugae* ovisacs by *L. osakensis* and *L. nigrinus* larvae was best described by a type II functional response curve.

The relationship between the *A. tsugae* ovisac density (N_0) and the number of *A. tsugae* ovisacs attacked (N_e) by *L. osakensis* larvae (1), or *L. nigrinus* larvae (2), accounting for prey depletion during the study, is illustrated in Fig. 5.

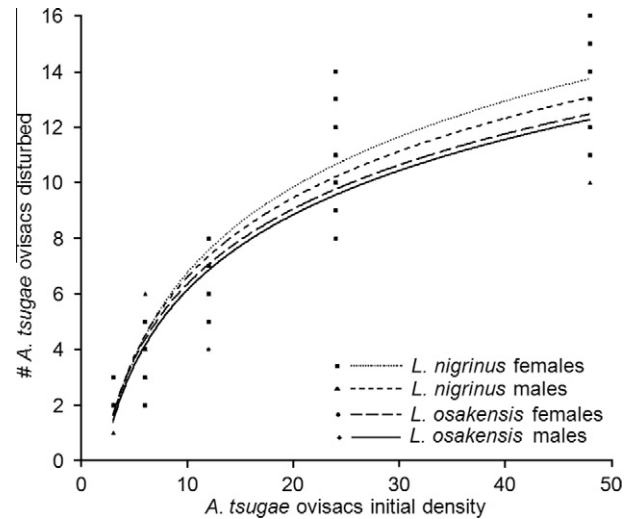


Fig. 2. Number of *A. tsugae* ovisacs disturbed as a function of *A. tsugae* ovisacs density. *L. osakensis* males (solid line [$N_e = N_0 (1 - \exp [0.013(3.55N_e - 72)])$]), *L. osakensis* females (long dashes line [$N_e = N_0 (1 - \exp [0.015(3.77N_e - 72)])$]), *L. nigrinus* males (short dashes line [$N_e = N_0 (1 - \exp [0.021(4.33N_e - 72)])$]) and *L. nigrinus* females (dotted line [$N_e = N_0 (1 - \exp [0.018(3.63N_e - 72)])$]).

Table 2

Comparison of attack rates and handling times of *L. osakensis* and *L. nigrinus* males and females. Significant differences ($P < 0.05$) highlighted. df – degree of freedom, Lo – *L. osakensis*, and Ln – *L. nigrinus*.

Species compared	df	Attack rate		Handling time	
		t	P	t	P
Lo female/Ln female	75	1.2355	0.2205	0.3448	0.7312
Lo male/Ln male	93	3.388	0.001	2.3476	0.021
Lo female/Lo male	74	0.9502	0.3451	0.5776	0.5653
Ln female/Ln male	93	1	0.3199	2.1546	0.0338

The attack rates for *L. osakensis*, and *L. nigrinus* larvae were 0.018 and 0.019, and the handling times were 12.1 and 24.2 h, respectively. Attack rates were not significantly different ($t = 0.11$, $P = 0.91$), but *L. osakensis* larvae handling time was significantly less than handling time by *L. nigrinus* larvae ($t = 6.52$, $P < 0.0001$).

3.4. Behavioral information of adult predators

3.4.1. A. tsugae killed

Males of *L. osakensis* and *L. nigrinus* killed significantly more *A. tsugae* adults than the females ($F_{1,69} = 5.80$, $P = 0.0187$ and $F_{1,87} = 18.00$, $P < 0.0001$, respectively). There were no differences between species within the same sex (Females – $F_{1,87} = 1.58$, $P = 0.2129$; Males – $F_{1,69} = 0.04$, $P = 0.8455$) (Fig. 6). The numbers of *A. tsugae* killed by both *L. osakensis* and *L. nigrinus* males and females were also dependent on the density offered, but there was no interaction between the effects. For comparisons of *A. tsugae* adults killed across species and sexes, significantly more *A. tsugae* were killed at the two highest densities (24 and 48 *A. tsugae* ovisacs) when compared with the two lowest densities (3 or 6 *A. tsugae* ovisacs) (Table 4).

3.4.2. Predator location

The location of the predators at the end of the trial did not differ among the species tested ($\chi^2 = 3.852$, $P = 0.1457$). Most frequently hid in the foliage, with the exception of *L. osakensis* males that wandered in the container outside of the foliage more frequently

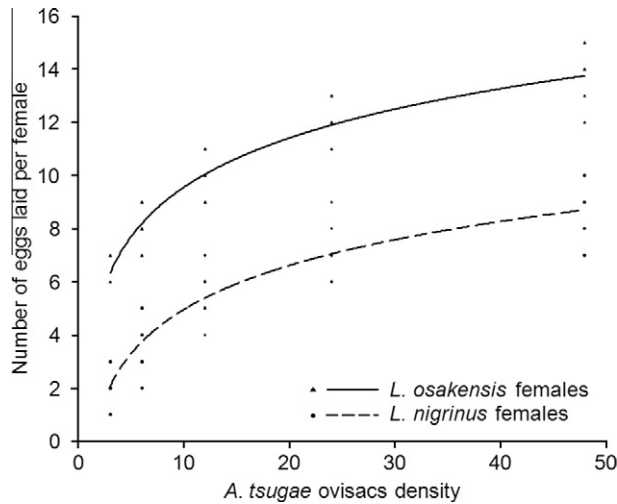


Fig. 3. Number of eggs laid as a function of number of *A. tsugae* ovisacs for *L. osakensis* (solid line) and *L. nigrinus* (dashed line) females. Lines represent the best regression for each species.

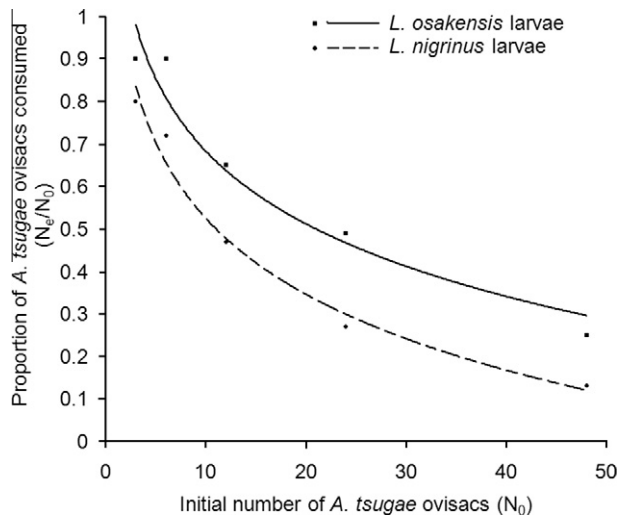


Fig. 4. Relationship between proportion of *A. tsugae* ovisacs disturbed and ovisac density for *L. osakensis* (solid line) and *L. nigrinus* (dashed line) larvae.

than all others (*L. osakensis* females ($\chi^2 = 14.187$, $P = 0.0002$), *L. nigrinus* females ($\chi^2 = 26.970$, $P < 0.0001$), and *L. nigrinus* males ($\chi^2 = 11.374$, $P = 0.0007$)) (Fig. 7A).

The location of the individuals at the end of the trial was consistent across densities ($\chi^2 = 4.588$, $P = 0.3322$) (Fig. 7B).

3.4.3. Oviposition site preferences

Oviposition site preference was different for each species ($\chi^2 = 262.132$, $P < 0.0001$). *L. nigrinus* laid eggs mostly in the wool of the *A. tsugae* ovisac, while most of the *L. osakensis* eggs were laid under the *A. tsugae* adult within the ovisac. *L. osakensis* was also the only species to lay eggs on the bark, outside of an *A. tsugae* ovisac (Fig. 8).

4. Discussion

The attack response of *L. osakensis* and *L. nigrinus* adults was best described by a type II functional response. This type of response indicates that these predators increase their prey consumption with increasing prey availability at a decreasing rate to

Table 3

Maximum likelihood analysis of the response of *L. osakensis* and *L. nigrinus* larvae to varying densities of *A. tsugae* ovisacs. Significant negative linear parameter ($P < 0.05$) highlighted. Lo – *L. osakensis* and Ln – *L. nigrinus*.

Species	Source	Parameter	Estimate	SE	χ^2	P
Lo	Intercept	Constant (P_0)	3.8591	0.9131	17.86	<0.0001
	N_0	Linear (P_1)	−0.4177	0.1551	7.25	0.0071
	N_0^2	Quadratic (P_2)	0.0147	0.00713	4.22	0.0399
	N_0^3	Cubic (P_3)	−0.00017	0.00009	3.54	0.0600
Ln	Intercept	Constant (P_0)	2.2950	0.6477	12.56	0.0012
	N_0	Linear (P_1)	−0.2883	0.1191	5.86	0.0154
	N_0^2	Quadratic (P_2)	0.00833	0.00572	2.12	0.1454
	N_0^3	Cubic (P_3)	−0.00009	0.000074	1.36	0.2435

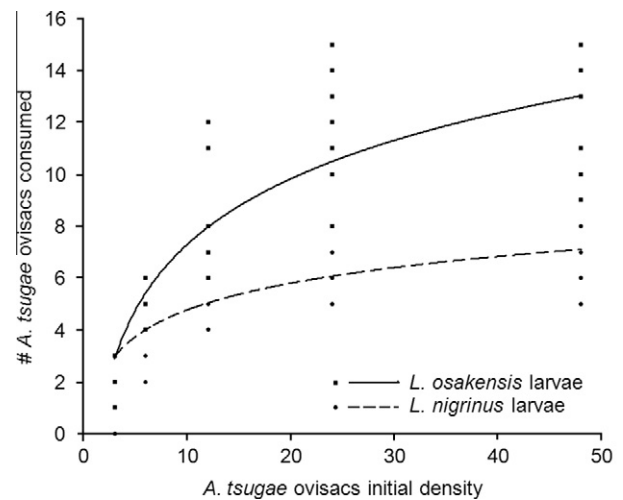


Fig. 5. Number of *A. tsugae* ovisacs disturbed as a function of *A. tsugae* ovisacs density offered for *L. osakensis* (solid line [$N_e = N_0 (1 - \exp [0.018(12.08N_e - 168)])$]) and *L. nigrinus* (dashed line [$N_e = N_0 (1 - \exp [0.019(24.16N_e - 168)])$]) larvae. Lines represent the best regression for each species.

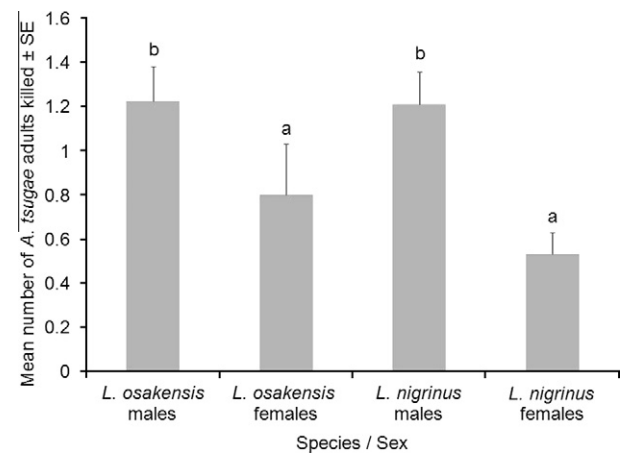


Fig. 6. Mean number of *A. tsugae* adults killed \pm SE by *L. osakensis* and *L. nigrinus* males and females. Species/sex with different letters were significantly different ($P < 0.05$).

a maximum. These findings are consistent with the type of functional response exhibited by many other insect predators (Hassell et al., 1977; Santos, 1975). The functional response of *L. osakensis* and *L. nigrinus* males and females to *A. tsugae* density was, in

Table 4

Comparison of *A. tsugae* adults killed by the predators at different initial *A. tsugae* densities. Significant differences ($P < 0.05$) highlighted.

Initial <i>A. tsugae</i> densities compared	<i>A. tsugae</i> adults killed (Density 1) \pm SE	<i>A. tsugae</i> adults killed (Density 2) \pm SE	<i>P</i>
Density 1 vs Density 2			
3 vs 6	0.54 \pm 0.13	0.61 \pm 0.12	0.9781
3 vs 12	0.54 \pm 0.13	1.20 \pm 0.14	0.0509
3 vs 24	0.54 \pm 0.13	1.56 \pm 0.18	0.0023
3 vs 48	0.54 \pm 0.13	–4.41	0.0002
6 vs 12	0.61 \pm 0.12	1.20 \pm 0.14	0.1331
6 vs 24	0.61 \pm 0.12	1.56 \pm 0.18	0.0055
6 vs 48	0.61 \pm 0.12	–4.25	0.0004
12 vs 24	1.20 \pm 0.14	1.56 \pm 0.18	0.6206
12 vs 48	1.20 \pm 0.14	–2.43	0.1119
24 vs 48	1.56 \pm 0.18	–1.10	0.8041

general, very similar. *L. nigrinus* males showed the most distinct response, but its higher attack rate is possibly balanced out by the longer handling time.

The values for attack rate and handling time should be used only as comparative values since the calculations assume that the individuals in the trials are always searching, attacking and consuming prey. That is probably not the case, especially for the adults. Another important activity for adult females is oviposition, while males spend considerable time just wandering, possibly looking for the females.

The seemingly great difference in handling time between adults and larvae could be explained by the difference in activity. Adult handling time involves disturbance of the ovisac that includes eating some eggs and/or the adult, while larval handling time is the consumption of all the eggs and the adult within the ovisac.

It is important to note a particular aspect of this study with *A. tsugae* that might not be comparable with functional response studies with other prey/predator systems – the prey unit. In other studies with aphid predators, the prey unit considered is one individual nymph or adult (Jafari and Goldasteh, 2009; Lee and Kang, 2004; Messina and Hanks, 1998; Sarmiento et al., 2007). However, in this study the prey unit is composed of what would be

considered several individual prey units in those aphid predator studies since an *A. tsugae* ovisac includes one adult plus hundreds of eggs.

As with the functional response, the numerical response of both species also increased with prey density, but at a lower rate approaching a plateau. However, *L. osakensis* females consistently exhibited a higher numerical response, laying significantly more eggs than *L. nigrinus* females across all densities.

The attack response of both *L. osakensis* and *L. nigrinus* larvae was also best described by a type II functional response. The functional response of *L. osakensis* and *L. nigrinus* larvae to *A. tsugae* density was significantly different, with *L. osakensis* larvae showing about half the handling time of *L. nigrinus* larvae. In terms of impact on *A. tsugae* populations, since *L. osakensis* larvae consume an ovisac in half the time *L. nigrinus* takes to do the same, they can consume more ovisacs than *L. nigrinus*.

L. osakensis and *L. nigrinus* males killed significantly more *A. tsugae* adults than the females. Unlike males, females use prey for oviposition in addition to feeding. Larvae of these species feed almost exclusively on *A. tsugae* eggs. Therefore, to be reproductively successful, females need to have food for themselves and for their progeny (Zilahi-Balogh, 2001; Zilahi-Balogh et al., 2003). To provide a continual supply of prey eggs for the progeny, the female would have to leave the adult prey alive. Males do not have such concerns and the best source of nutrition is the adult.

Males and females of both species killed significantly fewer *A. tsugae* adults at the lowest density than at the highest. In both species, adults feed on all stages of *A. tsugae* (Zilahi-Balogh, 2001). By not killing the adult prey at lower densities the predator gets a more continuous source of food, as the adult prey can continue to lay eggs.

It is not clear why *L. osakensis* males were observed wandering around the container outside of the foliage. This may be possibly related to the search of new feeding sites or of females forming.

L. osakensis and *L. nigrinus* females showed different preferences for oviposition sites. Eggs of both species were generally not visible unless the *A. tsugae* ovisac was disturbed. Occasionally *L. osakensis* deposited eggs on the bark. This could be a problem since eggs are

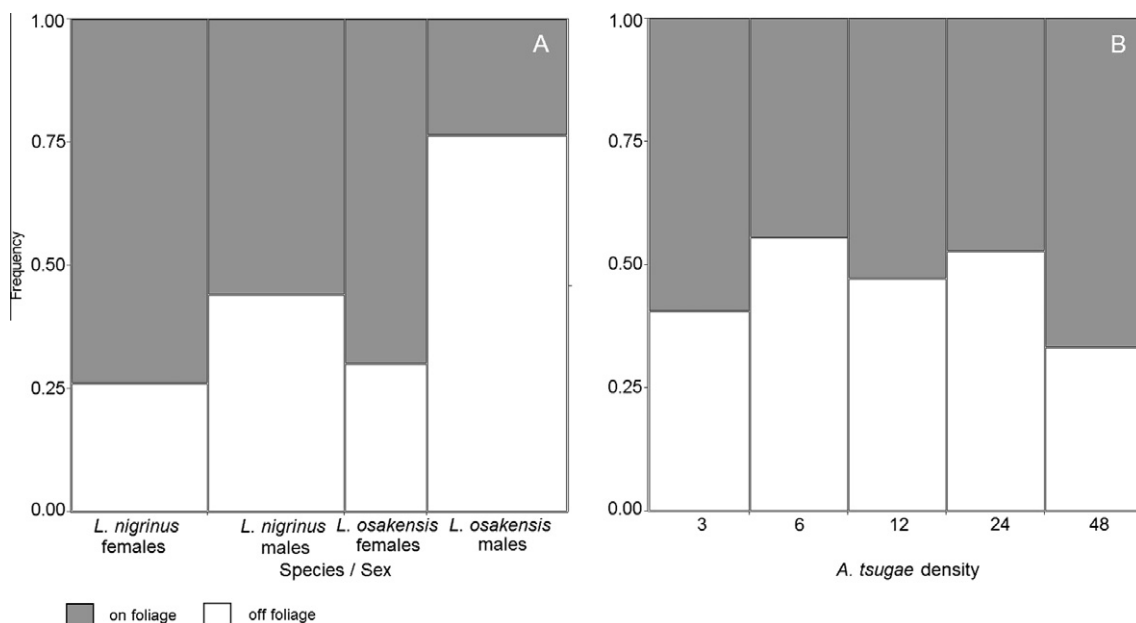


Fig. 7. Frequency of predators in/out of foliage at end of trial by species and sex of predator (A) or initial *A. tsugae* ovisacs density offered (B). *L. osakensis* males (LoM) and females (LoF) and *L. nigrinus* males (LnM) and females (LnF).

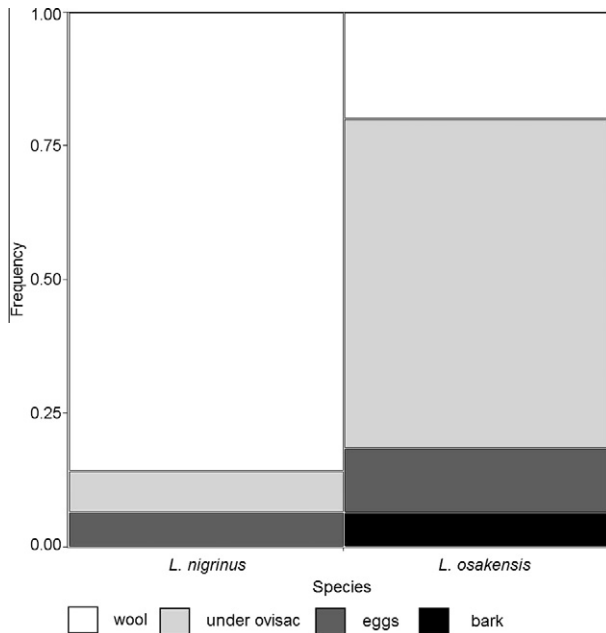


Fig. 8. Frequency of egg placement by each predator species (*L. osakensis* and *L. nigrinus*) in *A. tsugae* wool, under *A. tsugae* adult, amid *A. tsugae* eggs or in the bark.

more exposed to adverse environment conditions and predators in this location, but it occurred infrequently and in most cases *L. osakensis* females hid the eggs better than *L. nigrinus* by placing them under the *A. tsugae* adult.

5. Conclusion

L. osakensis has the potential to be a valuable addition to the *A. tsugae* predator community already introduced in the eastern US. Not only does it have several similarities with *L. nigrinus*, considered one of the most effective predators introduced, but surpasses it in some aspects. *L. osakensis* and *L. nigrinus* adults showed a similar functional response, but *L. osakensis* also exhibited a higher numerical response than *L. nigrinus*. The larvae showed significantly different functional responses, with *L. osakensis* being able to consume more ovisacs than *L. nigrinus* given the same amount of time. The roaming behavior of *L. osakensis* males might be related to the search of mates. Several studies have indicated that results obtained in the laboratory may not translate to the field, but they can indicate the potential (Munyanze and Obrycki, 1997; O'Neil, 1989, 1997).

For *L. osakensis* and *L. nigrinus* both adults and larvae are predatory. Both life stages exhibit a type II functional response. Having data from both larvae and adults for both species allow for a clearer differentiation in the potential impact of each species (Koch et al., 2003).

Additionally, the difference observed in the number of *A. tsugae* killed by males and females of both species also illustrates the need to consider the sexes separately since the different requirements for each sex can potentially translate into different levels of impact on the prey population.

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References

- Anonymous, 2010. Hemlock Woolly Adelgid Infestations 2010. USDA Forest Service, Durham NH.
- Cowles, R.S., 2009. Optimizing dosage and preventing leaching of imidacloprid for management of hemlock woolly adelgid in forests. *Forest Ecology and Management* 257, 1026–1033.
- Cowles, R.S., Montgomery, M.E., Cheah, C.J., 2006. Activity and residues of imidacloprid applied to soil and tree trunks to control hemlock woolly adelgid (Hemiptera: Adelgidae) in forests. *Journal of Economic Entomology* 99, 1258–1267.
- Evans, A., Gregoire, T., 2007. A geographically variable model of hemlock woolly adelgid spread. *Biological Invasions* 9, 369–382.
- Hassell, M.P., Lawton, J.H., Beddington, J.R., 1977. Sigmoid functional responses by invertebrate predators and parasitoids. *Journal of Animal Ecology* 46, 249–262.
- Havill, N.P., Montgomery, M.E., 2008. The role of arboreta in studying the evolution of host resistance to the hemlock woolly adelgid. *Arnoldia* 65, 2–9.
- Havill, N.P., Montgomery, M.E., Yu, G., Shiyake, S., Caccone, A., 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. *Annals of the Entomological Society of America* 99, 195–203.
- Holling, C.S., 1959. The components of predation as revealed by a study of small-mammal predation of the European pine sawfly. *The Canadian Entomologist* 91, 293–320.
- Jafari, R., Goldasteh, S., 2009. Functional response of *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae) on *Aphis fabae* (Scopoli) (Homoptera: Aphididae) in laboratory conditions. *Acta Entomologica Serbica* 14, 93–100.
- Juliano, S.A., 2001. Nonlinear curve fitting. Predation and functional response curves. In: Scheiner, S.M., Gurevitch, J. (Eds.), *Design and Analysis of Ecological Experiments*. Oxford University Press, Inc., New York, pp. 178–196.
- Kizilinski, M.L., Orwig, D.A., Cobb, R.C., Foster, D.R., 2002. Direct and indirect ecosystem consequences of an invasive pest on forests dominated by eastern hemlock. *Journal of Biogeography* 29, 1489–1503.
- Koch, R.L., Hutchison, W.D., Venette, R.C., Heimpel, G.E., 2003. Susceptibility of immature monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae: Danainae), to predation by *Harmonia axyridis* (Coleoptera: Coccinellidae). *Biological Control* 28, 265–270.
- Lamb, A.B., McAvoy, T., Dellinger, T., Salom, S.M., Kok, L.T., 2007. The Japanese Laricobius (*L. osakanus*): an update. HWA Biological Control Technical Committee Meeting, Annapolis, MD, p. 7.
- Lamb, A.B., Salom, S.M., Kok, L.T., 2005. Guidelines for rearing *Laricobius nigrinus* Fender. In: Onken, B., Reardon, R. (Eds.), *Third symposium on hemlock woolly adelgid in the Eastern United States*. USDA Forest Service, FHTET 2005-01, Asheville, NC, pp. 309–317.
- Lamb, A.B., Salom, S.M., Kok, L.T., Mauseel, D.L., 2006. Confined field release of *Laricobius nigrinus* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in Virginia. *Canadian Journal of Forest Research* 36, 369–375.
- Lamb, A.B., Shiyake, S., Salom, S.M., Montgomery, M.E., Kok, L.T., 2008. Evaluation of the Japanese *Laricobius* sp. N. and other natural enemies of hemlock woolly adelgid in Japan. In: Onken, B., Reardon, R. (Eds.), *Fourth symposium on hemlock woolly adelgid in the eastern United States*. USDA Forest Service, FHTET 2008-01, Hartford, CT, pp. 29–36.
- Lee, J.-H., Kang, T.-J., 2004. Functional response of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) to *Aphis gossypii* Glover (Homoptera: Aphididae) in the Laboratory. *Biological Control* 31, 306–310.
- Mauseel, D.L., Salom, S.M., Kok, L.T., Davis, G.A., 2010. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. *Environmental Entomology* 39, 440–448.
- McClure, M.S., Cheah, C.A., 1999. Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. *Biological Invasions* 1, 247–254.
- McDonald, R., Mauseel, D., Salom, S., Kok, L., Montgomery, M., Luker, G., Steury, S., Spears, G., Skeate, S., Graham, J., Hamstead, B., 2008. Recovery of hemlock woolly adelgid predators in the high country of northwestern North Carolina, 2004–2008. In: Onken, B., Reardon, R. (Eds.), *Fourth Symposium on hemlock woolly adelgid in the eastern United States*. USDA Forest Service, FHTET 2008-01, Hartford, CT, pp. 53–60.
- Messina, F.J., Hanks, J.B., 1998. Host plant alters the shape of the functional response of an aphid predator (Coleoptera: Coccinellidae). *Environmental Entomology* 27, 1196–1202.
- Montgomery, M.E., Bentz, S.E., Olsen, R.T., 2009. Evaluation of hemlock (*Tsuga*) species and hybrids for resistance to *Adelges tsugae* (Hemiptera: Adelgidae) using artificial infestation. *Journal of Economic Entomology* 102, 1247–1254.
- Munyanze, J., Obrycki, J.J., 1997. Functional response of *Coleomegilla maculata* (Coleoptera: Coccinellidae) to Colorado potato beetle eggs (Coleoptera: Chrysomelidae). *Biological Control* 8, 215–224.
- O'Neil, R.J., 1989. Comparison of laboratory and field measurements of the functional response of *Podisus maculiventris* (Heteroptera: Pentatomidae). *Journal of the Kansas Entomological Society* 62, 148–155.

- O'Neil, R.J., 1997. Functional response and search strategy of *Podisus maculiventris* (Heteroptera: Pentatomidae) attacking colorado potato beetle (Coleoptera: Chrysomelidae). *Environmental Entomology* 26, 1183–1190.
- Orwig, D.A., Foster, D.R., 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. *Journal of the Torrey Botanical Society* 125, 60–73.
- Orwig, D.A., Foster, D.R., Mausel, D.L., 2002. Landscape patterns of hemlock decline in New England due to the introduced hemlock woolly adelgid. *Journal of Biogeography* 29, 1475–1487.
- Salom, S.M., Kok, L.T., Lamb, A.B., Jubb, C., Onken, B.P., 2008. Biological control of hemlock woolly adelgid: What is it going to take to make it work? In: Onken, B.P., Reardon, R.C. (Eds.), *Fourth symposium on hemlock woolly adelgid in the eastern united states*. USDA Forest Service FHTET 2008-01, Hartford, CT, pp. 11–17.
- Santos, M.A., 1975. Functional and numerical responses of the predatory mite, *Amblyseius fallacis*, to prey density. *Environmental Entomology* 4, 989–992.
- Sarmento, R.A., Pallini, A., Venzon, M., Souza, O.F.F.d., Molina-Rugama, A.J., Oliveira, C.L.d., 2007. Functional response of the predator *Eriopis connexa* (Coleoptera: Coccinellidae) to different prey types. *Brazilian Archives of Biology and Technology* 50, 121–126.
- SAS Institute Inc., 2008a. The GLIMMIX procedure. SAS/STAT 9.2 User's Guide. SAS Institute, Inc., Cary, NC, pp. 2078–2419.
- SAS Institute Inc., 2008b. SAS/STAT 9.2 User's guide. SAS Institute, Inc., Cary, NC.
- SAS Institute Inc., 2009. JMP® 8 User Guide, second ed. SAS Institute, Inc., Cary, NC.
- Solomon, M.E., 1949. The natural control of animal populations. *Journal of Animal Ecology* 18, 1–35.
- Story, H., 2010. Assessing performance and compatibility of three *Laricobius* species as predators of hemlock woolly adelgid, *Adelges tsugae*. Department of Entomology, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA, p. 75.
- USDAFS, 2005. Hemlock woolly adelgid (Pest Alert) NA-PR-09-05. USDA Forest Service, Northern Area State & Private Forestry, Newtown Square, PA.
- Vieira, L.C., McAvoy, T.J., Chantos, J., Lamb, A.B., Salom, S.M., Kok, L.T., 2011. Host range of *Laricobius osakensis* (Coleoptera: Derodontidae), a new biological control agent of hemlock woolly adelgid (Hemiptera: Adelgidae). *Environmental Entomology* 40, 324–332.
- Zilahi-Balogh, G.M.G., 2001. Biology of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and its potential as a biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae) in the eastern United States. Department of Entomology, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA, p. 110.
- Zilahi-Balogh, G.M.G., Kok, L.T., Salom, S.M., 2002. Host specificity of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a potential biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). *Biological control* 24, 192–198.
- Zilahi-Balogh, G.M.G., Salom, S.M., Kok, L.T., 2003. Development and reproductive biology of *Laricobius nigrinus*, a potential biological control agent of *Adelges tsugae*. *BioControl* 48, 293–306.

Chapter 4 **Field-cage evaluation of the survival, feeding and reproduction of**
Laricobius osakensis* (Coleoptera: Derodontidae), a predator of *Adelges tsugae
(Hemiptera: Adelgidae)

Abstract

The hemlock woolly adelgid, *Adelges tsugae* Annand, is a serious, non-native pest of hemlock in the eastern North America. *Laricobius osakensis* Montgomery and Shiyake was identified as a key predator in Japan, where *A. tsugae* is native. Performance of adult and immature stages of *L. osakensis* was evaluated in sleeve cages on adelgid infested *Tsuga canadensis* (L.) Carrière) in plant hardiness zones 5b and 6b in the mountains of southwestern Virginia, US. Adults fed on the adelgid and laid eggs during all biweekly sample periods from December 2010 to May 2011, including winter when the temperature averaged below 0° C. In cages with one adult pair (one male and one female), predation of *A. tsugae*/predator was 0.3 to 0.9/day during December and January, increased to 2.5/day in February, and then declined to 0.15/day in early May. The oviposition rate lagged the changes in feeding by 2-4 weeks, increasing from 0.02 eggs/day, from December to mid-January, to a peak of 1.5 eggs/day in early April, then declining to 0.4 eggs/day in late April. Mortality was 20% in cages left undisturbed for two months during the winter; even though temperatures were as low as -18° C (cages examined biweekly had higher mortality, likely due to disturbance). In cages left undisturbed for two months during winter or early spring, 34 and 37 progeny were recovered, respectively. During each bimonthly period, a pair of adults and their progeny

consumed 2.5 and 2.4 adult adelgids or ovisacs per day, respectively. Twenty-eight days after eggs were placed in the cages (on 11 March or 27 April 2012), 48% and 95% of the recovered larvae had reached maturity and each larva had destroyed 43 and 39 ovisacs, respectively. This research indicates that *L. osakensis* has the potential to be an effective biological control agent of *A. tsugae* in most of the area where it is a pest in the eastern US.

Keywords: Biological control; Field cages; Predation; *Laricobius osakensis*; *Adelges tsugae*; Hemlock woolly adelgid

4.1 Introduction

Hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) is an introduced pest from Japan that is threatening eastern (*Tsuga canadensis* (L.) Carrière) and Carolina (*T. caroliniana* Engelmann) hemlock forests in the eastern United States (Havill et al. 2006; Orwig et al. 2002). This pest can colonize all hemlock species, being relatively innocuous for Asian and western US hemlock species due to the action of both host resistance and natural enemies, but can be fatal to eastern and Carolina hemlocks (Havill and Montgomery 2008; McClure and Cheah 1999; Montgomery et al. 2009a). Since its initial detection near Richmond, VA in the early 1950s, *A. tsugae* has spread throughout the eastern US, covering more than 50% of the geographic range of eastern hemlock in the US (USDA-FS 2012).

Due to environmental concerns, operational problems, and the high cost involved in chemical control of *A. tsugae* in a forest settings, long-term sustainable efforts to control this pest have focused on biological control. A coordinated effort is being made to establish a complex of natural enemies, adapted to the various environmental conditions in which *A. tsugae* is present, that can reduce *A. tsugae* populations below damaging levels (Onken and Reardon 2011b). So far three species of predators have been released for the control of *A. tsugae*: *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) in 1995, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) in 2003, and *Scymnus sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae) in 2004 (Cheah et al. 2004a). However, despite evidence of the establishment of *S. tsugae* and *L. nigrinus* in several locations, additional predators are

needed since measurable impact has not yet been achieved by these predators (Davis et al. 2012; Hakeem et al. 2010).

Laricobius osakensis was found to be a key predator of *A. tsugae* in Japan (Lamb et al. 2011), the source of the introduction of *A. tsugae* to the eastern US (Havill et al. 2006). This predator is a highly-specialized natural enemy of *A. tsugae*, as it is only able to develop on this prey (Vieira et al. 2011). Emergence of *L. osakensis* adults starts in October and ends in December (Salom and Lamb 2008). *Laricobius osakensis* adult females start oviposition one to two weeks prior to the start of oviposition by *A. tsugae* (Vieira et al. in press). *Laricobius osakensis* larvae feed mostly on eggs but larger larvae (3rd and 4th instars) also feed and develop on *A. tsugae* nymphs (pers. obs.), and most larvae have already reached maturity and dropped to the soil to pupate by the end of May (Vieira et al. in press). In laboratory studies, *L. osakensis* showed both a higher numerical response and functional response than *L. nigrinus* (Vieira et al. 2012). Overall, these studies indicate that *L. osakensis* has the potential to be a valuable addition to the natural enemy community previously released for the biological control of *A. tsugae*. *Laricobius osakensis* has recently received regulatory approval for release from quarantine (USDA-APHIS-PPQ 2010); thus, making pre-release evaluations in the field possible.

While laboratory assays can provide some indication of the potential impact of a natural enemy, field evaluations allow for the assessment of the efficacy of a natural enemy in more realistic conditions—they have to survive variable climatic conditions, deal with competitors and/or their own natural enemies (generalist predators), and be able to find their prey/host (O'Neil 1989).

The present study evaluates the survival, feeding, reproduction, and development of *L. osakensis* adults and larvae on *A. tsugae* populations in sleeve cages in Virginia, from late fall to mid-spring.

4.2 Materials and Methods

4.2.1 Experimental subjects

The *L. osakensis* evaluated were progeny of beetles collected in October 2009 (adult trials) or 2010 (larval trials) from *T. diversifolia* in Shiga Kogen, Japan, and then reared in a containment facility for at least one complete generation (Lamb et al. 2005a; Salom et al. 2012). Following emergence from aestivation in October-December, 2010, until they were used in the field trials, the F1 generation adults were maintained on field-collected *A. tsugae*-infested eastern hemlock twig cuttings in environmental chambers at 4° C, 12:12 (L:D), and 65-75% RH until February, and then 9° C, 12:12 (L:D), and 65-75% RH. Trials of larvae, conducted in spring 2012, were F2 progeny of *L. osakensis* collected in Japan in October 2010 and reared as stated above.

4.2.1.1 Adult sex determination

Adult *L. osakensis* exhibit some degree of sexual dimorphism—coloration ranges from coppery brown to black, but distinctive coppery brown individuals are usually females and dark brown or black individuals are usually males (Montgomery et al.

2011b). To improve the accuracy of this approach, only individuals with pronounced colorings indicative of each sex were selected. From January to May, when *L. osakensis* was ovipositing in the laboratory, sex was verified by isolating individuals in 55×20.3 mm² polystyrene Petri dishes with *A. tsugae*-infested twigs. Petri dishes were kept in an environmental chamber at 9° C, 12:12 (L:D) and 75-87% RH for 3 days. After the trials, *A. tsugae* ovisacs were searched for predator eggs. If predator eggs were found, the individual was identified as a female, and in the absence of eggs, the individual was identified as a male, if it did not have female coloration.

4.2.1.2 Adult species confirmation

After field trials were started, it was discovered that the laboratory colony was a mixture of *L. osakensis* and a morphologically similar species, *L. naganoensis* Leschen (Leschen 2011). The two species can be distinguished by microscopic examination and dissection of male genitalia (Leschen 2011). However, this method is not exact and does not allow for the identification of the species of the females; therefore, the identity of the beetles used in these experiments was confirmed using DNA sequence data. After use in the experiments, adult specimens were kept in dry storage or placed in 100% ethanol, until they were dissected under a microscope. The head, prothorax, elytra, and genitalia were kept as voucher specimens and the meso- and metathorax with membranous wings were used for the molecular analysis.

Genomic DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, California, USA) and PCR was used to amplify the partial cytochrome oxidase

subunit I (COI). PCR was carried out following the protocol developed by Davis et al. (2011) with some modifications—15.5 µL sterile water, 4.8 µL MgCl₂ (25 mM), and 41 cycles. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Sequencing reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems 3730 automated sequencer. Edition and alignment of partial cytochrome oxidase subunit I (COI) sequences were performed in Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI).

All experimental subjects used in the trials were confirmed to be *L. osakensis*, except for one *L. nigrinus* used in the adult predators' evaluation (Table 4.1). The presence of the *L. nigrinus* specimen was probably a consequence of it being reared in the same facility as *L. osakensis*. It was not possible to trace and exclude the data represented by this specimen, but is too low to influence the results.

Vouchers for all specimens sequenced were placed in the Entomology Museum at Virginia Tech. These are labeled “10-LOSG1” (adult trials) or “11 LOSG1-F” (adult females that laid eggs for larval trials), followed by a two digit number. The label indicates the year the specimen emerged (10 or 11), the assumed species (LOS), the generation of the specimen in relation to the wild progenitors (G1), the sex of the specimen (F), and the specimen individual number.

4.2.2 Adult feeding, reproduction, and survival

4.2.2.3 Location and timing of experiments

Evaluation of *L. osakensis* adult feeding, reproduction and survival was conducted in Saltville, VA (36.886675° N, 81.855183° W), located in plant hardiness zone 6b (USDA-ARS 2012), from 20 Dec. 2010 to 9 May 2011. This site has an elevation of 636 m and was located on a southwestern slope. Eastern hemlock trees dominated the overstory vegetation at this site. Trees were selected for high levels of infestation and low lying branches that could be easily reached. The trees used for the experiments were 10-50 cm dbh (diameter at breast height), heavily infested, yet still producing new growth. Because the datalogger placed in the field was vandalized, temperature data (Fig. 4.1a) were retrieved from the closest meteorological station (Poor Valley, Saltville, VA, station code - KVASALTV2) at www.wunderground.com. The mean temperature increased month to month (Fig. 4.1a). The minimum and maximum temperatures registered during the sample period were -17.8° C in January 2011 and 32.7° C in May 2011, respectively. During the study, total precipitation was 586 mm, with several snow events. Monthly precipitation increased until March 2011 and decreased afterward.

At the beginning of the study on 20 Dec 2010, 2nd instar *A. tsugae* sistens were present in the field. By mid-February, *A. tsugae* was in the adult stage and had started ovipositing. By the end of April, the sistens adults had died and eggs and nymphs of the next generation (progrediens) were present. Observations in the laboratory and in the field in Japan indicate that all *L. osakensis* adults would have emerged from aestivation by the end of December and would be found on *A. tsugae* infested foliage, in diminishing numbers, until the beginning of May (Salom and Lamb 2008; Vieira et al. in press). The

biweekly sample intervals thus capture the effects of weather and adelgid life history, while the first two-month sample interval covers the heart of winter to the beginning of *A. tsugae* egg laying and the second two-month covers the period of peak egg production by the sistens generation of *A. tsugae*.

4.2.2.2 Experimental procedure

At the beginning of each sample period, five trees were selected and pre-counts of *A. tsugae* were made on four branches of each tree. Starting from the tip of each branch, *A. tsugae* were counted on 62 cm of branch or less until a pre-set minimum density or above was reached. The branch was marked with flagging tape where the count ended. The tree and branch codes as well as the number of *A. tsugae* ovisacs counted were registered on the flag. To ensure that the counted adelgids were not disturbed by the sleeve cage, it was secured to the branch above (proximal) of the flag at a point where the branch forked and was devoid of adelgid. On each tree, two branches were caged with a pair of predators inside (a male and a female *L. osakensis*) and the other two branches were caged without predators (control). Since it was not possible to find four branches with exactly the same *A. tsugae* density on a tree, the two branches with the lowest and the two branches with the highest densities were grouped and the two treatments randomly assigned within the paired branches.

Cages were rectangular bags (72 × 55 cm) made of polyester fabric (white chiffon). The branches were vigorously shaken before the application of either treatment (with or without predators) to dislodge any native predators, such as spiders, that might

be present. At the end of the sample period, the branch was cut proximal to the cage and taken to the laboratory to determine predator survival, reproduction, and feeding.

Predator feeding was based on the difference in the number of dead *A. tsugae* in cages with predators and cages without predators. Adult *L. osakensis* feeds by wounding its prey and sucking the hemolymph. Prey is not completely consumed usually and the dried cadavers of the prey remain (personal observation). Reproduction was based on the number of eggs and larvae recovered. For samples where both adults and larvae were present (second biweekly sample), feeding by larvae was estimated by counting the number of disturbed ovisacs, which usually had all eggs consumed and only egg chorions remaining. Adult predators consume prey eggs and large predator larvae (3rd and 4th instar) can consume prey adult. However, adult predators do not consume all eggs in an ovisac like the larvae, and predator larvae do not consume prey adults without consuming all the eggs in the ovisac as well (Vieira et al. 2012).

4.2.2.4 Evaluation at biweekly intervals

For this study, only branches with a density of 140-190 *A. tsugae* were used. Every two weeks the caged branches were clipped and taken to the laboratory, and cages (with predators and controls) were set up on new branches the following day. The number of surviving predators of each sex, predator eggs, and dead *A. tsugae* adults were counted. The adult beetles that survived were used in the new cages, and adult beetles were added to replace those that died. Specimens in the first sample period and those

added in the second sample period were tracked (from cage to cage) during the entire trial.

4.2.2.4 Evaluation at bimonthly intervals

For this study, branches with an initial density of 400-485 *A. tsugae* were used. After 2 months, the caged branches were clipped and taken to the laboratory. The number and sex of live and dead predators, the number of dead and live *A. tsugae* adults, the number of disturbed and undisturbed ovisacs, and the number of immature predators (eggs and larvae) on the branches were counted. The following day, a new set of cages with predators and controls was set up in the field. As in the evaluation at biweekly intervals, surviving adult predators and additional beetles to replace the ones that died were used in the second bimonthly sample period.

4.2.3 Larval feeding, development, and survival

4.2.3.1 Location and timing of experiments

Evaluation of *Laricobius osakensis* larval feeding and development was conducted from 11 Mar. 2012 to 03 Jun. 2012 in Mountain Lake, VA (37.373517° N, 80.525258° W, elevation 1218 m). Eastern hemlock trees also dominated the overstory vegetation at this site, which was located in proximity of a creek. Similar to the adult experiments, trees were selected for high levels of infestation and low lying branches that

could be easily reached. The trees used for the experiments were medium size (30-75 cm dbh), highly infested, but still fairly healthy. Temperature data were obtained from Mountain Lake Biological Research Station, about 1.5 Km from the sample site. Temperature during the trials ranged from -0.9° C on March 2012 to 31.8° C on May 2012 with monthly means ranging from 14.2° C and 20.2° C (Fig. 4.1b). Total precipitation was 366 mm during the study.

There were two trials, one beginning 11 Mar. 2012 and the other 27 April 2012, each lasting 4-6 weeks. The first trial began 1 to 2 weeks before *A. tsugae* sistens adults started laying eggs in the field and by the end of the trial adelgid eggs were still plentiful. The second trial began when the adelgid egg production was declining, and by the end of the trial most of the eggs had hatched and progrediens 1st instar nymphs were present.

4.2.3.2 Experimental procedure

Evaluation of larval development and predation began with the placement in the field of *L. osakensis* eggs produced by ten predator adult females in the laboratory over a nine day period and kept in storage at 4° C for 23 to 31 days. Because of the unintended inclusion of *L. naganoensis* in the colony, it was necessary to store the eggs while the species of the egg's female parent was confirmed using molecular analysis, as previously outlined. For transfer to the field, the eggs were affixed to a bare hemlock twig by the wool material from an adelgid ovisac.

Adelgid ovisacs were counted on nine branches of five trees in the same manner as for the trials of adult predators, except that the number of ovisacs ranged from 150 to

177. Six branches were randomly caged with predator eggs and three without predator eggs (control). Branches on a tree with the most similar *A. tsugae* density were grouped in sets of three, with two branches in each group treated with predator eggs treatment, and the remaining branch serving as the control.

For the predator treatments, a small twig with seven *L. osakensis* eggs of the same age (same day laid) was affixed with 34 gauge craft wire against the underside of a twig of the branch before it was enclosed in the cage.

Cages were harvested at different times because it was not certain when the larvae would mature—ideally cages would be harvested when 50% of the larvae were mature. One third of the cages were clipped and taken to the laboratory after three weeks or one month, and the remaining two thirds were removed from the field one week afterward. Counts were made on the number of dead and live larvae, with the state of larval development (immature or mature) noted, and the number of disturbed and undisturbed *A. tsugae* ovisacs. All disturbed ovisacs were counted; larval disturbance of ovisacs can be easily differentiated from those disturbed by other means. An ovisac disturbed by feeding of *Laricobius* larvae has more circular and looser wool covering, whereas an ovisac brushed by foliage or the cages has wool pulled from the ovisac. The number of disturbed ovisacs was used instead of the number of consumed ovisacs (all eggs consumed) because *A. tsugae* was ovipositing during the entire first sample period. Consequently, in this sample period, despite the larvae eating all the eggs before moving to a new ovisac, the *A. tsugae* adult left alive continued to lay eggs after the larvae left. The number of disturbed ovisacs was thus a better approximation of feeding.

For the second sample period, the number of live progrediens nymphs was also assessed. Recognition of mature *L. osakensis* larvae was based on size (>1.5 mm) and presence off the foliage on the bottom of the cage—mature larvae leave the foliage to pupate in the soil.

Larval developmental time (from the day egg was laid to larval maturity) was assessed only for the experiment set up on 11 Mar 2012, since the date of oviposition was known, and some of the larvae that reached the pre-pupal stage were still alive. It was not possible to assess development time for the experiments started on 27 Apr 2012 because temperatures were much higher during this period. For the latter experiment, when branches were recovered, larvae had already completed development and were all dead. Developmental time estimates include the time the eggs spent in cold storage in the laboratory (23 to 31 days).

4.2.5 Data Analysis

Statistical treatments were performed in SAS (SAS_Institute_Inc. 2008b) and JMP (SAS_Institute_Inc. 2009). All data were tested for normality and equality of variance.

Differences between the number of dead *A. tsugae* or disturbed ovisacs in cages with and without predators, for each sample date, were analyzed with a paired *t*-test. To compare differences in predation among sample dates, a predation rate (prey killed/day/predator) was calculated to account for the small differences in the duration of each sample interval and number of predators in the sample. It was presumed that the

mortality due to factors other than predators would be the same in the cages with and without predators. Since sequential samples had new beetles added to replace those that did not survive from the previous sample, each sample was treated as unique rather than a measurement of the same beetles. Differences in predation at different sample periods were determined with a one-way ANOVA followed by Tukey's HSD. The number of dead *A. tsugae* was also compared between cages with the same treatment (with or without predators) at different intervals in the two-month experiments using a one-way ANOVA.

Larval predation was calculated as the difference between the number of disturbed *A. tsugae* ovisacs in the cages with predator larvae and the number of disturbed *A. tsugae* ovisacs in cages without predator larvae. Larval predation for each sample date was compared with a paired *t*-test. The effect of larval predation on the following generation (progreddiens) was determined as the difference between the number of live progreddiens in cages with predator larvae and cages without predator larvae in the experiments set up on 27 Apr 2012, and analyzed with a paired *t*-test. In all analyses, the natural pairs were branches from the same tree with the same density that received the different treatments (control or predator adults/eggs). Cages seeded with *L. osakensis* eggs from which no larvae were recovered were excluded from the analysis.

Oviposition was analyzed as a function of date using the GLIMMIX procedure in SAS assuming a Poisson distribution (SAS_Institute_Inc. 2008b). The adjusted Tukey-Kramer HSD test was used to determine significant differences in oviposition between dates.

Survival of adult predators was recorded for each individual as a binomial response (alive = 1, dead = 0) and was analyzed by date and sex using the GLIMMIX procedure (SAS_Institute_Inc. 2008b). The adjusted Tukey-Kramer HSD was used to determine significant differences in survival between dates and sexes.

4.3 Results

4.3.1 Adult feeding, reproduction, and survival

4.3.1.1 Evaluation of adults at biweekly intervals

Cages with a pair of *L. osakensis* adults had significantly more dead *A. tsugae* than cages without the predators in all biweekly sample periods from 20 Dec 2010 to 9 May 2011 ($t = 10.03$, $df = 87$, $P < 0.0001$) (Table 4.2). The predation varied according to the sample period, being greater in February and beginning of March 2011, and lesser in mid-January and April 2011 (Table 4.2).

Oviposition started by the end of January, had its peak by the end of March, and decreased thereafter (Fig. 4.2). A maximum of 35 eggs were laid by a female during a biweekly period.

Survival of *L. osakensis* was examined two ways—survival of all beetles (both re-used and replaced beetles) at each sampling date and survival of a set of individuals throughout the experiment. In the evaluation of survival at each sampling date, no difference was found on the survival of *L. osakensis* males and females ($F(1,162) = 0.28$,

$P = 0.5957$), but some differences were found between the sampling periods ($F(8,162) = 4.38, P < 0.0001$). Paired comparisons indicated that survival was fairly consistent across the entire experimental period, but was significantly less at the end (sample periods starting on 10 and 24 Apr 2011) (35 %) than on 08 Mar 2011 (80 %) (Fig. 4.3). Survival was tracked for individuals first used in the first and third sample periods to the end of the experiment in May (Fig. 4.4). Some of these specimens survived almost the entire experimental period from December 2010 to May 2011. However, for both groups there is a large decline in survival in the two sample periods following the data of introduction into the field—70 and 45 % mortality for individuals first used in 20 Dec 2010 and 23 Jan 2011, respectively. After the second group went through this two sample periods (one month) of higher mortality, survival in this (55 %) and the first group (30 %) was not statistically different ($\chi^2(1, N = 35) = 0.89, P = 0.3454$). Survival continued to diminish in following sample periods but to a lesser extent (Fig. 4.4).

4.3.1.2 Evaluation of adults at bimonthly intervals

In the bimonthly evaluation, cages with the *L. osakensis* adult pair had significantly fewer live and significantly more dead *A. tsugae* than in cages without predators in both bimonthly sample periods (Table 4.3). The number of dead *A. tsugae* adults was significantly greater in the second sample period than in the first sample period both in cages with ($F(1, 18) = 16.36, P = 0.0008$) and without ($F(1, 18) = 10.97, P = 0.0039$) predators.

The predation rate (prey killed/day/predator) in the first and second sample periods was 1.2 ± 0.3 and 1.2 ± 0.3 , respectively, being similar during both sample periods ($F(1, 18) = 0.05$, $P = 0.83$). However, in the second sample, resources were exhausted in the cages with predators. There were no live adelgids and only two ovisacs with any eggs remained in all of the cages with predators.

Each cage with predators had an average of 37 eggs and 34 larvae at the end of the first and second sample period respectively. Individual females produced a maximum of 59 and 69 progeny in the first and second bimonthly periods, respectively. The average number of progeny produced per female/day was 0.6 ± 0.1 and 0.5 ± 0.1 in the first and second sample periods, respectively, which were not significantly different ($F(1, 18) = 0.09$, $P = 0.7691$).

Survival of the pair of adult beetles placed in the cages was significantly greater during the first than the second bimonthly period, where no adults survived ($F(1, 36) = 76$, $P < 0.0001$). The high mortality in the second sample period was likely related to exhaustion of available resources. During the first sample period, 70 and 90 % of the males and females survived, respectively, but this difference was not significant ($F(1, 36) = 0.34$, $P = 0.5635$).

4.3.2 Larval feeding, development, and survival

Of the 210 eggs placed in the field at each sample period, an average of 21.4 and 28.0 % hatched and developed for sample periods starting at 11 Mar 2012 and 27 Apr 2012, respectively. The low egg hatch is likely a consequence of handling and storage of

the eggs in the laboratory. Of the larvae recovered from the 11 Mar 2012 trial, only 2 (6.7 %) died before reaching maturity. All were still immature after 21 days, but 25.9 % had reached maturity after 28 days in the field. For the 27 Apr 2012 trials, only 3 larvae (5.2 %) did not reach maturity; all remaining larvae were mature and dead after 28 or 38 days in the field (Table 4.5). It took an average of 48.1 ± 0.7 days for *L. osakensis* to develop from egg (day egg was laid) to mature larvae in March/April 2012, with a minimum and a maximum of 46 and 54 days, respectively.

In the 11 Mar 2012 trials, after 21 days in the field, the larvae were still immature (probably 3rd or 4th instar) and each preyed upon an average of 23.0 ± 1.9 ovisacs. But after an additional week in the field (28 days total), most larvae were mature or close to maturity (probably 4th instar) and each preyed upon an average of 42.7 ± 2.6 ovisacs. For the second sample period, starting on 27 Apr 2012, after either 27 days or 38 days in the field all larvae were dead and mature, and 40.0 ± 3.2 or 38.7 ± 2.7 ovisacs on average were eaten, respectively (Table 4.4).

Cages with the *L. osakensis* larvae had a significantly greater number of disturbed *A. tsugae* ovisacs than cages without predators, in all sample periods ($t = -29.73$, $DF = 102$, $P < 0.0001$) (Table 4.5). Disturbed ovisacs were registered for both cages with and without predator larvae. However, it was evident that the disturbance in cages with predators was caused by predator larval activity. The disturbance of the ovisacs seen in the control cages was a result of some ovisacs rubbing against something, probably the fabric of the cage. Additionally, no other predators in any stage were found in the control cages. Predation was similar during all sample periods ($F = 0.0004$, $DF = 1$, $P = 0.98$). Cages with predator larvae had significantly less adult progrediens than cages without

predators ($t = 10.27$, $DF = 21$, $P < 0.0001$), with an average \pm SE of 50.6 ± 7.2 and 150.1 ± 9.2 progreddiens, respectively.

4.4 Discussion

Adult predation impact was evident throughout both biweekly and bimonthly experiments. It varied according to the sample period in the biweekly experiments, probably as a function of variation in temperature, live prey availability, prey stage, and predator mortality. Temperature is usually a driving force for insects, with most terrestrial insects being inactive at temperatures below 4°C (Schowalter 2011), but both *L. osakensis* and *A. tsugae* are unusual in feeding during the winter months when temperatures are above freezing. Adult predation decreased due to lower temperatures in December through January (Fig. 4.1a). The low predation in the sample period starting on 08 Jan 2011 corresponded with very low temperatures experienced during that 15-day period. Predation peaked from February to the beginning of March, corresponding to an optimal range of temperatures, prey availability, and prey stage. During this time *A. tsugae* adults are fully developed, starting to oviposit, and their chance of survival is high. Temperature during these months is likely optimal for both predator and prey survival and activity. The low predation in April and May has to be interpreted with caution since it can be an artifact of the type of prey assessed. The number of dead/alive *A. tsugae* adults is a measure of predation more reliable than the number of eggs or nymphs, since the number of adults does not vary from the beginning to the end of each sample period, and is also easier to assess. However, adult *L. osakensis* also feed on *A.*

tsugae eggs and nymphs, leading to an underestimation of the total predator impact. This underestimation was especially noticeable in April and May, since most *A. tsugae* adults had died naturally but the eggs and eclosed nymphs remained a food source. The increase in predation from winter to spring was also observed in cage studies with *L. nigrinus* (Lamb et al. 2005b).

For the entire duration of both bimonthly evaluations, the predation rate of an adult beetle was 1.2 *A. tsugae* sistens adults/day, which is near the average rate of the biweekly data. The similarity in the predation rate in both bimonthly sample periods was likely more related to the impact measure selected (dead *A. tsugae* adults) and prey limitation than to a true lack of differences. Almost all *A. tsugae* adults, and all predator adults and larvae were dead by the end of the second sample period. This suggests that if sufficient prey was available predation would be much higher. Additionally, the higher natural mortality of *A. tsugae* adults at the end of the sistens generation life cycle, as seen in the cages without predators, also made it difficult to detect predator impact. The bimonthly predation rates found for *L. osakensis* seem much lower than the 3.3 and 5.8 adelgids/day/adult predator rates reported for *L. nigrinus* in sleeve cage studies at 90-day intervals for Nov to Jan and Feb to April, respectively (Lamb et al. 2005b). However, these rates do not account for natural mortality (mortality in control cages), attributing all mortality in cages with predators to predation. Accounting for natural mortality, predation rates for *L. nigrinus* were 1.2 and 2.0 adelgids/day/adult predator for Nov to Jan and Feb to April, respectively, similar to the bimonthly predation rates found for *L. osakensis* in this study. The discrepancy between the spring predation rate of *L. osakensis* (1.2 adelgids/day/adult predator) and *L. nigrinus* (2.0 adelgids/day/adult

predator) was likely motivated by different prey availability in the two studies. The spring cage evaluation of *L. nigrinus* ended earlier and food was still plentiful, more than one-half of the initial number of adelgids remained, whereas the spring evaluation of *L. osakensis* ended later in the season and all adelgids were dead either from predation or natural mortality. Additionally, while the *L. nigrinus* studies lasted longer, the initial densities used were twice that of the ones used in this study. Given sufficient prey, *L. osakensis* predation in the second bimonthly period would likely match that of *L. nigrinus*. Similar predation rates were also previously found in laboratory studies (Vieira et al. 2012).

Oviposition in the biweekly experiments showed a pattern similar to predation but with a delay. In early February there was a significant increase in predation. The following sample period showed a significant increase in oviposition. This increase also coincided with the onset of oviposition by *A. tsugae* sistens adults. Peak oviposition on 24 Mar 2011 was reached one month after peak predation on 20 Feb 2011. The delay between increases in predation and oviposition is likely related to adult female predators' nutritional requirements for oviposition as well as reproductive phenology. Since egg production is nutritionally costly for the females, it is likely they have to increase their food intake in preparation for egg production (Chapman et al. 1998). The lower predation at peak oviposition might be related to female behavior. In previous studies (Vieira et al. 2012), *L. osakensis* females did not feed on ovisacs in which they oviposited. If adult prey was limited, they would feed on adelgid eggs in other ovisacs and even on some of the eggs in the ovisacs they had laid an egg. With both behaviors, females left prey for their progeny.

The oviposition results of this study are comparable in many respects to those of similar cage studies with *L. nigrinus* (Lamb et al. 2005b). Oviposition of both *L. nigrinus* and *L. osakensis* varied throughout the season and peaked in mid-April. Both species laid some eggs before February, but *L. osakensis* produced 8 eggs/female from Dec to Jan, whereas *L. nigrinus* produced only 0.2 eggs/adult from Nov to Jan. The start of oviposition by *L. osakensis* before the onset of *A. tsugae* sistentes oviposition has also been observed in predators' native range (Lamb et al. 2011). Total spring fecundity of *L. osakensis* (34 progeny/female) was higher than the 20 progeny/female by *L. nigrinus*. The higher oviposition by *L. osakensis* in relation to *L. nigrinus* reflects what would happen under similar conditions, since the range of temperatures in this study (-17.8 to 32.7° C) and that with *L. nigrinus* (-16 to 32.7° C) were similar. The average temperature in January and February was also lower in this study (-2.6° C) than in the study with *L. nigrinus* (-0.5° C). The higher oviposition of *L. osakensis* in relation to *L. nigrinus* was also seen in laboratory studies (Vieira et al. 2012).

The lack of differences in reproduction during the two sample periods in the bimonthly experiments was probably because of limited resources causing mortality of the adults in the second sample period. When the adults and progeny exhausted the food resources, greater reproduction would only reduce the chance of survival of the progeny. Additionally, all adults died in the second sample period, so none of the females oviposited during the entire sample period. That only eggs were found at the end of the first bimonthly period suggests that the eggs were laid near the end of the period and these developed slowly because of the cool temperatures. In contrast, only dead, mostly mature, larvae were found at the end of the second bimonthly period, suggesting that

most of the eggs were laid by the predator during the early part of the period and these developed rapidly with the warmer temperatures. There are two possible reasons why the larvae died—the larvae ran out of food and died of starvation before reaching maturity or larvae reached maturity but died because the cage prevented them from entering the soil to complete development.

Survival of almost all of the adult specimens in the first bimonthly period indicates that *L. osakensis* can withstand low winter temperatures at high elevations in Virginia's mountains. The sharp decrease in survival one month following initial introduction of the individuals into the field and the lower winter survival in the biweekly study than in the bimonthly study, indicate that the need for acclimation of the insects after being maintained at 4° C (Dec to Feb) or 12° C (Mar to May) in the laboratory. *Laricobius osakensis* survival (80%) after 2 months in the field was similar to that found for *L. nigrinus* (88.9 % November to January) (Lamb et al. 2005b). Since *L. osakensis* was exposed to -18°C and *L. nigrinus* to -16°C in the field cages (Lamb et al. 2005b), both species seem cold-adapted and able to survive equally cold winters in more northern latitudes at lower elevations (Mausel et al. 2010).

Larvae produced from eggs laid in the cages placed in the field on 20 Feb 2010 were almost all mature or dead before reaching maturity when the cages were collected on 23 April. In these cages, the impact from both predator adults and larvae was evident. Most *A. tsugae* adults in the cages with predators were dead with all the eggs consumed. In most cages, only 1 or 2 *A. tsugae* ovisacs had intact eggs.

Larvae in the cages in which laboratory eggs were seeded reached maturity in 48 days, with each larva feeding on > 40 *A. tsugae* ovisacs. Thus, a larva feeds on about 1.3

ovisacs/day, which is about the predation rate of an adult over its lifetime. The significant increase in the predation from three to four weeks in the field shows the considerable activity of *L. osakensis* larvae in the 3rd and 4th instars. It was also clear that the predator activity, larval in this case, had an impact on the next prey generation. The larvae directly eliminate the eggs that would be part of the next generation. The carry-over effect of predator activity on the next generation (progrediens) was also observed for *L. nigrinus* (Lamb et al. 2005b; Lamb et al. 2006). The development times from egg to mature larvae under field conditions are likely much shorter than the ones determined in this study, which were affected by the time the eggs spent in cold storage.

Based on the results reported here, it is possible to estimate the total impact of one *L. osakensis* female and its progeny. A female emerging in December and dying at the beginning of May can consume an average total of 163 *A. tsugae* adults (Table 4.2) and lay an average of 103 eggs in her lifetime (Fig. 4.2). *Laricobius osakensis* adults can emerge as early as October, so the impact might be slightly higher. If all the eggs produced by the female hatch and develop to mature larvae, these larvae will consume an average total of 4035 ovisacs ($103 \text{ larvae} \times 39.18 \text{ ovisacs/larva}$) (Table 4.4). A fertilized female thus has the potential to exert control over 4198 *A. tsugae*.

Overall, *L. osakensis* can survive, reproduce, develop, and impact *A. tsugae* populations in sleeve cages in plant hardiness zones 5b and 6b in Virginia. The earlier onset of oviposition and higher fecundity of *L. osakensis* are indications that *L. osakensis* has the potential to be a more effective biological control than *L. nigrinus*. Future experiments should focus on the performance of this predator in open releases, evaluating its impact, establishment, and dispersal.

Table 4.1 Voucher specimens for each study determined to species by sequence analysis.

Each voucher is labelled with “10-LOG1- followed by a unique sample number.

Sample number	Study	Species
01, 03-09, 11-12, 17-18, 22, 40-69	Adults (Biweekly)	<i>Laricobius osakensis</i>
02	Adults (Biweekly)	<i>Laricobius nigrinus</i>
23, 26-27, 30-33, 35-37	Adults (Bimonthly)	<i>Laricobius osakensis</i>
F4, F7-F15, F22- F31	Larvae	<i>Laricobius osakensis</i>

Table 4.2 Comparison of the number of dead *A. tsugae* in cages with and without (control) *L. osakensis* adults at the end of each biweekly interval (*t* statistic, degrees of freedom (DF) and *P*-value), and biweekly predation rates.

Sample interval (mm.dd.yy)	Dead <i>A. tsugae</i> (mean \pm SE)					Predation rate ¹ (prey/day/ predator)
	Cages with predators	Cages without predators	<i>t</i>	DF	<i>P</i> -value	
12.20.10 – 01.07.11	51.7 \pm 2.5	29.4 \pm 1.4	8.39	9	< 0.0001	0.6 \pm 0.1 bc
01.08.11 – 01.22.11	69.7 \pm 3.8	60.0 \pm 4.2	4.28	9	0.002	0.3 \pm 0.1 c
01.23.11 – 02.05.11	79.4 \pm 3.6	54.2 \pm 4.0	7.80	9	< 0.0001	1.0 \pm 0.1 bc
02.06.11 – 02.19.11	122.9 \pm 10.6	61.8 \pm 7.0	10.02	9	< 0.0001	2.3 \pm 0.2 a
02.20.11 – 03.07.11	129.2 \pm 9.9	53.6 \pm 9.2	6.35	9	< 0.0001	2.5 \pm 0.4 a
03.08.11 – 03.23.11	89.8 \pm 6.7	46.6 \pm 4.2	6.38	9	0.0001	1.4 \pm 0.2 b
03.24.11 – 04.09.11	105.5 \pm 7.3	73.6 \pm 6.6	4.48	9	0.0015	1.0 \pm 0.2 bc
04.10.11 – 04.23.11	155.0 \pm 9.0	150.0 \pm 9.6	2.27	9	0.0496	0.2 \pm 0.1 c

04.24.11 – 05.09.11	159.0 ± 5.7	154.5 ± 5.4	3.72	7	0.0075	0.1 ± 0.0 c
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¹ Predation rates with different letters were significantly different at *P*-value < 0.05 using Tukey Kramer HSD test.

Table 4.3 Comparison of mean number of live *A. tsugae* adults, dead *A. tsugae* adults, and consumed *A. tsugae* ovisacs in cages, with and without (control) *L. osakensis* adults at the end of each bimonthly interval (*P*-value based on paired t-test, DF = 9).

Sample interval (mm.dd.yy)	Dependent variable	<i>A. tsugae</i> (mean \pm SE)		<i>P</i> -value Between treatments
		Cages with predators	Cages without predators	
12.20.10 – 02.20.11	Live adults	193.3 \pm 26.1	348.8 \pm 10.6	0.0010
	Dead adults	332.4 \pm 27.5	176.9 \pm 11.5	0.0010
	Ovisacs consumed	0 \pm 0	0 \pm 0	nd
02.20.11 – 04.23.11	Live adults	0 \pm 0	146.6 \pm 35.4	0.0025
	Dead adults	450.8 \pm 8.9	304.2 \pm 36.7	0.0025
	Ovisacs consumed	404.2 \pm 45.8	0 \pm 0	< 0.0001

Table 4.4 Percentage of eggs that hatched, dead and alive immature and mature *L. osakensis* larvae recovered from sleeve cages at Mountain Lake, VA.

Sample interval (mm.dd.yy)	Eggs that hatched	Immature larvae (%)		Mature larvae (%)	
		Dead	Alive	Dead	Alive
03.11.12 – 04.01.12	15 (21.4 %)	0	100	0	0
03.11.12 – 04.08.12	30 (21.4 %)	7.4	44.5	22.2	25.9
04.27.12 – 05.23.12	18 (32.1 %)	5.6	0	94.4	0
04.27.12 – 06.03.12	40 (26.0 %)	5	0	95	0

Table 4.5 Comparison of the number of disturbed *A. tsugae* ovisacs in sleeve cages in the field with and without *L. osakensis* larvae (*t* statistic, degrees of freedom (DF) and *P*-value), and predation.

Sample interval (mm.dd.yy)	Disturbed <i>A. tsugae</i> ovisacs (mean \pm SE)					Predation (prey/larva) ¹
	Cages with predators	Cages without predators (total)	<i>t</i>	DF	<i>P</i> -value	
03.11.12 – 04.01.12	53.6 \pm 11.6	6.7 \pm 0.4	4.07	7	0.0047	23.0 \pm 1.9 a
03.11.12 – 04.08.12	89.2 \pm 7.9	9.1 \pm 0.3	10.37	14	< 0.0001	42.8 \pm 2.6 b
04.27.12 – 05.25.12	97.7 \pm 14.3	10.7 \pm 1.1	5.89	7	0.0006	40.0 \pm 3.2 b
04.27.12 – 06.03.12	111.9 \pm 9.1	10.4 \pm 1.3	10.63	13	< 0.0001	38.7 \pm 2.7 b

¹ Predation rates with different letters were significantly different at *P*-value < 0.05 using Tukey Kramer HSD test.

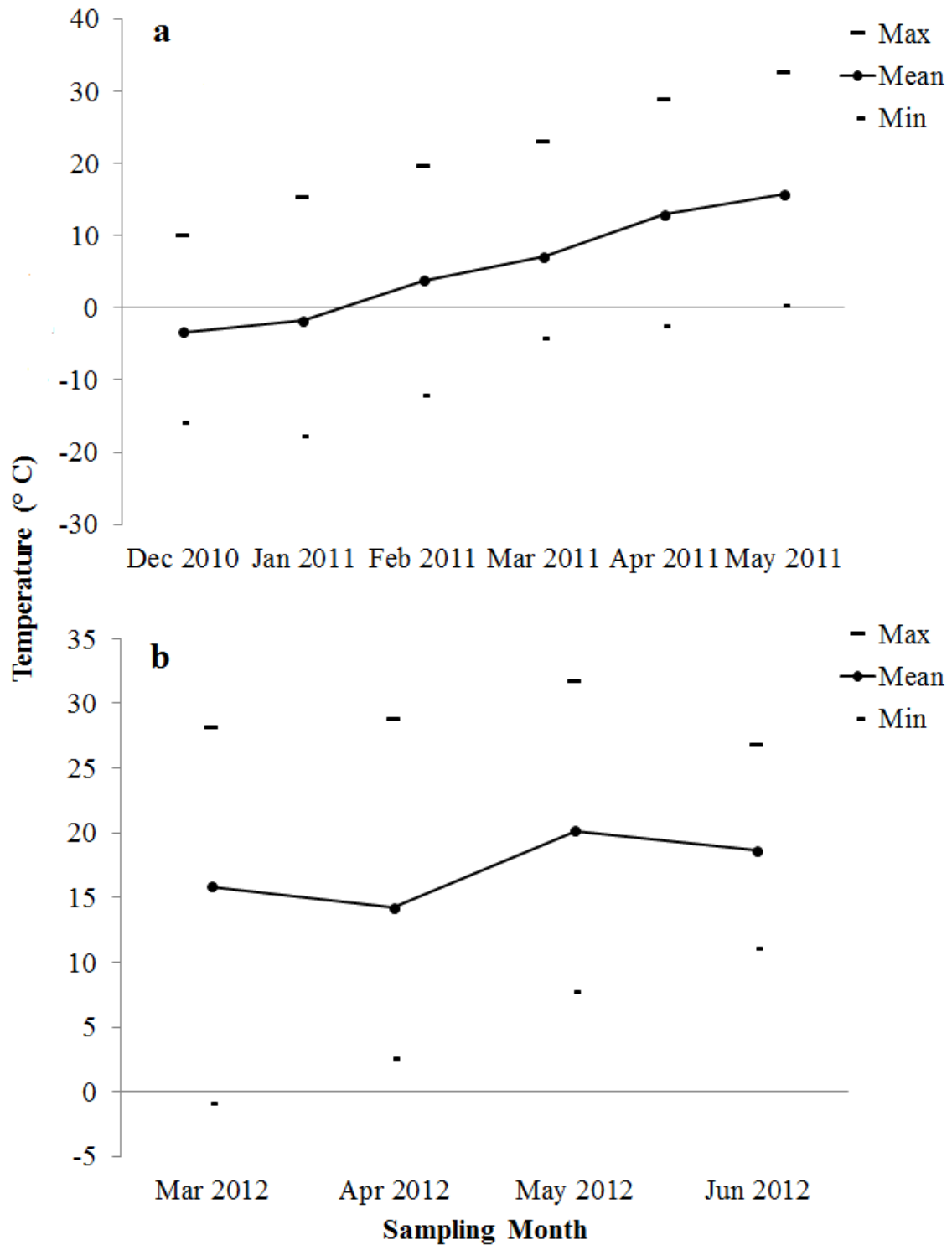


Figure 4.1 Mean, minimum (Min), and maximum (Max) temperatures for each sample month at Saltville, VA (a) and Mountain Lake, VA (b).

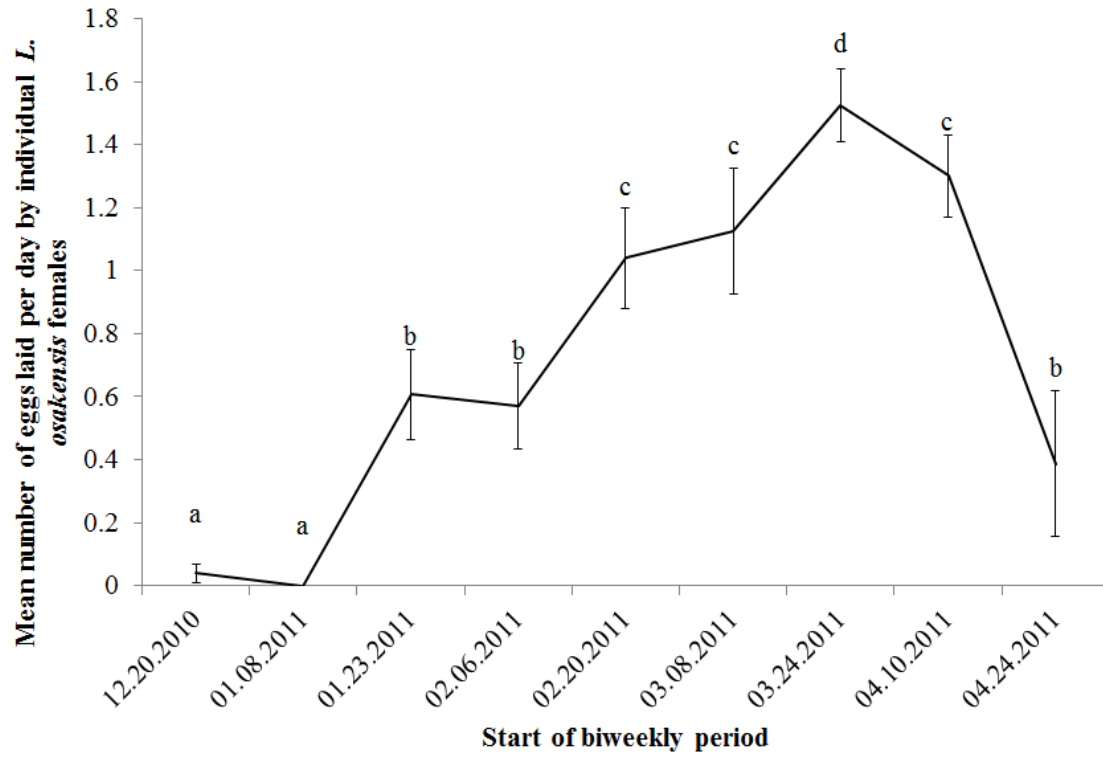


Figure 4.2 Number of eggs laid by individual *L. osakensis* females per day (\pm SE) in sleeve cages in the field during each biweekly interval in Saltville, VA. Different letters indicate a significantly different oviposition rate at P -value < 0.05 using Tukey Kramer HSD test.

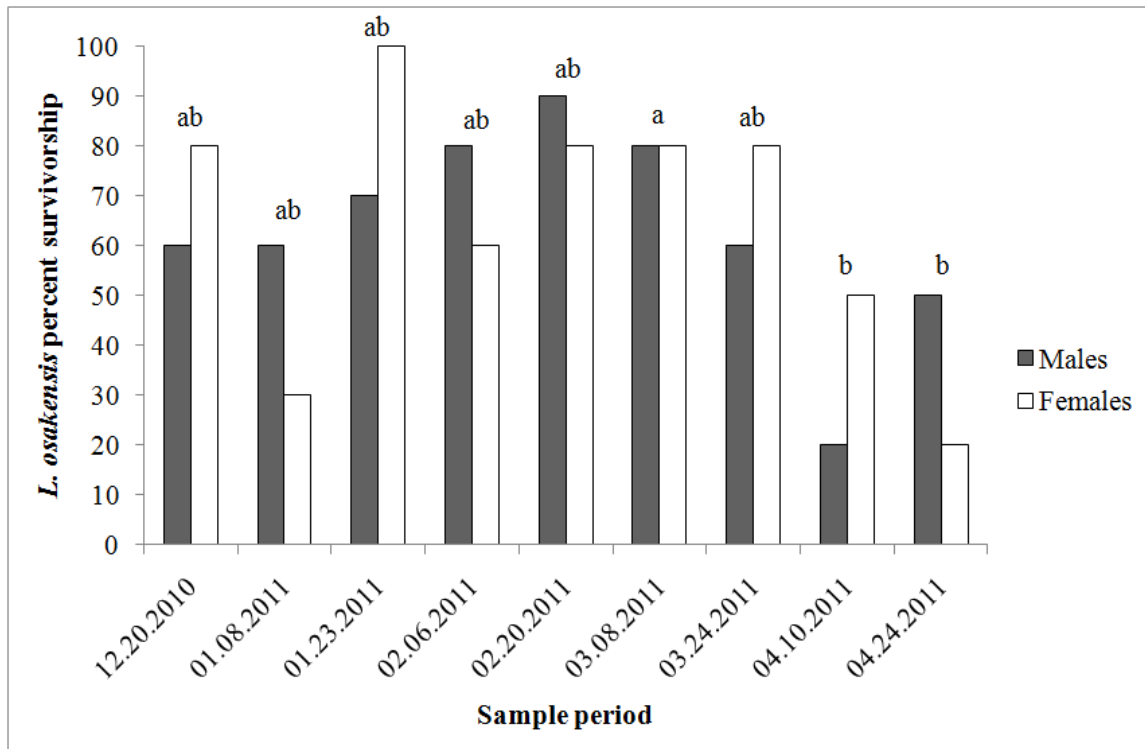


Figure 4.3 Survival of *L. osakensis* adult females and males during each biweekly interval in sleeve cages in Saltville, VA. Different letters indicate a significantly different survival rate at P -value < 0.05 using Tukey Kramer HSD test.

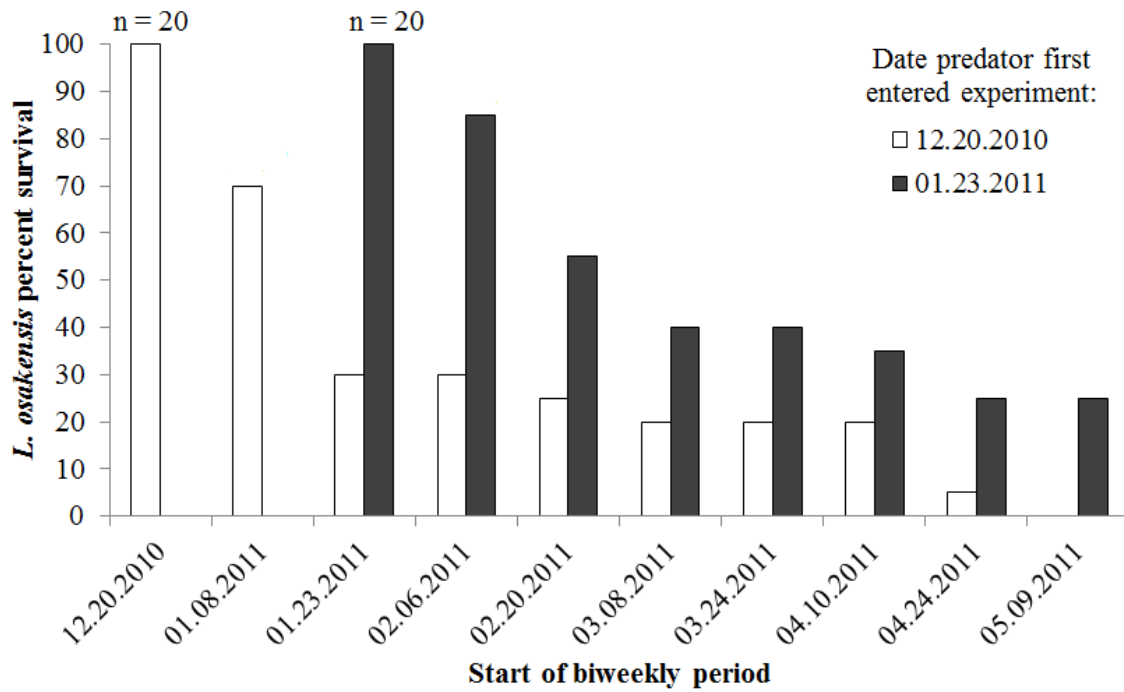


Figure 4.4 Survival of adult *L. osakensis* first placed in the field on 20 Dec 2010 or 23 Jan 2011 for the biweekly sleeve cage studies, to the end of the experiment on 09 May 2011, in Saltville, VA.

Chapter 5 **Behavior and daily activity patterns of *Laricobius osakensis* Montgomery & Shiyake and *L. nigrinus* Fender (Coleoptera: Derodontidae), two specialist predators of *Adelges tsugae* Annand (Hemiptera: Adelgidae)**

Abstract

Adelges tsugae Annand (Hemiptera: Adelgidae) is an exotic pest of hemlocks in the eastern US. The implementation of classical biological control has focused on building a natural enemy community, and is considered the most sustainable option for managing this pest in forest areas. *Laricobius nigrinus* Fender, native to western North America and *Laricobius osakensis* Montgomery & Shiyake, native to Japan, are highly specialized predators of *A. tsugae* already introduced or in the process of being introduced into the eastern US for the control of this pest. Previous studies on potential interactions have focused mostly on whether the two predators impact each other's effectiveness or not, but there are no studies on how *L. osakensis* interacts with its prey, in the presence of a congener individual. The behavior and daily activity patterns of both predators were examined using digital video recording in the laboratory. The daily activity patterns of adult females and males of each species were recorded in single-predator, and same sex/different species and different sex/same species (just winter) paired-predators assays under simulated winter and spring conditions. Behavior was qualitatively similar, but varied quantitatively by species. *L. osakensis* was more active than *L. nigrinus* and had a lower association to the tree host. Interactions between specimens were generally low and were not detrimental to either species. A greater level of interactions was observed

in male-female pairings, followed by male-male pairings, with the lowest level of interactions observed in female-female pairings. Intrasexual copulation attempts were observed between males and between females. The presence of the other species did not interfere with the level of activity or of oviposition of either species.

Keywords: *Laricobius osakensis*, *Laricobius nigrinus*, predator interactions, biological control

5.1 Introduction

There are two opposing schools of thought in classical biological control in relation to the use of a single predator or multiple predators. The first is careful screening of candidate natural enemy species, only using the most effective. This would limit the presence of natural enemies that are inferior biological control agents, but superior competitors (Heinz and Nelson 1996). The second assumes it is not cost- and time-effective to test all the natural enemies before selection, and there is little interference when releasing all available natural enemies (Heinz and Nelson 1996). The development of governmental policies regulating all activities related to the development and implementation of classical biological programs in the US have reflected influences of both schools—with a great emphasis in finding the best natural enemies and minimizing risks, but also the acknowledgement that resources are limited and that biological control is highly time sensitive. Biological control has to be implemented before the resource has been destroyed by the pest (Plant Protection Act (2000)). To assist in determining the best approach (single or multiple predator) for any given biological control effort, the true role of interspecific interactions has to be assessed. These types of experiments in general are fairly uncommon (Heinz and Nelson 1996); however, in the biological control program for *Adelges tsugae* Annand (Hemiptera: Adelgidae) there has been a diligent examination of this issue (Fischer et al. 2011, Flowers et al. 2005, 2006, 2007, Havill et al. 2011, Story et al. 2012).

Adelges tsugae is an exotic pest of eastern, *Tsuga canadensis* (L.) Carrière, and Carolina hemlocks, *T. caroliniana* Engelman, in the eastern US (Montgomery et al. 2009a; Orwig et al. 2002). First detected in Richmond in the early 1950's, it has spread to more than half of the geographic distribution of eastern hemlock, at times being responsible for the death of more than 90% of hemlocks in one location (Spaulding and Rieske 2010; USDA-FS 2012). This species is native to all ranges of hemlock with the exception of the eastern US (Havill et al. 2006). The origin of introduction into the eastern US has been identified as Japan (Havill et al. 2006). In its native range, *A. tsugae* is an innocuous inhabitant of hemlocks, its population being kept in control by the natural resistance of the host trees combined with the presence of a community of natural enemies. In contrast, in the eastern US the hemlock species are highly susceptible and there are no natural enemies specific to *A. tsugae* present (Cheah and McClure 2000). The only factor limiting the species dispersal is low winter temperatures, but even this factor is possibly being overcome by the pest (Butin et al. 2005).

Taking into consideration the large areas needing treatment and the sensitivity of hemlock ecosystems, biological control has been identified as the most sustainable option for the control of *A. tsugae* early on (USDA-FS 2005).

Sasajiscymnus tsugae (Sasaji and McClure) (Coleoptera: Coccinellidae), a specific predator of *A. tsugae* native to Japan, was the first predator introduced. Releases started in 1995 and continue to this date. However, even after establishment of the predator in some areas, it was apparent that this predator is unable to control *A. tsugae* on its own fast enough (Hakeem et al. 2010). It became clear that the success of this biological control program would rely on the establishment of a natural enemy community that is

able to control *A. tsugae* populations in the eastern US. With the need of using several predators as an effective unit, it became essential to make sure that all species introduced are effective natural enemies of *A. tsugae* but do not interfere with the action of others.

Laricobius nigrinus Fender is a specific predator of *A. tsugae* found in western North America (Zilahi-Balogh et al. 2002). This species exhibits: a good phenological synchrony with *A. tsugae* (Zilahi-Balogh et al. 2002), a functional and numerical response to varying prey densities (Vieira et al. 2012), and promising results in terms of establishment success and impact on *A. tsugae* populations (Davis et al. 2010; Mausel et al. 2010). Releases of *L. nigrinus* started in 2003 (Lamb et al. 2006) and are ongoing.

Scymnus camptodromus Yu & Liu (Coleoptera: Coccinellidae), *Scymnus sinuanodulus* Yu & Yao (Coleoptera: Coccinellidae), and *Scymnus ningshanensis* Yu & Yao (Coleoptera: Coccinellidae) were found frequently in association with *A. tsugae* in China. After laboratory trials with three species, *S. camptodromus* was dropped as a potential candidate due to rearing difficulties with this species, and *S. sinuanodulus* and *S. ningshanensis* releases started in 2004 (Cheah et al. 2004a).

Laricobius osakensis Montgomery & Shiyake is a specific predator of *A. tsugae* native to Japan (Vieira et al. 2011). As with its congener *L. nigrinus*, this predator shows a good phenological synchrony with *A. tsugae* (Lamb et al. 2011). It also shows a higher functional and numerical response than *L. nigrinus* (Vieira et al. 2012). Releases for this species started in December 2012.

In Flowers et al. (2007) the interactions between two specialist predators, *S. tsugae* and *L. nigrinus*, and the generalist *Harmonia axyridis* Pallas (Coleoptera: Derodontidae) were evaluated. Prey searching behavior by the generalist was different from the two

specialist predators. There are clear similarities between the specialist predators derived from specialization on the same prey, however, the two predators naturally do not interact with one another due to differences in temporal pattern. *Laricobius nigrinus* is a winter to spring predator, while *S. tsugae* is a spring to summer predator. Both species also maintain spatial separation when put together. This study provided some insights on *L. nigrinus* behavior, but only assessed females during part of their active period.

The finding of *Laricobius rubidus* LeConte (Coleoptera: Derodontidae), a native predator of *Pineus strobi* Hartig (Hemiptera: Adelgidae), also feeding on *A. tsugae* alongside *L. nigrinus*, and evidence that these species are hybridizing in some areas (Havill et al. 2011), has fueled the research of potential interactions between these two species and species being evaluated for introduction, such as *L. osakensis*. Preliminary studies on the hybridization of the three species indicate that while all species copulate, *L. osakensis* does not produce viable eggs paired with any of the other two species (Fischer et al. 2011).

As closely related congeneric species, the requirements of males and females of these species are the same or very similar, creating a greater potential for interference between the predator species. Intraguild predation, egg production, and survivorship were evaluated among *L. rubidus*, *L. nigrinus*, and *L. osakensis*. These species do not seem to negatively affect each other's impact on the prey, and when they do, interference is similar for intra and interspecifics (Story et al. 2012). This study did not capture: 1) the behavior pattern of how the predators interact with the prey, the environment, and other predators; 2) the behavioral pattern of females and males of both species throughout its period of activity (winter and spring); and 3) if there are potential indirect negative

effects of natural enemy effectiveness by potential aggression. The study reported here aims to fill these gaps in knowledge. For that purpose, the behavior and daily activity patterns of both predators were examined using digital video recording in the laboratory. The daily activity patterns of adult females and males of each species were recorded in single-predator and same sex/different species paired-predators assays under simulated winter and spring conditions.

5.2 Materials and Methods

5.2.1 Predator source

Laricobius osakensis adults used in the experiments were progeny of wild *L. osakensis* collected from *T. diversifolia* in Shiga Kogen, Japan in October 2011 that emerged in October/November of 2012. The *L. nigrinus* adults used in the experiments were progeny of wild *L. nigrinus* collected from *T. heterophylla* in urban areas in Seattle, Tacoma and Olympia, WA on November 2011 that emerged in September/October of 2012. Specimens of both species were maintained on field collected *A. tsugae*-infested eastern hemlock twig cuttings in environmental chambers at 4° C 12:12 (L:D) and 65-75% RH.

5.2.2 Specimen sex determination

5.2.2.1 Pre-oviposition period (only for *L. osakensis*)

Because both species are specific predators that are synchronized with *A. tsugae*, females usually only start ovipositing with the presence of *A. tsugae* sistens adults with eggs (Vieira et al. 2012; Zilahi-Balogh et al. 2003). This prevents the determination of sex by oviposition trials from emergence to the beginning of oviposition, 4 (*L. osakensis*) to 5 (*L. nigrinus*) months after emergence. Since *L. osakensis* exhibits some level of sexual dimorphism (Montgomery et al. 2011b), this was used to identify males and females in the pre-oviposition period. *Laricobius osakensis* coloration ranges from coppery brown to black. However, distinctive coppery brown specimens are usually females and dark brown or black specimens are males. Only specimens with these extreme colorings were selected with this method. Specimens were later dissected to confirm sex.

5.2.2.2 Oviposition period

Oviposition trials were conducted in 55 × 20.3 mm polystyrene Petri dishes with *A. tsugae*-infested twigs. One specimen was assigned to each Petri dish and allowed to feed and oviposit for 3 days. Petri dishes were placed in an environmental chamber at 9° C, 12:12 L:D, and 75-87% RH. In the subsequent analysis of the *A. tsugae* ovisacs, specimens were identified as a female if eggs were found and as males in the absence of eggs.

After the experiments, the specimens used were killed and preserved in 100% ethanol. *Laricobius osakensis* and *L. nigrinus* specimens were dissected to confirm sex.

5.2.3 Species determination

In fall 2011, it was discovered that the *L. osakensis* colony had a small cohort of the morphologically undistinguishable species, *L. naganoensis* Leschen (Leschen 2011). As a result, *L. osakensis* specimens were preserved in individual vials with 100% ethanol for post-experimental molecular analysis.

Laricobius osakensis adults were dissected under a microscope. The head, prothorax, elytra, and genitalia were kept as voucher specimens, and the meso- and metathorax with membranous wings were used for molecular analysis.

Genomic DNA extraction was performed with the use of a Qiagen DNeasy Blood & Tissue Kit (Qiagen, California, USA). PCR for the amplification of the partial cytochrome oxidase subunit I (COI) was performed in 30 µL reactions containing 3.0 µL 10× PCR Buffer, 2.4 µL dNTPs (10 mM), 3.0 µL MgCl₂ (25 mM), 1.0 µL BSA (10 mg/ml), 1.0 µl of forward primer LepF1 (10 mM), 1.0 µl of reverse primer LepR1 (10 mM), 0.3 µL Taq DNA polymerase, and 1.0 µL of DNA template. Thermal cycles were 95° C for 5 min. followed by 35 cycles of 45 s at 95° C, 45 s at 48° C, and 1 min at 72° C, with a final extension of 72° C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA), sequencing reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and the sequence was analyzed on an Applied Biosystems

3730 automated sequencer. Edition and alignment of partial cytochrome oxidase subunit I (COI) sequences was performed in Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI).

All specimens from the *L. osakensis* colony used in the study were confirmed to be *L. osakensis* (Table 5.1). There was a high level of genetic variation among the specimens used, as seen from the variation in relation to the reference *L. osakensis* sequence.

5.2.4 Video set up

Laricobius osakensis and *L. nigrinus* male and female behavior, daily activity pattern, and interactions were documented in the laboratory using intermittent digital video recording. Predator arenas consisted of 55 x 20.3 mm Petri dishes with two 2 cm hemlock twigs without needles, infested with at least 15 *A. tsugae* adults. The prey density was determined as to not limit the food resource for two predators in one day (Vieira et al. 2011). As the insects are very small, the needles had to be removed to allow for adequate observation of the insects. Although the removal of the needles is not consistent with what would be experienced in the wild, it fit the purpose of the assay by promoting contact between the beetles in the paired assays due to the reduced space and the scarcity of hiding places (no needles). While the assay is very artificial, it can provide valuable information on how these species interact when resources are limited and localized. Each predator species/sex was evaluated alone (individual assays) and combined with a specimen of the other species of the same sex (1 *L. osakensis* female + 1

L. nigrinus female or 1 *L. osakensis* male + 1 *L. nigrinus* male) (paired assays). Same sex pairings were analyzed because potential negative interactions are likely to be more evident in same sex pairings, as females or males of both species have similar requirements, and preliminary analysis of different sex interactions consisted mostly of copulation attempts. The first oviposition trials for *L. nigrinus* were found not to be very accurate, as a result, a lot of the specimens classified as males and paired with *L. osakensis* were in fact non-ovipositing females. Since there were enough replicates of this situation (1 *L. osakensis* male + 1 *L. nigrinus* female) in the winter, these different sex pairings were also included in the analysis. Predators were evaluated in environmental chambers under two sets of conditions termed as 1) winter: 4° C, 10:14 L:D, and 40-60 % RH and 2) spring: 12° C, 14:10 L:D, and 40-60 % RH. This range of conditions would be experienced by both predator species in the field. Two light sources with timers were placed on each side of the arena inside the environmental chamber to illuminate the arena for filming, one was a full-spectrum white light (day) and the other a red light (night). Ten replications of each treatment were completed.

Video capture was done using a one-chip MiniDV camcorder (Panasonic PV-GS35) that was mounted on a stand 5 cm above the arena. Automatic settings were used to control camcorder exposure, gain and white-balance level. A USB connection was used to join the camcorder to a computer. The webcam mode on the camcorder was used and video capture was controlled using the WebcamXP 5.5.1.2 software. One minute video recordings were captured every 15 min. over 24 h, making a total of 96 observations per day. Video segments were converted to Windows Media Video (.wmv)

format using YouTube Downloader & Converter 3.7 software and visualized using VLC media player 2.0.2 Twoflower software.

5.2.4.1 Individual predator assays

Predator behavior was classified into five general types: 1) inactive, 2) intensive behavior, 3) extensive behavior, 4) feeding, and 5) oviposition. This information was combined with the location of the specimen as: 1) on foliage and 2) outside of foliage. In each video segment, the time (in seconds) allotted to each behavior, except for oviposition, was registered. Inactive behavior was defined as a specimen motionless for more than 10 seconds. Intensive behavior was defined as a specimen moving slowly with frequent stops, turns and movement of head side to side, or small steps and head movement from side to side (antennal probing). Extensive behavior was defined as a specimen moving (regardless of speed) in a straight line, without stops and head pointed forward, or a specimen flying, defined as a specimen lifting the elytra and unfolding the membranous wings. Feeding was defined as a specimen placing head or mouthparts within a *A. tsugae* ovisac with some movement of the head pulling and pushing. Oviposition was determined indirectly by microscopic examination of twigs in search of eggs at the conclusion of the trial. While oviposition could sometimes be determined by observing the beetle inserting its ovipositor into an ovisac after close inspection, it was hard to distinguish this behavior given the size of the beetle and footage definition. Oviposition was recorded as the number of eggs the female laid in a 24 h period. Individuals of both species sometimes fell on their backs and remained that way for the

entirety of the video segment and sometimes through most of the video segments.

Specimens that were on their backs for 50% of the footage or more were excluded from the analysis. For the remaining specimens, the video segments where the specimens were on their backs were removed from the calculations.

Patterns of daily activity were determined by the time in seconds (excluding the time the specimen was on its back) each species allocated to each behavior classification during the day and the night, over the 24 h evaluation period. The pattern was compared between males and females, season, light conditions, and between species.

Behavioral pattern was variable even for the same individual. The overall level of activity was more consistent and gives a better indication of the potential impact of each species/sex on the pest population as all behaviors are involved in finding or interacting with the prey. Activity was determined as the total time spent feeding and searching intensively and extensively on and outside the host material. Inactivity was determined as the total time spent inactive on and outside the host material. Activity was compared between day and night conditions, males and females, seasons, and species.

Some differences in the association with the host material were noticed. To assess these potential differences, the time spent on and outside the host material (regardless of the behavior) was also compared between day and night conditions, males and females of the same species, and between species.

5.2.4.2 Paired predators assays

Behavior was scored for each of the two predators following the same classification identified in the individual trials, and the type and duration of interactions between the specimens was registered. The total time spent interacting with the other species was calculated, and compared between seasons and sex.

As previously mentioned, the behavior of each individual was highly variable, so the variation on the pattern might not be the best indicator of interference from the presence of the other species. Interference from the presence of the other specimen was evaluated by excluding the interactions and comparing the time a specific specimen was active in paired conditions with the time that same specimen was active in individual trials. One of the predators was marked with non-toxic pen Marvy Uchida Opaque Stix, however it was not possible to see the marking under night (red light) conditions, and the small size of the beetle and the position of the beetle in the assay (upside down on the lid) also made it hard to see the marker even in day conditions. It was still possible to differentiate the beetles based on their final positions, tracking the position of each specimen from the end of the trial to the beginning, and from differences in size.

5.2.5 Statistical Analysis

All data were tested for normality and equality of variance. Data in general did not follow a normal distribution but variance was homogeneous. The effects of species, sex, and season on the behavioral pattern (all behavior classifications except oviposition) in individual trials were evaluated by a multivariate analysis of variance (MANOVA). If significant differences were found, univariate analyses were also performed. A

parametric test was used because it is considered to be more accurate than the non-parametric alternative when the assumption violated is normality (Finch 2005). The effect of light conditions (day/night) on the behavioral pattern in individual trials was evaluated by a repeated measures MANOVA. If significant differences were found, univariate analyses were also performed. Differences between the time spent on and outside the host material and between inactive and active were assessed using a paired t-test, accounting for the correlation between the variables (measured in the same individual). The effect of species, sex, and season on the time spent on the host material, and on time spent inactive, were evaluated by an ANOVA. The effect of light conditions on time spent on the host material, and on time spent inactive was evaluated by a paired t-test. Generalized Linear Model following a Poisson distribution was used to assess the effect of species and season on oviposition.

The effect of species, season, and light conditions on the behavioral pattern in paired assays was evaluated by a MANOVA. If significant differences were found, univariate analyses were also performed. The effect of sex pairing (female-female or male-male), season, and light conditions in the incidence of the different interaction types was evaluated by Wilcoxon tests. The effect of the presence of the specimen of the other species on individual activity and oviposition was evaluated by a paired t-test. The response pairs were the time the same individual was active in the individual assay and in the paired assay, and the number of eggs laid in the paired trial and the sum of the eggs laid by the same females in the individual trials, respectively. Statistical treatments were performed in JMP (SAS_Institute_Inc. 2009).

5.3 Results

5.3.1 Individual predator assays

5.3.1.1 Qualitative analysis

Adult predator behavior had similar characteristics for both species. Extensive behavior occurred primarily outside foliage and consisted primarily of linear movement of the specimens with few turns. Specimens of both species moved slower in winter conditions than in spring conditions, but speed was variable among specimens of the same species. Both species walked frequently along the bottom and upper rim of the Petri dish, with *L. osakensis* having a preference for the upper rim (Video 5.1). Flying was also included into extensive behavior, but was a rare occurrence for both species. The specimens climbed to an elevated point in the assay (the top rim of the Petri dish), lifted the elytra, unfolded the membranous wings, and dropped from that point.

Intensive search consisted of close inspection of particular points in the Petri dish (Video 5.2) or successive inspection of *A. tsugae* ovisacs (Video 5.3). Inspection consisted mostly of frequent tapping of the antennae around the point or prey of interest. Intensive search on foliage was followed by feeding or oviposition, but also inactive.

Feeding behavior was exhibited by both species as pulling the basal (closer to the twig) woolly covering of an ovisac with their mandibles, moving their heads side to side while pushing to gain access to the eggs and adult *A. tsugae* within, and head movement up and down while consuming the prey. When attacking *A. tsugae* adults, predators

frequently made one single wound to drink the exuding hemolymph. Predators rarely completely drained the *A. tsugae* adults they fed upon.

Before oviposition, the female walked on and tapped the ovisac with her antennae. After inspection, the female stood on or close to the ovisac, extended the posterior abdominal segments into a functional ovisac, inserted the tip of the extended abdomen into the side of the *A. tsugae* ovisac, and deposited one or more eggs inside. Oviposition behavior was hard to observe because of the image quality. It can also be confused with intense search of the foliage as it is frequently preceded and followed by this behavior and it can be very fast—the extension of the abdomen, egg deposition and retraction of the abdomen can take less than 10 s. When the behavior was observed, search of predator eggs within the *A. tsugae* ovisacs confirmed or disproved that the behavior observed was oviposition.

While inactive, specimens were completely stationary and with their heads lowered while inactive. Inactivity usually occurred in concealed locations—on the side or under the twig—for both species. The specimens would actively avoid illumination hiding the head under the twig or an ovisac. However, *L. osakensis* was also inactive frequently outside of the foliage, mostly at the top rim of the Petri dish (Video 5.4). Grooming was also included into this behavior because it was very hard to consistently detect in the footage.

5.3.1.2 Quantitative analysis

The daily activity patterns were affected by some of the independent variables (Wilk's Lambda $F(42, 63) = 2.41, P < 0.0001$). They were significantly different in the winter and spring (season) ($F(6, 63) = 3.57, P = 0.0044$) and for *L. osakensis* and *L. nigrinus* (species) ($F(6, 63) = 13.63, P < 0.0001$), but not for males and females (sex) ($F(6, 63) = 0.67, P = 0.6721$). No interactions were found between the independent variables. Light conditions also affected the daily activity patterns ($F(11, 63) = 13.37, P < 0.0001$). Feeding and oviposition were significantly greater in the spring than in winter (Table 5.2). Intensive behavior, inactivity on host material, and oviposition were greater, but intensive behavior outside host material was lesser for *L. nigrinus* than for *L. osakensis* (Table 5.2, Fig. 5.1). Inactivity on host material was significantly lesser, but feeding, intensive behavior on and outside foliage, and extensive behavior on and outside foliage were significantly greater, during the day than at night (Table 5.2).

In terms of location, *L. osakensis* spent significantly more time outside (4026.65 ± 292.07 s) than on (1733.35 ± 292.07 s) host material ($t = 3.93, DF = 32, P = 0.0004$). Light conditions did not affect, but both sex and season affected the location preference (Table 5.3). A greater amount of time was spent on the host material by females than males, and during the spring than in winter. *Laricobius nigrinus* species spent significantly more time on (3644.45 ± 269.17 s) than outside host material (2114.77 ± 169.17 s) ($t = -2.89, DF = 36, P = 0.0064$). Sex, season and light conditions did not affect the location preference (Table 5.3). *Laricobius nigrinus* was on host material a significantly greater amount of time than *L. osakensis* was ($F(1, 68) = 23.23, P < 0.0001$).

Laricobius osakensis was active (4317.18 ± 192.86 s) for a greater amount of time than it was inactive (1442.82 ± 192.86 s) ($t = -7.45$, $DF = 32$, $P < 0.0001$). Light conditions and season had a significant effect on activity, with greater activity during the day than at night and in the spring than in the winter (Table 5.3). Active (3196.93 ± 213.98 s) and inactive (2563.07 ± 213.98 s) time were generally equivalent for *L. nigrinus* ($t = -1.48$, $DF = 36$, $P = 0.1473$), but varied with light conditions and season, with a significantly greater level of activity during the day than at night, and in spring than in winter (Table 5.3). *Laricobius osakensis* activity level was significantly greater than that of *L. nigrinus* ($F(1, 64) = 13.65$, $DF = 1$, $P = 0.0005$).

5.3.2 Paired predator assays

5.3.2.1 Qualitative analysis

The interactions between the specimens fitted into two categories: antennal probing, characterized by one or both specimens analyzing the other with the antennae (Video 5.5); and copulation attempt, characterized by one specimen holding the side of the pronotum with the mandibles and mounting the other specimen. If the specimen attempting copulation was a male and the other specimen did not try to evade the attempt, which happened more frequently, the specimen attempting the copulation was usually seen holding on the side of the other specimen and extending the abdomen to expose the aedeagus and insert it in the other specimen. Copulation attempts were also seen by females (confirmed by dissection) of both species. For females, one specimen also held

the side of the pronotum with the mandibles and mounted the other specimen. The duration of this interaction was usually short and afterwards each would continue with the behavior exhibited before the interaction. Both types of interactions in general resulted from random crossing of paths, although males were sometimes seen walking towards and even chasing the other specimen to attempt copulation.

5.3.2.2 Quantitative analysis

Similar to the individual assays, the daily activity pattern in the paired assays was affected by the season ($F(6, 63) = 11.72, P < 0.0001$) and species ($F(6, 63) = 11.10, P < 0.0001$), but not sex ($F(6, 63) = 1.99, P = 0.0813$) (Wilk's Lambda $F(42, 63) = 4.00, P < 0.0001$). No interactions were found between the independent variables. Light conditions also affected the daily activity pattern ($F(11, 63) = 18.28, P < 0.0001$). Intensive behavior on and outside host material, and feeding were significantly greater, and inactivity outside host material was significantly lesser, in spring than in winter (Table 5.4). Inactivity on host material was greater, but extensive behavior and intensive behavior outside host material was lesser, for *L. nigrinus* than for *L. osakensis* (Table 5.4, Fig. 5.2). Inactivity outside host material was significantly lesser, but intensive behavior on and outside foliage, and feeding were significantly greater, during the day than at night (Table 5.4).

As in the individual assays, *L. osakensis* spent significantly more time outside (4033.17 ± 280.58 s) than on (1726.83 ± 280.58 s) host material ($t = -4.11, DF = 33, P = 0.0002$). Light conditions did not affect the location preference (Table 5.5). Sex and

season affected the location preference. A greater amount of time was spent on the host material by females than males, and during the spring than in winter (Table 5.5).

Laricobius nigrinus species spent significantly more time on (3553.40 ± 265.86 s) than outside (2206.31 ± 265.86 s) the host material ($t = -2.53$, $DF = 36$, $P = 0.0158$). Sex and season did not affect location preference, but light conditions did. A greater amount of time was spent on the host material during the day than at night (Table 5.5).

Laricobius nigrinus was on host material a significantly greater amount of time than *L. osakensis* was ($F(1, 69) = 22.35$, $P < 0.0001$).

As in the individual assays, *L. osakensis* was active (4322.76 ± 192.88 s) for a greater amount of time than it was inactive (1437.24 ± 192.88 s) ($t = -7.48$, $DF = 33$, $P < 0.0001$). Light conditions and season had a significant effect on activity, with greater activity during the day than at night and in the spring than in the winter (Table 5.5). Active (3195.09 ± 236.57 s) and inactive (2390.04 ± 236.57 s) time were in general equivalent for *L. nigrinus* ($t = -1.87$, $DF = 36$, $P = 0.0701$), but also varied with light conditions and season, with a significantly greater level of activity during the day than at night, and in spring than in winter (Table 5.5). *Laricobius osakensis* activity level was significantly greater than that of *L. nigrinus* ($F(1, 64) = 13.65$, $P = 0.0005$).

5.3.2.3 Interactions and interference evaluation

Light conditions did not affect the level of either type of interaction: copulation attempts ($Z = -0.23908$, $P = 0.811$) or antennal probing ($Z = -1.29469$, $P = 0.1954$). The level of interactions was generally low, but some males interacted up to 35.35 % of the

time. No significant differences were found in the amount of time spent antennal probing the other species in same sex pairings in the spring, and in same sex pairings or different sex pairings in the winter (Table 5.6). The level of copulation attempts was greater in male-male pairings than in female-female pairings in winter and spring (Table 5.6). In male-female pairings the level of copulation attempts was greater than in both male-male and female-female pairings in the winter (Table 5.6).

The level of activity was similar in individual and paired assays for *L. osakensis* and *L. nigrinus* males and females in spring and winter (Table 5.7).

The average number of eggs laid by females in paired trials (winter: 0.91 ± 0.37 , spring: 5.5 ± 0.78) and by both females in individual trials (winter: 1.09 ± 0.48 , spring: 5.0 ± 0.65) was similar in the winter ($S = 3.0$, $P = 0.5313$) and the spring ($S = -7.5$, $P = 0.1875$).

5.4 Discussion

The theory of optimal foraging contends that a predator adjusts its foraging effort according to the resource availability (MacArthur and Pianka 1966). For a predator whose prey has an aggregated distribution, the search activity of the predator is usually divided into two phases: a superficial sampling over a large area (extensive search) and, after finding the prey patch, a thorough inspection of a localized region (intensive search) (Bond 1980). As it was expected, since *L. osakensis* and *L. nigrinus* are specific predators of *A. tsugae* (Vieira et al. 2012; Zilahi-Balogh et al. 2002), both species exhibited this adaptation for predation on aggregated prey.

The change from extensive to intensive search has been described for several aphidophagous predators and is usually related to the detection of resource cues (Banks 1957; Bond 1980; Chandler 1969; Dixon 1959; Ferran et al. 1994; Nakamuta 1985). The intense movement of the head and antennae for *L. osakensis* and *L. nigrinus* indicates that olfaction is probably a key sense in resource finding for both species. This finding is consistent with previous studies describing: the numerous olfactory sensilla found along the length of the antennae of *L. nigrinus* (Broeckling and Salom 2003), olfaction as a potential key sense in prey finding (Flowers et al. 2007), and the orientation of *L. nigrinus* to host tree odors (Wallin et al. 2011). The same characteristics are also likely to be found in *L. osakensis*. It was also noticeable that in most cases where there were significant differences between diurnal and nocturnal activity, the highest level of activity was registered during the day for both species. This provides some indication that vision might also play a relevant part in prey location, which contradicts Flowers et al. (2007) that found a greater nocturnal activity for *L. nigrinus*, but is consistent with studies on the visual ability and searching behavior of *L. nigrinus*, which indicated a greater level of searching activity during the day (Mausel et al. 2011). The high nocturnal activity in Flowers et al. (2007) might be related to the experimental conditions in that study. In Flowers et al. 2007 the beetles were tested under each light condition (day or night) continuously for 24 h. As it has been observed for several other insects, these abnormal conditions might lead to a disruption in the circadian rhythm of the beetles (Aschoff 1979; Hammack and Burkholder 1976; Romero et al. 2010; Saunders 1997).

Intensive search for *L. osakensis* and *L. nigrinus* was characterized by tapping the antennae on the point of interest; this indicates that contact chemoreceptors might be used

by these species for the evaluation of prey quality for feeding or oviposition. In addition to the olfactory sensilla, contact chemoreceptive sensilla also have been found on *L. nigrinus* antennae (Broeckling and Salom 2003).

When outside the foliage, both species frequently climbed to the highest point in the container—the lid. This climbing behavior has been observed as part of prey searching behavior (Mausel et al. 2011) and matches the dispersal pattern of *L. nigrinus* in the field characterized by the initial movement of the predator up the canopy of the release tree and, only after reaching the upper part, dispersal to other trees (Davis et al. 2012).

The even distribution between activity and inactivity of *L. nigrinus* in the spring was seen by Flowers et al. (2007). This study confirms this pattern in spring but also in winter. The clear association of *L. nigrinus* with the tree host is consistent with findings of a fairly low level of dispersal immediately after their release in the field (Davis et al. 2010).

No differences were found in the specific behavior pattern of males and females of *L. osakensis* or *L. nigrinus*. However, in contrast with *L. nigrinus*, significant differences were found between the location preference of *L. osakensis* males and females. The behavioral differences between females and males were probably a reflection of different factors directing behavior for each sex and different ways to achieve those goals. Female behavior is likely driven by the purpose of producing eggs and laying them as effectively as possible, thus the association with the host material. Conversely, male behavior is likely driven by finding as many females as possible to

mate with, being advantageous for the males to “roam” outside of the host material for this purpose (Evans 2003).

The tendency of *L. osakensis* to be outside the foliage can either reflect the normal behavior of this species in its native environment or it might be interpreted as a low attractiveness of this predator to the prey. In Wallin et al. (2011), comparison of preferences to tree host volatiles between native range and first generation laboratory reared *L. nigrinus* were observed. Field collected beetles in general selected a tree host (mostly western hemlock) they were collected from. In contrast, greater than 50% laboratory reared beetles did not exhibit any preference. This suggests that the attractiveness to the tree host for *L. nigrinus* is related to the physical ability to detect tree host volatiles and its previous experience with a specific host. The lack of preference of a lot of the *L. nigrinus* specimens can potentially be related to a suboptimal ability of these specimens to detect the new plant (did not evolve with) host volatiles. It is likely that *L. osakensis* would have the same problem, especially as second generation laboratory reared specimens. However, it is unlikely that this is driving the behavior because the *L. nigrinus* laboratory reared specimens used in this study would have the same limitation but were nonetheless associated with the foliage. *Laricobius osakensis* did not show any difficulties in returning to the foliage after being outside. Additionally, there were strong indications that vision is also an important sense in prey searching. Studies on the orientation of *L. osakensis* to the host tree would help elucidate this matter. The preference of *L. osakensis* for being outside the foliage is probably a result of the greater activity of this predator and might be indicative of a faster dispersal by this species.

Both species responded to season, with an increase in the feeding. However, the effect of season is more evident in regards to oviposition. *Laricobius osakensis* and *L. nigrinus* females laid eggs mostly under spring conditions. Oviposition in spring is related to the synchrony of these species with *A. tsugae*. Laying eggs at this time guarantees that the progeny will have optimal temperature for development and food available as *A. tsugae* starts oviposition in spring as well (Zilahi-Balogh et al. 2003).

In general, *L. osakensis* activity was greater than *L. nigrinus* regardless of sex, season or light conditions, but it is likely that *L. nigrinus* narrows the gap from *L. osakensis* with a slight increase of activity in spring (as seen by oviposition). *Laricobius osakensis* showed a greater intensive behavior outside the host material, and a lesser activity (intensive behavior) and inactivity on the host material than *L. nigrinus*, evidencing the greater activity and the lesser association to the host material of *L. osakensis*. The higher oviposition rate of *L. osakensis* is consistent with previous findings indicating a greater numerical response of this species (Vieira et al. 2012). The overall greater activity level of *L. osakensis* indicates an adaptation of this species to colder climates. The greater activity of *L. osakensis* in relation to *L. nigrinus* would indicate a greater impact under the same conditions but, because of *L. osakensis* adaptation to colder climates, there seems to be a trade-off between level of activity and the life span of the specimen (pers. obs.). To ensure the success of this predator in impacting *A. tsugae* populations and establishing in the release sites, there should be a careful climate matching to optimize the activity and longevity of the beetles.

In the paired trials both species initiated contact with the other. No direct negative interactions (aggression) were observed. Intrasexual mating was observed for

males and females. Copulations attempts between males were very frequent. Male-male mating attempts have been observed in several insect species (Clarke et al. 1985; Harari et al. 2000; Peschke 1987; Scott 1986). Several potential justifications for this behavior have been proposed by Harari et al. (2000): 1) female mimicry by less competitive males to avoid aggression, 2) larger males displaying dominance over weaker males, 3) sperm transfer by which the sperm of the smaller males 'hitchhikes' with the sperm of larger males, and 4) poor sex recognition. Since the copulation attempts between the males ended fairly quickly, probably as soon as the male attempting copulation realizes the other specimen is also a male, the male-male mating attempts in *L. osakensis* and *L. nigrinus* are likely a result of poor sex recognition. When males did find females, the duration of the interactions was significantly longer as seen by the greater proportion of time spent on copulation attempts in the intersexual pairings. Copulation attempt by females was rare, but females of both species attempted it, and it was previously observed among *L. nigrinus* females. Female-female mating is less frequent, but while its benefits are generally unknown, it has been observed in other insect species (Harari and Brockmann 1999; Robertson 1985). Potential justifications for this behavior have been proposed by Harari et al. (2000): 1) females mimic male behavior to reduce harassment by males, 2) females mount other females in order to appear larger and thereby attract larger (better) males for mating, 3) females that are normally larger than the males mimic males in order to only have the larger males attempt mating (are the only ones that can face the large female mounting). We propose an additional alternative, 4) females mimic male behavior to induce egg production on other females. Egg production induced by copulation has been seen in several mammals (Larivière and Ferguson 2003). The

selection of this last option as the justification for *L. osakensis* and *L. nigrinus* female behavior is related to the fact that virgin females of both species paired with other virgin females of the same or different species lay unfertilized eggs (Fischer pers. comm.).

The differences between species in the paired assays were consistent with the findings from individual assays, relating to location (on or outside host material) and activity level. The differences between species found in the paired assays were consistent with the differences found in individual assays. The behavioral pattern of either species does not seem to be affected by the presence of the other species.

Neither predator species attacked the other, but it should also be considered that a change in activity due to the presence of the other individual could potentially affect their effectiveness as biological control. In our findings, activity levels were similar in individual and paired trials. The presence of the other predators did not affect the predators' behavior in relation to the prey. The presence of another female also did not seem to affect the oviposition of either species.

5.5 Conclusion

Laricobius osakensis and *L. nigrinus* behaviors were generally similar, however *L. osakensis* showed an overall greater level of activity and a lesser association to the tree host, which might be indicative of this species' adaptation to cooler climates and a potentially greater level of dispersal, respectively. Interactions between specimens were not detrimental to either species, consisting of either antennal probing of the other specimen or copulation attempts. The presence of the other species is unlikely to

significantly affect the impact of each species as it did not change the level of activity or of oviposition of either species. Based on these results, it seems unlikely either predatory species would negatively impact the other if released in the same sites. For the successful implementation of biological control for *A. tsugae*, provided there is careful matching of *L. osakensis* with the appropriate climatic conditions, previous releases of *L. nigrinus* should not constrain the addition of *L. osakensis*.

Table 5.1 Specimens sample name, sex, the species determined by sequence analysis, and the number of different nucleotides from the reference *L. osakensis* sequence.

Sample name	Sex	Species Determined	Number of different nucleotides
11-LOSG1-F7, 21, 30	Female	<i>Laricobius osakensis</i>	0
11-LOSG1-F1, 2, 6, 16, 18	Female	<i>Laricobius osakensis</i>	1
11-LOSG1-F4, 5, 8, 14, 19, 26, 27, 34	Female	<i>Laricobius osakensis</i>	2
11-LOSG1-F3, 9, 11, 13, 15, 20, 22, 28, 29, 33, 37	Female	<i>Laricobius osakensis</i>	3
11-LOSG1-F10, 17, 24, 25, 31, 32	Female	<i>Laricobius osakensis</i>	4
11-LOSG1-F12, 23, 35	Female	<i>Laricobius osakensis</i>	5
11-LOSG1-M7, 13, 19	Male	<i>Laricobius osakensis</i>	0
11-LOSG1-M1, 12, 20	Male	<i>Laricobius osakensis</i>	1
11-LOSG1-M3, 4, 22	Male	<i>Laricobius osakensis</i>	2

11-LOSG1-M5, 10, 11, 14, 16, 17, 23	Male	<i>Laricobius osakensis</i>	3
11-LOSG1-M9, 15, 18	Male	<i>Laricobius osakensis</i>	5
11-LOSG1-M2, 6, 24	Male	<i>Laricobius osakensis</i>	6
11-LOSG1-M8	Male	<i>Laricobius osakensis</i>	7

Table 5.2 Effect of season (winter and spring), species (*L. osakensis* and *L. nigrinus*) and light conditions (day and night) on behavior (inactive on foliage, inactive outside foliage, intensive behavior on foliage, extensive behavior, feeding, and oviposition) in individual assays.

Independent Variable	Behavior	<i>F</i>	DF	<i>P</i> -value ¹
Season	Inactive on foliage	1.16	1	0.2848
	Inactive outside foliage	2.97	1	0.0893
	Intensive behavior on foliage	0.23	1	0.6353
	Intensive behavior outside foliage	3.35	1	0.0716
	Extensive behavior	0.10	1	0.7507
	Feeding	4.38	1	0.0400
	Oviposition	18.06	1	< 0.0001
Species	Inactive on foliage	31.50	1	< 0.0001
	Inactive outside foliage	0.81	1	0.3708
	Intensive behavior on foliage	5.16	1	0.0262
	Intensive behavior outside foliage	26.95	1	< 0.0001
	Extensive behavior	3.05	1	0.0850
	Feeding	2.23	1	0.1400
	Oviposition	17.22	1	< 0.0001
Light Conditions	Inactive on foliage	5.12	1	0.0268
	Inactive outside foliage	1.73	1	0.1924
	Intensive behavior on foliage	10.14	1	0.0022

Intensive behavior outside foliage	4.99	1	0.0287
Extensive behavior	4.56	1	0.0363
Feeding	22.73	1	< 0.0001

¹ Highlighted (bold) values were significantly different at $P \leq 0.05$.

Table 5.3 Comparison of the mean time \pm SE (in seconds) spent on the host material and active by *L. osakensis* (*Lo*) and *L. nigrinus* (*Ln*) in individual assays for different light conditions, sexes, and seasons.

Species	Independent Variable	Levels	Mean time on host \pm SE (in seconds) ¹	Mean time active \pm SE (in seconds) ²
<i>Lo</i>	Light conditions	Day	863.67 \pm 151.24 a	2387.99 \pm 99.31 a
		Night	850.08 \pm 157.22 a	1951.08 \pm 120.93 b
	Sex	Male	1252.99 \pm 368.05 b	4042.91 \pm 268.35 a
		Female	2243.73 \pm 433.70 a	4591.45 \pm 268.35 a
	Season	Winter	1012.74 \pm 347.83 b	3764.77 \pm 251.99 b
		Spring	2411.57 \pm 406.36 a	4808.21 \pm 237.57 a
<i>Ln</i>	Light conditions	Day	1838.36 \pm 155.59 a	1750.62 \pm 127.83 a
		Night	1741.26 \pm 146.41 a	1475.86 \pm 128.45 b
	Sex	Male	3357.12 \pm 328.61 a	3218.88 \pm 366.08 a
		Female	3800.86 \pm 375.90 a	3185.04 \pm 269.43 a
	Season	Winter	3382.46 \pm 457.26 a	2521.84 \pm 280.02 b
		Spring	3868.07 \pm 313.99 a	3770.75 \pm 258.16 a

¹ Mean times on host with different letters within each independent variable were significantly different at $P \leq 0.05$.

² Mean times active with different letters within each independent variable were significantly different at $P \leq 0.05$.

Table 5.4 Effect of season (winter and spring), species (*L. osakensis* and *L. nigrinus*) and light conditions (day and night) on behavior (inactive on foliage, inactive outside foliage, intensive behavior on foliage, extensive behavior, and feeding) in paired assays.

Independent Variable	Behavior	<i>F</i>	DF	<i>P</i> -value ¹
Season	Inactive on foliage	0.18	1	0.6740
	Inactive outside foliage	11.34	1	0.0012
	Intensive behavior on foliage	18.25	1	< 0.0001
	Intensive behavior outside foliage	6.68	1	0.0119
	Extensive behavior	2.59	1	0.1120
	Feeding	8.70	1	0.0043
Species	Inactive on foliage	43.13	1	< 0.0001
	Inactive outside foliage	2.84	1	0.0963
	Intensive behavior on foliage	3.77	1	0.0563
	Intensive behavior outside foliage	31.10	1	< 0.0001
	Extensive behavior	5.96	1	0.0172
	Feeding	0.33	1	0.5646
Light Conditions	Inactive on foliage	3.29	1	0.0739
	Inactive outside foliage	14.09	1	0.0004
	Intensive behavior on foliage	5.02	1	0.0283
	Intensive behavior outside foliage	5.55	1	0.0213
	Extensive behavior	0.03	1	0.8700
	Feeding	29.76	1	< 0.0001

¹ Highlighted (bold) values were significantly different at $P \leq 0.05$.

Table 5.5 Comparison of the mean time \pm SE (in seconds) spent on the host material and active by *L. osakensis* (*Lo*) and *L. nigrinus* (*Ln*) in paired assays for different light conditions, sexes, and seasons.

Species	Independent Variable	Levels	Time on host \pm SE (in seconds) ¹	Time active \pm SE (in seconds) ²
<i>Laricobius osakensis</i>	Light conditions	Day	458.35 \pm 104.52 a	1266.20 \pm 87.42 a
		Night	296.99 \pm 78.37 a	958.50 \pm 68.62 b
	Sex	Male	1254.99 \pm 385.14 b	4045.98 \pm 263.53 a
		Female	2200.67 \pm 385.14 a	4616.84 \pm 271.64 a
	Season	Winter	988.52 \pm 374.62 b	3824.90 \pm 252.36 b
		Spring	2383.11 \pm 353.20 a	4791.33 \pm 244.82 a
<i>Laricobius nigrinus</i>	Light conditions	Day	774.45 \pm 125.96 a	995.69 \pm 87.99 a
		Night	461.37 \pm 76.63 b	675.44 \pm 56.68 b
	Sex	Male	3350.54 \pm 452.88 a	3411.37 \pm 379.69 a
		Female	3663.29 \pm 333.31 a	3054.51 \pm 306.11 a
	Season	Winter	3363.15 \pm 395.37 a	2600.52 \pm 311.48 b
		Spring	3715.12 \pm 364.51 a	3754.69 \pm 302.18 a

¹ Mean times on host with different letters within each independent variable were significantly different at $P \leq 0.05$.

² Mean times active with different letters within each independent variable were significantly different at $P \leq 0.05$.

Table 5.6 Comparison of the mean time (in seconds) (\pm SE) spent by specimens in *L. osakensis* and *L. nigrinus* paired assays (female-female (FF), male-male (MM) and male-female (MF)) on antennal probing of the other species and copulation attempts in spring (12° C, 14:10 L:D, 50-75% RH) and winter conditions (4° C, 10:14 L:D, 50-75% RH).

	Spring		Winter		
	FF ¹	MM ¹	FF ¹	MF ¹	MM ¹
Antennal probing	15.8 \pm 5.16 a	36.7 \pm 17.00 a	73.55 \pm 46.40 a	35.6 \pm 35.6 a	75.6 \pm 56.06 a
Copulation Attempt	68.9 \pm 31.56 b	451.1 \pm 163.82 a	0 \pm 0 c	1406.4 \pm 598.12 a	538.40 \pm 305.67 b

¹ Means within each row by season with different letters were significantly different at $P \leq 0.05$.

Table 5.7 Activity time (in seconds) of *Laricobius osakensis* females (*LoF*) and males (*LoM*), and *L. nigrinus* females (*LnF*) and males (*LnM*) in individual assays and paired assays in spring and winter.

Species/Sex	Season	Individual	Paired
<i>LoF</i>	Spring	4676.00 \pm 394.01	5130.09 \pm 162.09
	Winter	4497.98 \pm 205.84	4755.90 \pm 329.55
<i>LoM</i>	Spring	4917.29 \pm 254.55	4436.56 \pm 190.44
	Winter	3056.93 \pm 608.89	3407.33 \pm 575.27
<i>LnF</i>	Spring	3962.41 \pm 422.07	3776.15 \pm 340.18
	Winter	2351.62 \pm 300.27	2704.01 \pm 374.48
<i>LnM</i>	Spring	3096.00 \pm 700.75	3555.13 \pm 516.77
	Winter	3472.67 \pm 181.52	2825.32 \pm 804.27

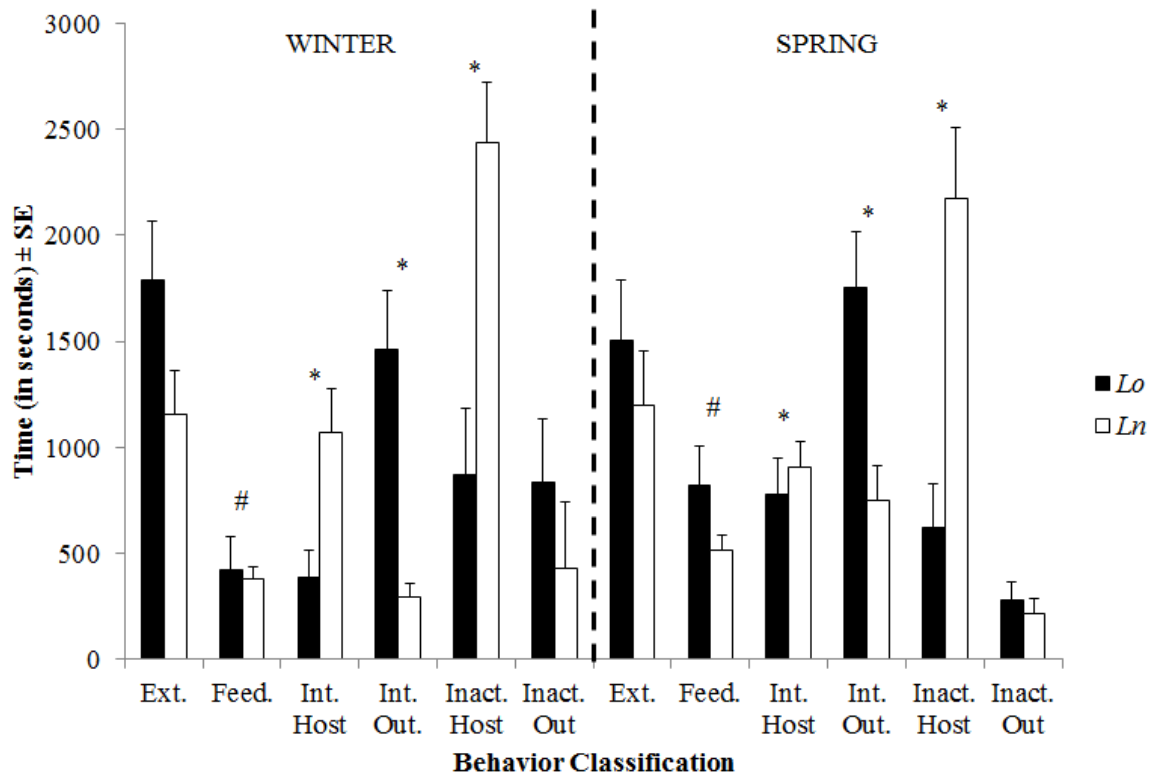


Figure 5.1 Behavioral evaluation of adult *Laricobius osakensis* (*Lo*) (n = 36) and *L. nigrinus* (*Ln*) (n = 37) in individual assays in the winter (4° C, 10:14 L:D, 50-75% RH) and spring (12° C, 14:10 L:D, 50-75% RH). Bars represent mean time (in seconds) ± SE spent on extensive behavior (Ext.), feeding (Feed.), intensive behavior on host material (Int. Host), intensive behavior outside host material (Int. Out.), inactive on host material (Inact. Host), and inactive outside host material (Inact. Out.). * Species significantly different at $P \leq 0.05$. # Seasons significantly different at $P \leq 0.05$.

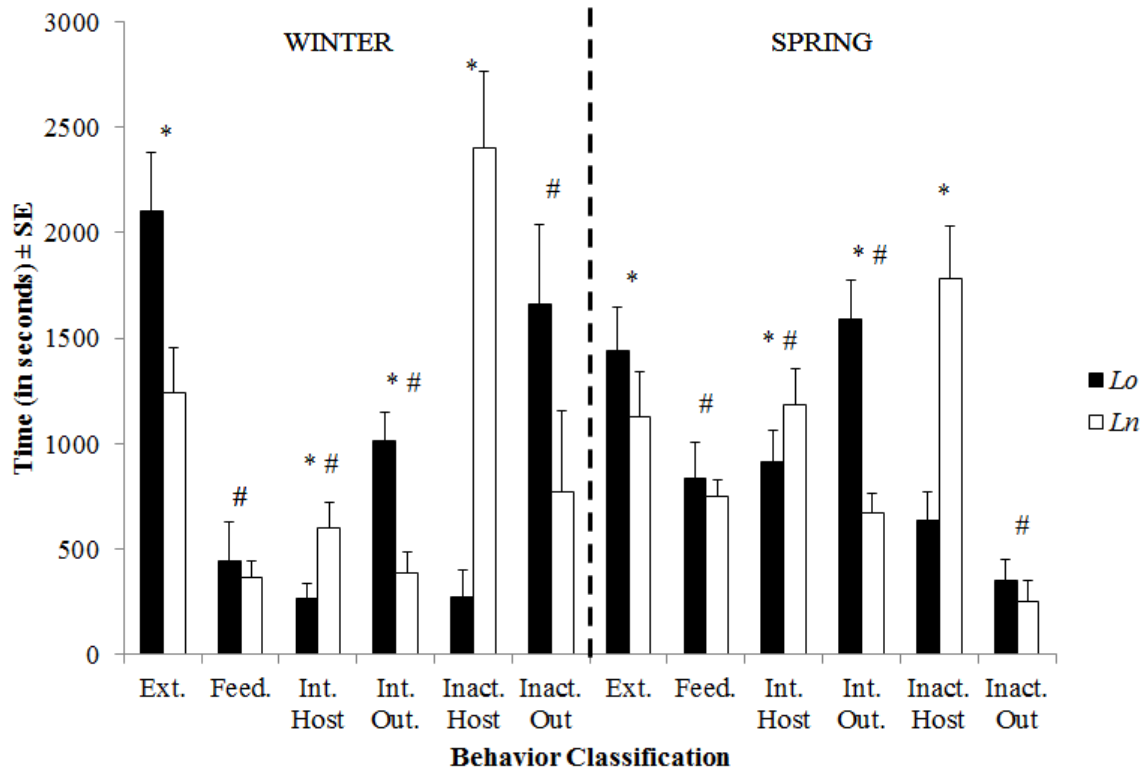


Figure 5.2 Behavioral evaluation of adult *Laricobius osakensis* (*Lo*) ($n = 36$) and *L. nigrinus* (*Ln*) ($n = 37$) in paired assays in the winter (4°C , 10:14 L:D, 50-75% RH) and spring (12°C , 14:10 L:D, 50-75% RH). Bars represent mean time (in seconds) \pm SE spent on extensive behavior (Ext.), feeding (Feed.), intensive behavior on host material (Int. Host), intensive behavior outside host material (Int. Out.), inactive on host material (Inact. Host), and inactive outside host material (Inact. Out.). * Species significantly different at $P \leq 0.05$. # Seasons significantly different at $P \leq 0.05$.

Chapter 6 Summary

Adelges tsugae Annand, is an exotic pest of eastern and Carolina hemlock in the eastern US and its devastation now affects more than 50% of the eastern hemlock range. The negative impacts on ecosystem structure and dynamics and on the dependent biodiversity are already apparent in several areas affected by this pest. If *A. tsugae* is not effectively controlled, the eastern US forest landscape we know today will likely be irrevocably changed. Research efforts towards the control of *A. tsugae* have focused on classical biological control. The pre-release evaluation of *Laricobius osakensis* Montgomery & Shiyake, a key predator of *A. tsugae* in Japan is described here. The overall goal of this research was to provide evidence that *L. osakensis* is an effective predator of *A. tsugae*.

Chapter 2 describes the host specificity of *L. osakensis*. *Laricobius osakensis* fed and oviposited significantly more on *A. tsugae* over all the other host species tested. Complete development was only possible on *A. tsugae*. This predator seems adequate for use as a biological control agent since it is not only very specific to the target pest, *A. tsugae*, but also does not seem to pose a threat to non-target species.

In chapter 3, the functional and numerical responses of *L. osakensis* adults and larvae of were compared to that of *L. nigrinus*. Adult functional responses were similar for both species but numerical response was higher in *L. osakensis*. *Laricobius osakensis* larval functional response was also higher than that of *L. nigrinus*. The introduction of this predator seems worthwhile as it has a higher potential for the control of *A. tsugae* than *L. nigrinus*, previously introduced.

In chapter 4, *L. osakensis* adult and immature survivorship, feeding, development, and reproduction were evaluated in the field. Adults were able to survive in the field during the entire study (December to May). Females laid eggs during the entire sample period, and peak oviposition was reached in March and April. Adult predation was significant but varied throughout the season. Larvae developed successfully in the field, with only 2 larvae at most dying before reaching maturity at any given sampling period. Larval predation was also significant but consistent. The impact of *L. osakensis* on *A. tsugae* and its adaptability to a new environment further supports the introduction of this predator for the control of *A. tsugae*.

In chapter 5, the behavior, interactions, and daily activity patterns of *L. nigrinus* and *L. osakensis* were examined in the laboratory. *Laricobius osakensis* was more active than *L. nigrinus* and had a lower association to the host. Interactions between specimens were generally limited and non-detrimental. The presence of the other species did not interfere with the level of activity or of oviposition of either species. With the higher activity at lower temperatures, it is possible that *L. osakensis* can be successfully introduced in colder locations in which *L. nigrinus* was unable to establish. It also seems that both predators can be introduced simultaneously without negatively affecting each other.

Future work

Now that the pre-release studies of *L. osakensis* are mostly concluded, the next step is the release and post-release evaluation. The release methodology needs to be evaluated to determine the best timing, predator and prey stage, climate matching, and the minimum predator release density. Afterward, predator establishment and impact needs to be monitored in the short and long term. Predator and prey density, and tree health should be monitored in the release sites to see if *L. osakensis* density increases and *A. tsugae* density decreases through time, and consequently if trees recover.

As it was seen with *L. nigrinus* (Mausel et al. 2010), regardless of how much more *L. osakensis* can eat, the success of the predator in controlling *A. tsugae* populations will come down to a battle of numbers (pest vs. predator). It is essential that *L. osakensis* collection and rearing is optimized to increase the number of predators released with the lowest cost possible. More information is needed prior to collection trips, namely, the location of *A. tsugae*-infested trees and whether the predator emergence is at its peak. For rearing, both laboratory and field insectary approaches should be considered.

References

- Adkins, J. K. and Rieske, L. K. (2013) Loss of a foundation forest species due to an exotic invader impacts terrestrial arthropod communities. *Forest Ecology and Management* 295: 126-135.
- Allen, D. C. (2000) Native insect pests of hemlock, Vol. 38: The New York Forest Owner (ed., pp. 1-2.
- Allen, M. C., Sheehan, J., Master, T. L. and Mulvihill, R. S. (2009) Responses of acadian flycatchers (*Empidonax virescens*) to hemlock woolly adelgid (*Adelges tsugae*) infestation in Appalachian riparian forests. *The Auk* 126: 543-553.
- Annand, P. N. (1924) A new species of *Adelges* (Hemiptera: Phylloxeridae). *Pan-Pacific Entomologist* 1: 79-82.
- Anonymous (1989) Balsam woolly aphid (*Adelges piceae*), *In*: ed.^eds. Insects and diseases of trees in the south, USDA Forest Service - Forest Health Protection, Protrotection Report R8-PR16, p. 98.
- Aschoff, J. (1979) Circadian rhythms: Influences of internal and external factors on the period measured in constant conditions. *Zeitschrift für Tierpsychologie* 49: 225-249.
- ATC (2009) Trail clubs, Vol. 2009: Appalachian Trail Conservancy, Harpers Ferry, WV.
- Aukema, J. E., Leung, B., Kovacs, K., Chivers, C., Britton, K. O., Englin, J., Frankel, S. J., Haight, R. G., Holmes, T. P., Liebhold, A. M., McCullough, D. G. and Von Holle, B. (2011) Economic impacts of non-native forest insects in the continental United States. *PLoS ONE* 6: e24587.

- Bair, M. W. (2002) Eastern hemlock (*Tsuga canadensis*) mortality in Shenandoah National Park: Proceedings: hemlock woolly adelgid in the eastern United States symposium February 5-7, 2002 (ed. by B Onken, R Reardon and J Lashomb) N. J. Agricultural Experiment Station, Rutgers - The State University of New Jersey and USDA, East Brunswick, NJ, pp. 61-66.
- Baker, J. R. (1994) Woolly alder aphid *In*: ed.^eds. Ornamentals and turf - Department of Entomology insect note, ENT/ort-06 North Carolina University - North Carolina Cooperative Extension Service, NC.
- Banks, C. J. (1957) The behaviour of individual coccinellid larvae on plants. *Animal Behaviour* 5: 12-24.
- Bond, A. B. (1980) Optimal foraging in a uniform habitat: The search mechanism of the green lacewing. *Animal Behaviour* 28: 10-19.
- Broeckling, C. D. and Salom, S. M. (2003) Antennal morphology of two specialist predators of hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). *Annals of the Entomological Society of America* 96: 153-160.
- Brooks, R. T. (2001) Effects of the removal of overstory hemlock from hemlock-dominated forests on eastern redback salamanders. *Forest Ecology and Management* 149: 197-204.
- Brooks, R. T. (2004) Early regeneration following the presalvage cutting of hemlock from hemlock-dominated stands. *Northern Journal of Applied Forestry* 21: 12-18.
- Butin, E., Elkinton, J., Havill, N. and Montgomery, M. (2003) Comparison of numerical response and predation effects of two coccinellid species on hemlock woolly adelgid (Homoptera: Adelgidae). *Journal of Economic Entomology* 96: 763-767.

- Butin, E., Montgomery, M. E., Havill, N. P. and Elkinton, J. (2002) Pre-release host range assessment for classical biological controls: experience with predators of the hemlock woolly adelgid: Proceedings: hemlock woolly adelgid in the eastern United States symposium February 5-7, 2002 (ed. by BP Onken, RC Reardon and J Lashomb) N. J. Agricultural Experiment Station, Rutgers - The State University of New Jersey and USDA, East Brunswick, NJ, pp. 205-213.
- Butin, E., Porter, A. H. and Elkinton, J. (2005) Adaptation during biological invasions and the case of *Adelges tsugae*. *Evolutionary Ecology Research* 7: 887-900.
- Butin, E. E., Havill, N. P., Elkinton, J. S. and Montgomery, M. E. (2004) Feeding preference of three lady beetle predators of the hemlock woolly adelgid (Homoptera: Adelgidae). *Journal of Economic Entomology* 97: 1635-1641.
- Chandler, A. E. F. (1969) Locomotory behaviour of first instar larvae of aphidophagous syrphidae (Diptera) after contact with aphids. *Animal Behaviour* 17: 673-678.
- Chapman, T., Takahisa, M., Smith, H. K. and Partridge, L. (1998) Interactions of mating, egg production and death rates in females of the Mediterranean fruitfly, *Ceratitis capitata*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 265: 1879-1894.
- Cheah, C. A. and McClure, M. S. (1998) Life history and development of *Pseudoscymnus tsugae* (Coleoptera: Coccinellidae), a new predator of the hemlock woolly adelgid (Homoptera: Adelgidae). *Environmental Entomology* 27: 1531-1536.
- Cheah, C. A. and McClure, M. S. (2000) Seasonal synchrony of life cycles between the exotic predator, *Pseudoscymnus tsugae* (Coleoptera: Coccinellidae) and its prey,

- the hemlock woolly adelgid *Adelges tsugae* (Homoptera:Adelgidae). Agricultural and Forest Entomology 2: 241-251.
- Cheah, C. A., Montgomery, M. E., Salom, S. M., Parker, B. L., Costa, S. and Skinner, M., eds. (2004a) Biological control of hemlock woolly adelgid. USDA Forest Service, Morgantown, WV. FHTET-2004-04.
- Cheah, C. A., Shields, K. S., Moore, P. M., Ogrodnick, J. E. and Bradford, G. L. (2004b) Reproductive strategies of *Scymnus* ladybeetle predators of HWA: Proceedings, XV U.S. Department of Agriculture interagency research forum on gypsy moth and other invasive species 2004 (ed. by KW Gottschalk) USDA Forest Service - Northeastern Research Station, General Technical Report NE-332, Annapolis, MD, p. 14.
- Cheyney, E. G. (1942) American silvics and silviculture. The University of Minnesota Press Minneapolis.
- Churchel, M. A., Hanula, J. L., Berisford, C. W., Vose, J. M. and Dalusky, M. J. (2011) Impact of imidacloprid for control of hemlock woolly adelgid on nearby aquatic macroinvertebrate assemblages. Southern Journal of Applied Forestry 35: 26-32.
- Clarke, S. C., Clarke, L. F. M. M., Collins, S. C., Gill, A. C. L. and Turner, J. R. G. (1985) Male-like females, mimicry and transvestism in butterflies (Lepidoptera: Papilionidae). Systematic Entomology 10: 257-283.
- Cleaves, D. A. (2008) Forest Service, USDA - Information collection; Valuation of hemlock decline on public forests in the southern Appalachian Mountains, Vol. 73: Federal Register (ed., Notices, pp. 36297-36298.

- Costa, S. D., Parker, B. L., Gouli, V., Brownbridge, M., Skinner, M. and Gouli, S. (2005) Insect-killing fungi as a component of hemlock woolly adelgid integrated pest management: Third symposium on hemlock woolly adelgid in the eastern United States, February 1-3 (ed. by B Onken and R Reardon) USDA - Forest Service, Asheville, NC, pp. 155-160.
- Costanza, R. (1998) The value of ecosystem services. *Ecological Economics* 25: 1-2.
- Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R. V., Paruelo, J., Raskin, R. G., Sutton, P. and van den Belt, M. (1997) The value of the world's ecosystem services and natural capital. *Nature* 387: 253-260.
- Cowles, R. S. (2009) Optimizing dosage and preventing leaching of imidacloprid for management of hemlock woolly adelgid in forests. *Forest Ecology and Management* 257: 1026-1033.
- Cowles, R. S., Montgomery, M. E. and Cheah, C. A. (2006) Activity and residues of imidacloprid applied to soil and tree trunks to control hemlock woolly adelgid (Hemiptera: Adelgidae) in forests. *Journal of Economic Entomology* 99: 1258-1267.
- Daley, M. J., Phillips, N. G., Pettijohn, C. and Hadley, J. L. (2007) Water use by eastern hemlock (*Tsuga canadensis*) and black birch (*Betula lenta*): implications of effects of the hemlock woolly adelgid. *Canadian Journal of Forest Research* 37: 2031-2040.
- Danoff-Burg, J. A. and Bird, S. (2002) Hemlock woolly adelgid and elongate hemlock scale: partners in crime?: Proceedings: hemlock woolly adelgid in the eastern

- United States symposium February 5-7, 2002 (ed. by B Onken, R Reardon and J Lashomb) N. J. Agricultural Experiment Station, Rutgers - The State University of New Jersey and USDA, East Brunswick, NJ, pp. 254-268.
- Davis, G. A., Havill, N. P., Adelman, Z. N., Caccone, A., Kok, L. T. and Salom, S. M. (2011) DNA barcodes and molecular diagnostics to distinguish an introduced and native *Laricobius* (Coleoptera: Derodontidae) species in eastern North America. *Biological Control* 58: 53-59.
- Davis, G. A., Salom, S. M., Brewster, C. C., Onken, B. P. and Kok, L. T. (2012) Spatiotemporal distribution of the hemlock woolly adelgid predator *Laricobius nigrinus* after release in eastern hemlock forests. *Agricultural and Forest Entomology* 14: 408-418.
- Davis, G. A., Salom, S. M., Kok, L. T., Brewster, C. C. and Onken, B. (2010) Dispersal dynamics and prey impact of *Laricobius nigrinus* in the eastern United States: Fifth symposium on hemlock woolly adelgid in the eastern United States (ed. by B Onken and R Reardon) Forest Health Technology Enterprise Team, Asheville, NC, pp. 3-4.
- Degraaf, R. M. and Chadwick, N. L. (1987) Forest type, timber size class, and New England breeding birds. *Journal of Wildlife Management* 51: 212-217.
- Dilling, C., Lambdin, P., Grant, J. and Buck, L. (2007) Insect guild structure associated with eastern hemlock in the southern Appalachians. *Environmental Entomology* 36: 1408-1414.

- Dilling, C., Lambdin, P., Grant, J. and Rhea, R. (2009) Community response of insects associated with eastern hemlock to imidacloprid and horticultural oil treatments. *Environmental Entomology* 38: 53-66.
- Dilling, C., Lambdin, P., Grant, J. and Rhea, R. (2010) Spatial and temporal distribution of imidacloprid in eastern hemlock in the southern Appalachians. *Journal of Economic Entomology* 103: 368-373.
- Dixon, A. F. G. (1959) An experimental study of the searching behaviour of the predatory coccinellid beetle *Adalia decempunctata* (L.). *Journal of Animal Ecology* 28: 259-281.
- Doccola, J. J., Bristol, E. J., Sifleet, S. D., Lojko, J. and Wild, P. M. (2007) Efficacy and duration of trunk-injected imidacloprid in the management of hemlock woolly adelgid (*Adelges tsugae*). *Arboriculture and Urban Forestry* 33: 12-21.
- Doccola, J. J., Castillo, P. and Taylor, C. (2002) Hemlock health and hemlock woolly adelgid management in the urban forest: Proceedings: hemlock woolly adelgid in the eastern United States symposium February 5-7, 2002 (ed. by B Onken, R Reardon and J Lashomb) N. J. Agricultural Experiment Station, Rutgers - The State University of New Jersey and USDA Forest Service, East Brunswick, NJ, pp. 288-301.
- Eisenback, B. M., Salom, S. M., Kok, L. T. and Lagalante, A. F. (2010) Lethal and sublethal effects of imidacloprid on hemlock woolly adelgid (Hemiptera: Adelgidae) and two introduced predator species. *Journal of Economic Entomology* 103: 1222-1234.

- Ellison, A. M., Bank, M. S., Clinton, B. D., Colburn, E. A., Elliott, K., Ford, C. R., Foster, D. R., Kloeppel, B. D., Knoepp, J. D., Lovett, G. M., Mohan, J., Orwig, D. A., Rodenhouse, N. L., Sobczak, W. V., Stinson, K. A., Stone, J. K., Swan, C. M., Thompson, J., Von Holle, B. and Webster, J. R. (2005a) Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. *Frontiers in Ecology and the Environment* 3: 479-486.
- Ellison, A. M., Chen, J., Díaz, D., Kammerer-Burnham, C. and Lau, M. (2005b) Changes in ant community structure and composition associated with hemlock decline in New England: Third symposium on hemlock woolly adelgid in the eastern United States, February 1-3 (ed. by B Onken and R Reardon) USDA - Forest Service, Ashville, NC, pp. 280-289.
- Eschtruth, A. K., Cleavitt, N. L., Battles, J. J., Evans, R. A. and Fahey, T. J. (2006) Vegetation dynamics in declining eastern hemlock stands: 9 years of forest response to hemlock woolly adelgid infestation. *Canadian Journal of Forest Research* 36: 1435-1450.
- Etzel, L. K. and Legner, E. F. (1999) Chapter 7. Culture and colonization, *In*: TS Bellows and TW Fischer, ed.^eds. *Handbook of biological control: principles and applications of biological control*, Academic Press, San Diego, CA, pp. 125-198.
- Evans, A. and Gregoire, T. (2007) A geographically variable model of hemlock woolly adelgid spread. *Biological Invasions* 9: 369-382.
- Evans, D. M., Aust, W. M., Dolloff, C. A., Templeton, B. S. and Peterson, J. A. (2011) Eastern hemlock decline in riparian areas from Maine to Alabama. *Northern Journal of Applied Forestry* 28: 97-104.

- Evans, E. W. (2003) Searching and reproductive behaviour of female aphidophagous ladybirds (Coleoptera: Coccinellidae): a review European Journal of Entomology 100: 1-10.
- FAO and IPPC (2006) International standards for phytosanitary measures - ISPM No. 3 Guidelines for the export shipment, import and release of biological control agents and other beneficial organisms (2005): FAO Food and Agriculture Organization of the United Nations, Rome, pp. 23-32.
- Farber, S. C., Costanza, R. and Wilson, M. A. (2002) Economic and ecological concepts for valuing ecosystem services. Ecological Economics 41: 375-392.
- Farjon, A. (1990) Pinaceae, drawings and descriptions of the Genera : *Abies*, *Cedrus*, *Pseudolarix*, *Keteleeria*, *Nothotsuga*, *Tsuga*, *Cathaya*, *Pseudotsuga*, *Larix* and *Picea*. Koeltz Scientific Books, Königstein, Federal Republic of Germany.
- Faulkenberry, M., Hedden, R. and Culin, J. (2009) Hemlock susceptibility to hemlock woolly adelgid attack in the Chattooga river watershed. Southeastern Naturalist 8: 129-140.
- Ferran, A., Ettifouri, M., Clement, P. and Bell, W. (1994) Sources of variability in the transition from extensive to intensive search in coccinellid predators (Homoptera: Coccinellidae). Journal of Insect Behavior 7: 633-647.
- Fidgen, J. G., McClellan, Q. C. and Salom, S. M. (2002) Efficacy and residual activity of two systemic insecticides for control of hemlock woolly adelgid on young eastern hemlocks: Proceedings hemlock woolly adelgid in the eastern United States symposium February 5-7, 2002 (ed. by B Onken, R Reardon and J Lashomb) N. J.

- Agricultural Experiment Station, Rutgers - The State University of New Jersey and USDA, East Brunswick, NJ, pp. 329-334.
- Finch, H. (2005) Comparison of the performance of nonparametric and parametric MANOVA test statistics when assumptions are violated. *Methodology* 1: 27-38.
- Fischer, M. J., Salom, S. M., Kok, L. T., Havill, N. P., Brewster, C. and Opell, B. (2011) Evaluation of hybridization between *Laricobius osakensis* and *Laricobius nigrinus*, predators of hemlock woolly adelgid (Adelgidae) Entomological society of America annual meeting (ed., Reno, NV, p. 153.
- Flowers, R. W., Salom, S. M. and Kok, L. T. (2005) Competitive interactions among two specialist predators and a generalist predator of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in the laboratory. *Environmental Entomology* 34: 664-675.
- Flowers, R. W., Salom, S. M. and Kok, L. T. (2006) Competitive interactions among two specialist predators and a generalist predator of hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae) in south-western Virginia. *Agricultural and Forest Entomology* 8: 253-262.
- Flowers, R. W., Salom, S. M., Kok, L. T. and Mullins, D. E. (2007) Behavior and daily activity patterns of specialist and generalist predators of the hemlock woolly adelgid, *Adelges tsugae* *Journal of Insect Science* 7: 1-20.
- Ford, C. R. and Vose, J. M. (2007) *Tsuga canadensis* (L.) Carr. mortality will impact hydrologic processes in southern Appalachian forest ecosystems. *Ecological Applications* 17: 1156-1167.

- Ford, C. R., Vose, J. M., Daley, M. and Phillips, N. (2007) Use of water by eastern hemlock: implications for systemic insecticide application. *Arboriculture and Urban Forestry* 33: 421-427.
- Fralish, J. S. and Franklin, S. B. (2002) Taxonomy and ecology of woody plants in North American forests (excluding Mexico and subtropical Florida). John Wiley New York.
- Frank, S. D. and Lebude, A. (2011) Season-long insecticide efficacy for hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), management in nurseries. *Florida Entomologist* 94: 290-295.
- Garrott, R. A., White, P. J. and White, C. A. V. (1993) Overabundance: an issue for conservation biologists? *Conservation Biology* 7: 946-949.
- Gatton, H., Zilahi-Balogh, G., Salom, S. and Kok, L. (2009) Biology and development of *Laricobius kangdingensis* Zilahi-Balogh and Jelínek, a newly discovered predator of *Adelges tsugae*. *BioControl* 54: 219-228.
- Gatton, H. A. (2005) Biology and host-range testing of *Laricobius kangdingensis* sp. n. (Coleoptera: Derodontidae), a newly discovered predator of hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae), Vol. Master of Science in Life Sciences: Department of Entomology (ed. Virginia Polytechnic Institute and State University, Blacksburg, Virginia, p. 60.
- Godman, R. M. and Lancaster, K. (1990) *Tsuga canadensis*, In: RM Burns and BH Honkala, ed.^eds. *Silvics of north America: Volume 1. Agricultural Handbook* 654., USDA, Forest Service, Washington, D. C. .

- Gómez, S., Orians, C. and Preisser, E. (2012) Exotic herbivores on a shared native host: tissue quality after individual, simultaneous, and sequential attack. *Oecologia* 169: 1015-1024.
- Gouli, S., Gouli, V., Teillon, H. B., Kassa, A., Skinner, M., Frank, C. and Parker, B. L. (2008) Controlling hemlock woolly adelgid with new forms of mycoinsecticides: Fourth symposium on hemlock woolly adelgid in the eastern United States (ed. by B Onken and R Reardon) USDA - Forest Service, Hartford, CT, pp. 179-187.
- Grassano, S. (2008) Whey-based fungal microfactories for in situ production of entomopathogenic fungi, Vol. Master of Science in Plant and Soil Science: Department of Plant and Soil Science (ed. University of Vermont, Burlington, VT, p. 67.
- Gray, D. R. and Salom, S. M. (1996) Biology of hemlock woolly adelgid in the southern Appalachians., Vol. 96-10: Proceedings of the first hemlock woolly adelgid review (ed. by SM Salom, TC Tigner and RC Reardon) Forest Health Technology Enterprise Team, Morgantown, pp. 26-35.
- Grimm, W. C. (2002) The illustrated book of trees : the comprehensive field guide to more than 250 trees of eastern North America. Rev. and updated, 1st ed. edn. Stackpole Books, Mechanicsburg, PA :.
- Grubin, S. M., Ross, D. W. and Wallin, K. F. (2011) Prey suitability and phenology of *Leucopis* spp. (Diptera: Chamaemyiidae) associated with hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific northwest. *Environmental Entomology* 40: 1410-1416.

- Hakeem, A., Grant, J. F., Lambdin, P. L., Buckley, D., Hale, F. A., Rhea, J. R., Wiggins, G. J. and Taylor, G. (2010) Recovery of *Sasajiscymnus tsugae*, released against hemlock woolly adelgid, *Adelges tsugae*, in the southern Appalachians. *Biocontrol Science and Technology* 20: 1069-1074.
- Hammack, L. and Burkholder, W. E. (1976) Circadian rhythm of sex pheromone-releasing behaviour in females of the dermestid beetle, *Trogoderma glabrum*: Regulation by photoperiod. *Journal of Insect Physiology* 22: 385-388.
- Harari, A. R. and Brockmann, H. J. (1999) Insect behaviour: Male beetles attracted by females mounting. *Nature* 401: 762-763.
- Harari, A. R., Brockmann, H. J. and Landolt, P. J. (2000) Intrasexual mounting in the beetle *Diaprepes abbreviatus* (L.). *Proceedings of the Royal Society of London. Series B: Biological Sciences* 267: 2071-2079.
- Havill, N., Davis, G., Fischer, M., Salom, S., Mausel, D. and Onken, B. (2011) Chapter 21: The introduction of *Laricobius nigrinus* as a biological control agent for the hemlock woolly adelgid: is there a threat to the native congener, *L. rubidus*?, In: B Onken and R Reardon, ed.^eds. *Implementation and status of biological control of the hemlock woolly adelgid*, Forest Health Technology Enterprise Team, Morgantown, WV (USDA Forest Service Publication FHTET-2011-04), pp. 212-217.
- Havill, N. P., Campbell, C. S., Vining, T. F., LePage, B., Bayer, R. J. and Donoghue, M. J. (2008) Phylogeny and biogeography of *Tsuga* (Pinaceae) inferred from nuclear ribosomal Its and chloroplast DNA sequence data. *Systematic Botany* 33: 478-789.

- Havill, N. P., Davis, G., Mausel, D. L., Klein, J., McDonald, R., Jones, C., Fischer, M., Salom, S. and Caccone, A. (2012) Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control. *Biological Control* 63: 359-369.
- Havill, N. P. and Foottit, R. G. (2007) Biology and evolution of Adelgidae. *Annual Review of Entomology* 52: 325-349.
- Havill, N. P. and Montgomery, M. E. (2008) The role of arboreta in studying the evolution of host resistance to the hemlock woolly adelgid. *Arnoldia - The Magazine of the Arnold Arboretum* 65: 2-9.
- Havill, N. P., Montgomery, M. E., Yu, G., Shiyake, S. and Caccone, A. (2006) Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. *Annals of the Entomological Society of America* 99: 195-203.
- Heal, G. M. (2000) *Nature and the marketplace: capturing the value of ecosystem services*. Island Press, Washington, DC.
- Heinz, K. M. and Nelson, J. M. (1996) Interspecific interactions among natural enemies of *Bemisia* in an inundative biological control program. *Biological Control* 6: 384-393.
- Holmes, T. P., Bell, K. P., Byrne, B. and S. Wilson, J. (2008) Economic aspects of invasive forest pest management, *In*: TP Holmes, JP Prestemon and KL Abt, eds. *The economics of forest disturbances*, Springer, pp. 381-406.
- Holmes, T. P., Murphy, E. A. and Bell, K. P. (2006) Exotic forest insects and residential property values. *Agricultural and Resource Economics Review* 35: 155-166.

- Holmes, T. P., Murphy, E. A., Bell, K. P. and Royle, D. D. (2010) Property value impacts of hemlock woolly adelgid in residential forests. *Forest Science* 56: 529-540.
- Hosie, R. C. (1969) *Native trees of Canada*. 7th Ed. edn. Queen's Printer, Ottawa.
- Ingwell, L., Brady, J., Fitzpatrick, M., Maynard, B., Casagrande, R. and Preisser, E. (2009) Intraspecific variation in *Tsuga canadensis* foliar chemistry. *Northeastern Naturalist* 16: 585-594.
- Iosue, F. V. (2008) The role of phytochemicals and leaf cushion morphology in host resistance to the hemlock woolly adelgid (*Adelges tsugae*), Vol. Master of Science in Biology: Department of Biology (ed. Villanova University, Villanova, PA, p. 52.
- Jetton, R. M., Dvorak, W. S. and Whittier, W. A. (2008a) Ecological and genetic factors that define the natural distribution of Carolina hemlock in the southeastern United States and their role in ex situ conservation. *Forest Ecology and Management* 255: 3212-3221.
- Jetton, R. M., Whittier, W. A., Dvorak, W. S. and potter, K. M. (2008b) Status of ex situ conservation efforts for carolina and eastern hemlock in the southeastern United States: Proceedings of the 4th symposium on hemlock woolly adelgid FHTET-2008-01 (ed. by B Onken and R Reardon) USDA Forest Service, Forest Health Technology Enterprise Team, Hartford, Connecticut, February 12-14, 2008, pp. 81-89.
- Jetton, R. M., Whittier, W. A., Dvorak, W. S. and Rhea, J. R. (2010) Status of gene conservation for eastern and Carolina hemlock in the eastern United States: Fifth

- symposium on hemlock woolly adelgid in the eastern United States (ed. by B Onken and R Reardon) USDA - Forest Service, Asheville, NC, pp. 93-99.
- Jones, A., Mullins, D., Jones, T. and Salom, S. (2012) Potential feeding deterrents found in hemlock woolly adelgid, *Adelges tsugae*. *Naturwissenschaften* 99: 583-586.
- Joseph, S. V., Braman, S. K. and Hanula, J. L. (2011a) Effects of fertilization of four hemlock species on *Adelges tsugae* (Hemiptera: Adelgidae) growth and feeding preference of predators. *Journal of Economic Entomology* 104: 288-298.
- Joseph, S. V., Hanula, J. L., Braman, S. K. and Byrne, F. J. (2011b) Effects of fertilizer and low rates of imidacloprid on *Adelges tsugae* (Hemiptera: Adelgidae). *Journal of Economic Entomology* 104: 868-878.
- Joseph, S. V., III, A. E. M., Dalusky, M. J., Asaro, C. and Berisford, C. W. (2011c) Phenology of the hemlock woolly adelgid (Hemiptera: Adelgidae) in northern Georgia. *Journal of Entomological Science* 46: 315-324.
- Kelty, M. J. (2000) Silviculture and stand dynamics of hemlock-dominated stands in southern New England: some lessons from early research: Proceedings: Symposium on sustainable management of hemlock ecosystems in eastern North America. (ed. by KA McManus, KS Shields and DR Souto) USDA - Forest Service, Durham, NH, pp. 11-13.
- Knoepp, J. D., Vose, J. M., Michael, J. L. and Reynolds, B. C. (2012) Imidacloprid movement in soils and impacts on soil microarthropods in southern Appalachian eastern hemlock stands. *Journal of Environmental Quality* 41: 469-478.

- Kohler, G. R., Stiefel, V. L., Wallin, K. F. and Ross, D. W. (2008) Predators associated with the hemlock woolly adelgid (Hemiptera: Adelgidae) in the Pacific Northwest. *Environmental Entomology* 37: 494-504.
- Kolk, A. and Starzyk, J. R. (1996) Larch woolly adelgid (*Adelges laricis* Vallot) and *Sacchiphantes abietis* (L.), *In*: ed.^eds. The atlas of forest insect pests (Atlas skodliwych owadów lesnych), <http://wiki.bugwood.org/Archive:Atlas>, Multico Publishing Company, Warszaw, Poland, p. 705.
- Lagalante, A. F., Montgomery, M. E., Calvosa, F. C. and Mirzabeigi, M. N. (2007) Characterization of terpenoid volatiles from cultivars of eastern hemlock (*Tsuga canadensis*). *Journal of Agricultural and Food Chemistry* 55: 10850-10856.
- Lamb, A., Montgomery, M. E., Vieira, L. C., Shiyake, S. and Salom, S. (2011) Chapter 7: *Laricobius osakensis*, a hemlock woolly adelgid predator from Japan., *In*: B Onken and R Reardon, ed.^eds. Implementation and status of biological control of the hemlock woolly adelgid, Forest Health Technology Enterprise Team, Morgantown, WV (USDA Forest Service Publication FHTET-2011-04), pp. 90-96.
- Lamb, A. B., Salom, S. M. and Kok, L. T. (2005a) Guidelines for rearing *Laricobius nigrinus* Fender: Third symposium on hemlock woolly adelgid in the eastern United States, February 1-3 (ed. by B Onken and R Reardon) USDA Forest Service, FHTET 2005-01, Ashville, NC, pp. 309-317.
- Lamb, A. B., Salom, S. M. and Kok, L. T. (2005b) Survival and reproduction of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a predator of hemlock

- woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae) in field cages. Biological Control 32: 200-207.
- Lamb, A. B., Salom, S. M. and Kok, L. T. (2007) Factors influencing aestivation in *Laricobius nigrinus* (Coleoptera: Derodontidae), a predator of *Adelges tsugae* (Hemiptera: Adelgidae). The Canadian Entomologist 139: 576-586.
- Lamb, A. B., Salom, S. M. and Kok, L. T. (2008a) 2008 Progress Report. Field Testing, Release, and Establishment of *Laricobius osakanus* (since changed to *osakensis*) sp. n., a Potential Biological Control Agent for the Hemlock Woolly Adelgid, p. 11.
- Lamb, A. B., Salom, S. M., Kok, L. T. and Mausel, D. L. (2006) Confined field release of *Laricobius nigrinus* (Coleoptera: Derodontidae), a predator of the hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in Virginia. Canadian Journal of Forest Research 36: 369-375.
- Lamb, A. B., Shiyake, S., Salom, S. M., Montgomery, M. E. and Kok, L. T. (2008b) Evaluation of the Japanese *Laricobius* sp. N. and other natural enemies of hemlock woolly adelgid in Japan: Fourth symposium on hemlock woolly adelgid in the eastern United States (ed. by B Onken and R Reardon) USDA Forest Service, FHTET 2008-01, Hartford, CT, pp. 29-36.
- Lancaster, K. F. (1985) Managing eastern hemlock: a preliminary guide. USDA - Forest Service, NA-FR-30.
- Larivière, S. and Ferguson, S. H. (2003) Evolution of induced ovulation in North American carnivores. Journal of Mammalogy 84: 937-947.

- Lawrence, J. F. and Hlavac, T. F. (1979) Review of the Derodontidae (Coleoptera: Polyphaga) with new species from North America and Chile. The Coleopterists Bulletin 33: 369-414.
- Legner, E. F. and Bellows, T. S. (1999) Chapter 5. Exploration for natural enemies, *In*: TS Bellows and TW Fischer, ed.^eds. Handbook of biological control: principles and applications of biological control, Academic Press, San Diego, CA, pp. 87-102.
- Lemieux, M. J., Beaulieu, J. and Bousquet, J. (2011) Chloroplast DNA polymorphisms in eastern hemlock: range-wide genogeographic analyses and implications for gene conservation. Canadian Journal of Forest Research 41: 1047-1059.
- Leschen, R. A. B. (2011) World review of *Laricobius* (Coleoptera: Derodontidae). Zootaxa 2908: 1-44.
- Loomis, J., Kent, P., Strange, L., Fausch, K. and Covich, A. (2000) Measuring the total economic value of restoring ecosystem services in an impaired river basin: results from a contingent valuation survey. Ecological Economics 33: 103-117.
- Lu, W. and Montgomery, M. E. (2001) Oviposition, development, and feeding of *Scymnus* (*Neopullus*) *sinuanodulus* (Coleoptera: Coccinellidae): A predator of *Adelges tsugae* (Homoptera: Adelgidae). Annals of the Entomological Society of America 94: 64-70.
- Lu, W., Souphanya, P. and Montgomery, M. E. (2002) Descriptions of immature stages of *Scymnus* (*Neopullus*) *sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae) with notes on life history. The Coleopterists Bulletin 56: 127-141.

- Luck, R. F. and Dahlsten, D. L. (1974) Bionomics of the pine needle scale, *Chionaspis pinifoliae*, and its natural enemies at south Lake Tahoe, Calif. Annals of the Entomological Society of America 67: 309-316.
- Luck, R. F., Shepard, B. M. and Kenmore, P. E. (1999) Chapter 9. Evaluation of biological control with experimental methods, *In*: TS Bellows and TW Fischer, ed.^eds. Handbook of biological control: principles and applications of biological control, Academic Press, San Diego, CA., pp. 225-242.
- MacArthur, R. H. and Pianka, E. R. (1966) On optimal use of a patchy environment. The American Naturalist 100: 603-609.
- Martineau, R. (1984) Insects harmful to forest trees. Multiscience Publications Ltd., Canadian Forestry Service, Environment Canada and the Canadian Government Publishing Centre, Supply and Services, Ottawa.
- Mausel, D. L. (2005) Observations on fecundity and natural enemies of hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) in Seattle, Washington. Pan-Pacific Entomologist 81: 97-98.
- Mausel, D. L., Salom, S. M. and Kok, K. T. (2011) Visual ability and searching behavior of adult *Laricobius nigrinus*, a hemlock woolly adelgid predator. Journal of Insect Science 11: 1-9.
- Mausel, D. L., Salom, S. M., Kok, L. T. and Davis, G. A. (2010) Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. Environmental Entomology 39: 440-448.

- Mausel, D. L., Salom, S. M., Kok, L. T. and Fidgen, J. G. (2008) Propagation, synchrony, and impact of introduced and native *Laricobius* spp. (Coleoptera: Derodontidae) on hemlock woolly adelgid in Virginia. *Environmental Entomology* 37: 1498-1507.
- Mayer, M., Chianese, R., Scudder, T., White, J., Vongpaseuth, K. and Ward, R. (2002) Thirteen years of monitoring the hemlock woolly adelgid In New Jersey forests: Proceedings: hemlock woolly adelgid in the eastern United States symposium February 5-7, 2002 (ed. by B Onken, R Reardon and J Lashomb) N. J. Agricultural Experiment Station, Rutgers - The State University of New Jersey and USDA, East Brunswick, NJ, pp. 50-60.
- McAvoy, T. J., Zilahi-Balogh, G. M. G., Salom, S. M., Kok, L. T. and Guoliang, Z. (2007) Development and feeding of *Tetrachleps galchanoides*, a predator of the hemlock woolly adelgid. *BioControl* 52: 339-350.
- McClure, M. S. (1978) Seasonal development of *Fiorinia externa*, *Tsugaspidotus tsugae* (Homoptera: Diaspididae), and their parasite, *Aspidiotiphagus citrinus* (Hymenoptera: Aphelinidae): Importance of parasite-host synchronism to the population dynamics of two scale pests of hemlock. *Environmental Entomology* 7: 863-870.
- McClure, M. S. (1987) Biology and control of hemlock woolly adelgid. *Bulletin of the Connecticut Agricultural Experiment Station*: 1-9.
- McClure, M. S. (1989) Evidence of a polymorphic life cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). *Annals of the Entomological Society of America* 82: 50-54.

- McClure, M. S. (1990) Role of wind, birds, deer, and humans in the dispersal of hemlock woolly adelgid (Homoptera: Adelgidae). *Environmental Entomology* 19: 36-43.
- McClure, M. S. (1991a) Density-dependent feedback and population cycles in *Adelges tsugae* (Homoptera: Adelgidae) on *Tsuga canadensis*. *Environmental Entomology* 20: 258-264.
- McClure, M. S. (1991b) Nitrogen fertilization of hemlock increases susceptibility to hemlock woolly adelgid. *Journal of Arboriculture* 17: 227-230.
- McClure, M. S. (1992a) Effects of implanted and injected pesticides and fertilizers on the survival of *Adelges tsugae* (Homoptera: Adelgidae) and on the growth of *Tsuga canadensis*. *Journal of Economic Entomology* 85: 468-472.
- McClure, M. S. (1992b) Hemlock woolly adelgid. *American Nurseryman* March 15, 1992: 82-89.
- McClure, M. S. (1995a) *Diapterobates humeralis* (Oribatida: Ceratozetidae): An effective control agent of hemlock woolly adelgid (Homoptera: Adelgidae) in Japan. *Environmental Entomology* 24: 1207-1215.
- McClure, M. S. (1995b) Using natural enemies from Japan to control hemlock woolly adelgid. *Frontiers of Plant Science* 47: 5-7.
- McClure, M. S. (1996) Biology of *Adelges tsugae* and its potential for spread in the northeastern United States: Proceedings of the first hemlock woolly adelgid review (ed. by SM Salom, TL Tignor and RC Reardon) USDA - Forest Service, Charlottesville, VA, October 12, 1995, pp. 16-25.
- McClure, M. S. (2002) The elongate hemlock scale, *Fiorinia externa* Ferris (Homoptera: Diaspididae): a new look at an old nemesis: Proceedings: hemlock woolly adelgid

- in the eastern United States symposium February 5-7 (ed. by B Onken, R Reardon and J Lashomb) N. J. Agricultural Experiment Station, Rutgers - The State University of New Jersey and USDA, East Brunswick, NJ.
- McClure, M. S. and Cheah, C. A. (1999) Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. *Biological Invasions* 1: 247-254.
- McEvoy, P. B. and Coombs, E. M. (2000) Why things bite back: unintended consequences of biological weed control, *In*: PA Follett and JJ Duan, ed.^eds. Nontarget effects of biological control, Kluwer Academic Publishers, Boston, Massachusetts, pp. 167-194
- McWilliams, W. H. and Schmidt, T. L. (2000) Composition, structure, and sustainability of hemlock ecosystems in eastern North America: Proceedings: symposium on sustainable management of hemlock ecosystems in eastern North America. Gen. Tech. Rep. NE-267 (ed. by KA McManus, KS Shields and DR Souto) U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station: 5-10, Newtown Square, PA.
- Mills, N. (2006) Interspecific competition among natural enemies and single versus multiple introductions in biological control. Trophic and guild in biological interactions control, *In*: J Brodeur and G Boivin, ed.^eds., Springer Netherlands, pp. 191-220.
- Montgomery, M. E., Bentz, S. E. and Olsen, R. T. (2009a) Evaluation of hemlock (*Tsuga*) species and hybrids for resistance to *Adelges tsugae* (Homoptera:

- Adelgidae) using artificial infestation. *Journal of Economic Entomology* 102: 1247-1254.
- Montgomery, M. E. and Keena, M. A. (2011) Chapter 5: *Scymnus* (*Neopullus*) lady beetles from China, *In*: B Onken and R Reardon, ed.^eds. Implementation and status of biological control of the hemlock woolly adelgid, Forest Health Technology Enterprise Team, Morgantown, WV (USDA Forest Service Publication FHTET-2011-04), pp. 53-76.
- Montgomery, M. E. and Lyon, S. M. (1995) Natural enemies of adelgids in North America: Their prospect for biological control of *Adelges tsugae* (Homoptera: Adelgidae). Proceedings of the First Hemlock Woolly Adelgid Review (ed. by SM Salom, TC Tigner and RC Reardon) USDA Forest Service, Forest Health Technology Enterprise Team, Morgantown West Virginia 96-10, Charlottesville, VA, pp. 89-102.
- Montgomery, M. E., McAvoy, T. J. and Salom, S. M. (2011a) Chapter 10: Other species considered, *In*: B Onken and R Reardon, ed.^eds. Implementation and Status of Biological control of the hemlock woolly adelgid, Forest Health Technology Enterprise Team Morgantown, WV (USDA Forest Service Publication FHTET-2011-04), pp. 116-121.
- Montgomery, M. E., McDonald, R. and Schwartzberg, L. (2009b) *Scymnus* (*Pullus*) *coniferarum* (Coleoptera: Coccinellidae): an adelgid predator native to the western United States: Proceedings 20th U. S. Department of Agriculture interagency research forum on invasive species (ed. by KA McManus and KW Gottschalk) USDA - Forest Service, Annapolis, MD, p. 89.

- Montgomery, M. E., Shiyake, S., Havill, N. P. and Leschen, R. A. B. (2011b) A New species of *Laricobius* (Coleoptera: Derodontidae) from Japan with phylogeny and a key for native and introduced congeners in North America. *Annals of the Entomological Society of America* 104: 389-401.
- Moore, C. C. and Holmes, T. P. (2008) Valuing forest protection programs to maximize economic benefit: U.S. Environmental Protection Agency - National Center for Environmental Economics, Washington, DC, p. 25.
- Morales, D. J. (1980) The contribution of trees to residential property value. *Journal of Arboriculture* 6: 305-308.
- Morin, R. S., Liebhold, A. M. and Gottschalk, K. W. (2009) Anisotropic spread of hemlock woolly adelgid in the eastern United States. *Biological Invasions* 11: 2341-2350.
- Morkeski, K. (2007) In-stream hemlock twig breakdown and effects of reach-scale twig additions on Appalachian headwater streams, Vol. Master of Science in Biological Sciences: Department of Biological Sciences (ed. Virginia Polytechnic Institute and State University Blacksburg, Virginia, M. S. thesis, 27 pp., p. 27.
- Nakamuta, K. (1985) Mechanism of the switchover from extensive to area-concentrated search behaviour of the ladybird beetle, *Coccinella septempunctata bruckii*. *Journal of Insect Physiology* 31: 849-856.
- NAPPO (2006) NAPPO Regional standards for phytosanitary measures (RSPM) - RSPM N°. 26 Guidelines for certification of commercial arthropod biological control agents moving into NAPPO member countries: The Secretariat of the North American Plant Protection Organization, Ontario, Canada, p. 15.

- NRCan (2002) Trees, insects and diseases of Canada's forests: Natural Resources Canada, Canadian Forestry Service, Sainte-Foy, Quebec.
- O'Neil, R. J. (1989) Comparison of laboratory and field measurements of the functional response of *Podisus maculiventris* (Heteroptera: Pentatomidae). Journal of the Kansas Entomological Society 62: 148-155.
- Onken, B. and Reardon, R., eds. (2011a) Implementation and status of biological control of the hemlock woolly adelgid. Forest Health Technology Enterprise Team, Morgantown, WV (USDA Forest Service Publication FHTET-2011-04).
- Onken, B. P. and Reardon, R. C. (2011b) Chapter 22: An overview and outlook for biological control of hemlock woolly adelgid, *In*: BP Onken and RC Reardon, ed.^eds. Implementation and status of biological control of the hemlock woolly adelgid, Forest Health Technology Enterprise Team, Morgantown, WV (USDA Forest Service Publication FHTET-2011-04), pp. 222-228.
- Orwig, D. A., Cobb, R. C., D'Amato, A. W., Kizlinski, M. L. and Foster, D. R. (2008) Multi-year ecosystem response to hemlock woolly adelgid infestation in southern New England forests. Canadian Journal of Forest Research 38: 834-843.
- Orwig, D. A. and Foster, D. R. (1998) Forest response to the introduced hemlock woolly adelgid in southern New England, USA. Journal of the Torrey Botanical Society 125: 60-73.
- Orwig, D. A., Foster, D. R. and Mausel, D. L. (2002) Landscape patterns of hemlock decline in New England due to the introduced hemlock woolly adelgid. Journal of Biogeography 29: 1475-1487.

- Orwig, D. A., Thompson, J. R., Povak, N. A., Manner, M., Niebyl, D. and Foster, D. R. (2012) A foundation tree at the precipice: *Tsuga canadensis* health after the arrival of *Adelges tsugae* in central New England. *Ecosphere* 3: art10.
- Oten, K. L. F. (2011) Host-plant selection by the hemlock woolly adelgid, *Adelges tsugae* Annand: sensory systems and feeding behavior in relation to physical and chemical host-plant characteristics, Vol. Doctor of Philosophy in Entomology: Department of Entomology (ed. North Carolina State University, Raleigh, NC, p. 265.
- Oten, K. L. F., Bauchan, G. R., Frampton, J. and Hain, F. P. (2012) Biophysical characteristics of the stem and petiole surface of six hemlock (*Tsuga*) species and a hybrid: implications for resistance to *Adelges tsugae*. *Botany* 90: 1170-1178.
- Paradis, A., Elkinton, J., Hayhoe, K. and Buonaccorsi, J. (2008) Role of winter temperature and climate change on the survival and future range expansion of the hemlock woolly adelgid (*Adelges tsugae*) in eastern North America. *Mitigation and Adaptation Strategies for Global Change* 13: 541-554.
- Parker, B. L., Skinner, M., Gouli, S., Ashikaga, T. and Teillon, H. B. (1998) Survival of hemlock woolly adelgid (Homoptera: Adelgidae) at low temperatures. *Forest Science* 44: 414-420.
- Parker, B. L., Skinner, M., Gouli, S., Ashikaga, T. and Teillon, H. B. (1999) Low lethal temperature for hemlock woolly adelgid (Homoptera: Adelgidae). *Environmental Entomology* 28: 1085-1091.
- Peattie, D. C. (1950) A natural history of trees of eastern and central North America. Houghton Mifflin, Boston.

- Peschke, K. (1987) Male aggression, female mimicry and female choice in the rove beetle, *Aleochara curtula* (Coleoptera, Staphylinidae). *Ethology* 75: 265-284.
- Pontius, J. A., Hallett, R. A. and Jenkins, J. C. (2006) Foliar chemistry linked to infestation and susceptibility to hemlock woolly adelgid (Homoptera: Adelgidae). *Environmental Entomology* 35: 112-120.
- Potter, K. M., Jetton, R. M., Dvorak, W. S., Frampton, J. and Rhea, R. (2010) Ex situ seed collection represents genetic variation present in natural stands of Carolina hemlock: Fifth symposium on hemlock woolly adelgid in the eastern United States (ed. by B Onken and R Reardon) USDA - Forest Service, Asheville, NC, pp. 181-190.
- Preisser, E., Lodge, A., Orwig, D. and Elkinton, J. (2008) Range expansion and population dynamics of co-occurring invasive herbivores. *Biological Invasions* 10: 201-213.
- Preisser, E. L. and Elkinton, J. S. (2008) Exploitative competition between invasive herbivores benefits a native host plant. *Ecology* 89: 2671-2677.
- Quimby, J. W. (1995) Value and importance of hemlock ecosystems in the United States: Proceedings of the first hemlock woolly adelgid review (ed. by SM Salom, TC Tigner and RC Reardon) USDA Forest Service, Forest Health Technology Enterprise Team-Morgantown, West Virginia, 96-10, Charlottesville, VA, pp. 1-8.
- Radville, L. (2012) Naturally-occurring resistance and a defensive hypersensitive response in the eastern hemlock (*Tsuga canadensis* (L.) Carriere) to the invasive hemlock woolly adelgid (*Adelges tsugae* Annand) Vol. Master of Science in

- Biological Sciences: Department of Biological Sciences (ed. University of Rhode Island, Kingston, RI, p. 40.
- Raupp, M. J., Webb, R. E., Szczepaniec, A., Booth, D. and Ahern, R. (2004) Incidence, abundance, and severity of mites on hemlocks following applications of imidacloprid. *Journal of Arboriculture* 30: 108-113.
- Reid, W. R., Parker, B. L., Gouli, S. Y., Skinner, M., Gouli, V. V. and Teillon, H. B. (2010) Fungi associated with the hemlock woolly adelgid, *Adelges tsugae*, and assessment of entomopathogenic isolates for management. *Journal of Insect Science* 10: 1-18.
- Rendell, E. G., Knoll, C. B. and Jr., D. T. C. (2003) The Pennsylvania manual - Volume 116. Commonwealth of Pennsylvania, Harrisburg, PA
- Roberts, A., Lamb, A., Onken, B. and Salom, S. (2011) Chapter 17: The hwa predator release and recovery database, *In*: B Onken and R Reardon, ed.^eds. Implementation and status of biological control of the hemlock woolly adelgid, Forest Health Technology Enterprise Team, Morgantown, WV (USDA Forest Service Publication FHTET-2011-04), pp. 176-191.
- Robertson, H. M. (1985) Female dimorphism and mating behaviour in a damselfly, *Ischnura ramburi*: females mimicking males. *Animal Behaviour* 33: 805-809.
- Rohr, J. R., Mahan, C. G. and Kim, K. C. (2009) Response of arthropod biodiversity to foundation species declines: The case of the eastern hemlock. *Forest Ecology and Management* 258: 1503-1510.

- Romero, A., Potter, M. F. and Haynes, K. F. (2010) Circadian rhythm of spontaneous locomotor activity in the bed bug, *Cimex lectularius* L. *Journal of Insect Physiology* 56: 1516-1522.
- ROSI (2008) Carolina hemlocks recreational area (NC), Vol. 2009: Recreation One-Stop Initiative.
- Ross, R. M., Bennett, R. M., Snyder, C. D., Young, J. A., Smith, D. R. and Lemarie, D. P. (2003) Influence of eastern hemlock (*Tsuga canadensis* L.) on fish community structure and function in headwater streams of the Delaware River basin. *Ecology of Freshwater Fish* 12: 60-65.
- Ross, R. M., Redell, L. A., Bennett, R. M. and Young, J. A. (2004) Mesohabitat use of threatened hemlock forests by breeding birds of the Delaware river basin in northeastern United States. *Natural Areas Journal* 24: 307-315.
- Royle, D. and Lathrop, R. (2000) The effects of site factors on the rate of hemlock decline: a case study in New Jersey: Proceedings: symposium on sustainable management of hemlock ecosystems in eastern North America (ed. by KA McManus, KS Shields and DR Souto) USDA - Forest Service, Durham, NH, p. 103.
- Sackett, T. E., Record, S., Bewick, S., Baiser, B., Sanders, N. J. and Ellison, A. M. (2011) Response of macroarthropod assemblages to the loss of hemlock (*Tsuga canadensis*), a foundation species. *Ecosphere* 2: art74.
- Salom, S., Mayfield, A. and McAvoy, T. (2011) Chapter 19: Integrating chemical and biological control, *In*: B Onken and R Reardon, ed.^eds. Implementation and status of biological control of the hemlock woolly adelgid, *Forest Health*

- Technology Enterprise Team, Morgantown, WV (USDA Forest Service Publication FHTET-2011-04), pp. 199-201.
- Salom, S. M., Kok, L. T., Lamb, A. B. and Jubb, C. (2012) Laboratory rearing of *Laricobius nigrinus* (Coleoptera: Derodontidae): A predator of the hemlock woolly adelgid (Hemiptera: Adelgidae). *Psyche* 2012: 9 pp.
- Salom, S. M., Kok, L. T., Lamb, A. B., Jubb, C. and Onken, B. P. (2008) Biological control of hemlock woolly adelgid: what is it going to take to make it work?: Fourth symposium on hemlock woolly adelgid in the eastern United States (ed. by BP Onken and RC Reardon) USDA Forest Service FHTET 2008-01, Hartford, CT pp. 11-17.
- Salom, S. M. and Lamb, A. B. (2008) Petition to permit the removal of *Laricobius osakensis* sp. nov. (Coleoptera: Derodontidae), a predator of hemlock woolly adelgid, from Quarantine in the Eastern United States: Department of Entomology, Virginia Tech, Blacksburg, p. 47.
- Sander, H., Polasky, S. and Haight, R. G. (2010) The value of urban tree cover: A hedonic property price model in Ramsey and Dakota Counties, Minnesota, USA. *Ecological Economics* 69: 1646-1656.
- SAS_Institute_Inc. (2007) JMP User's guide. SAS Institute Inc., Cary, NC.
- SAS_Institute_Inc. (2008a) The GLIMMIX procedure, *In*: ed.^eds. SAS/STAT 9.2 User's Guide, SAS Institute Inc., Cary, NC, pp. 2078-2419.
- SAS_Institute_Inc. (2008b) SAS/STAT 9.2 User's guide. SAS Institute Inc., Cary, NC.
- SAS_Institute_Inc. (2009) JMP® 8 User Guide, Second Edition: SAS Institute Inc., Cary, NC.

- Sasaji, H. and McClure, M. S. (1997) Description and distribution of *Pseudoscymnus tsugae* sp. Nov. (Coleoptera: Coccinellidae): an important predator of hemlock woolly adelgid in Japan. *Annals of the Entomological Society of America* 90: 563-568.
- Saunders, D. S. (1997) Insect circadian rhythms and photoperiodism. *Invertebrate Neuroscience* 3: 155-164.
- Schowalter, T. D. (2011) *Insect ecology: An ecosystem approach*. 3rd edn. Elsevier Inc., China.
- Scott, D. (1986) Sexual mimicry regulates the attractiveness of mated *Drosophila melanogaster* females. *Proceedings of the National Academy of Sciences of the United States of America* 83: 8429-8433.
- Scott, J. A. (1997) *The butterflies of north America: a natural history and field guide*. Stanford University Press, Stanford, California.
- Siderhurst, L. A., Griscom, H. P., Hudy, M. and Bortolot, Z. J. (2010) Changes in light levels and stream temperatures with loss of eastern hemlock (*Tsuga canadensis*) at a southern Appalachian stream: Implications for brook trout. *Forest Ecology and Management* 260: 1677-1688.
- Sivaramakrishnan, S. and Berlyn, G. P. (2000) The role of site conditions in survival of hemlocks infested with the hemlock woolly adelgid: amelioration through the use of organic biostimulants: *Proceedings: symposium on sustainable management of hemlock ecosystems in eastern North America* (ed. by KA McManus, KS Shields and DR Souto) USDA - Forest Service, Durham, NH, pp. 201-204.

- Skinner, M., Parker, B. L., Gouli, S. and Ashikaga, T. (2003) Regional responses of hemlock woolly adelgid (Homoptera: Adelgidae) to low temperatures. *Environmental Entomology* 32: 523-528.
- Small, M. J., Small, C. J. and Dreyer, G. D. (2005) Changes in a hemlock-dominated forest following woolly adelgid infestation in southern New England. *Journal of the Torrey Botanical Society* 132: 458-470.
- Snyder, C. D., Young, J. A., Lemarié, D. P. and Smith, D. R. (2002a) Influence of eastern hemlock (*Tsuga canadensis*) forests on aquatic invertebrate assemblages in headwater streams. *Canadian Journal of Fisheries and Aquatic Sciences* 59: 262-275.
- Snyder, C. D., Young, J. A., Lemarle, D. P. and Smith, D. R. (2002b) Influence of eastern hemlock (*Tsuga canadensis*) forests on aquatic invertebrate assemblages in headwater streams. *Canadian Journal of Fisheries & Aquatic Sciences* 59: 262.
- Souto, D. R., Luther, T. and Chianese, B. (1996) Past and current status of HWA in eastern and Carolina hemlock stands: Proceedings of the first hemlock woolly adelgid review (ed. by SM Salom, TC Tigner and RC Reardon) U. S. Department of Agriculture, Forest Service, Morgantown, WV, Charlottesville, VA, pp. 9-15.
- Spaulding, H. and Rieske, L. (2010) The aftermath of an invasion: structure and composition of central Appalachian hemlock forests following establishment of the hemlock woolly adelgid, *Adelges tsugae*. *Biological Invasions* 12: 3135-3143.
- Stadler, B., Müller, T., Orwig, D. and Cobb, R. (2005) Hemlock woolly adelgid in New England forests: canopy impacts transforming ecosystem processes and landscapes. *Ecosystems* 8: 233-247.

- Stimmel, J. F. (2000) Hemlock scale, *Abgrallaspis ithacae* (Ferris) Homoptera: Diaspididae: Regulatory Horticulture Volume 26: Entomology Circular No. 197 (ed. Pennsylvania Dept. of Agriculture - Bureau of Plant Industry).
- Stodola, K., Linder, E. and Cooper, R. (2013) Indirect effects of an invasive exotic species on a long-distance migratory songbird. *Biological Invasions*: 1-13.
- Story, H. M., Vieira, L. C., Salom, S. M. and Kok, L. T. (2012) Assessing performance and competition among three *Laricobius* (Coleoptera: Derodontidae) species, predators of hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). *Environmental Entomology* 41: 896-904.
- Sullivan, K. A. and Ellison, A. M. (2006) The seed bank of hemlock forests: implications for forest regeneration following hemlock decline. *Journal of the Torrey Botanical Society* 133: 393-402.
- Swartley, J. C. (1984) *The cultivated hemlocks*. Timber Press, Oregon, USA.
- Swartzentruber, B. A. and Master, T. L. (2005) The effects of hemlock woolly adelgid infestation on breeding populations of three species of eastern hemlock-dependent songbirds in the Delaware Water Gap National Recreation Area: Third symposium on hemlock woolly adelgid in the eastern United States, February 1-3 (ed. by B Onken and R Reardon) USDA - Forest Service, Ashville, NC, p. 353.
- Tattar, T. A., Dotson, J. A., Ruizzo, M. S. and Steward, V. B. (1998) Translocation of imidacloprid in three tree species when trunk- and soil-injected. *Journal of Arboriculture* 24: 54-56.

- Tingley, M. W., Orwig, D. A., Field, R. and Motzkin, G. (2002) Avian response to removal of a forest dominant: consequences of hemlock woolly adelgid infestations. *Journal of Biogeography* 29: 1505-1516.
- Tredici, P. D. and Kitajima, A. (2004) Introduction and cultivation of chinese hemlock (*Tsuga chinensis*) and its resistance to hemlock woolly adelgid (*Adelges tsugae*). *Journal of Arboriculture* 30: 282-287.
- Tree-SaversTM (2012) *Sasajiscymnus tsugae* (St Beetle) Price Guide: Tree-Savers, Greentown, PA.
- Tyrrell, L. E. and Crow, T. R. (1994) Structural characteristics of old-growth hemlock-hardwood forests in relation to age. *Ecology* 75: 370-386.
- U.S (2000) Plant Protection Act of 2000, 7 U.S.C. § 7701 et seq.: United States Federal Government.
- USDA-APHIS-PPQ (2010) Decision and finding of no significant impact for field release of *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae), a predatory beetle for biological control of hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in the continental United States. June 2010: http://www.aphis.usda.gov/plant_health/ea/downloads/Laricobius-osakensis-FONSI-June2010.pdf. (Accessed 25 January 2013).
- USDA-ARS (2012) USDA Plant Hardiness Zone Map: United States Department of Agriculture - Agricultural Research Service.
- USDA-FS (1992) Hemlock looper (Pest Alert) NA-PR-05-92: USDA Forest Service Northern Area State & Private Forestry and Region 8, Augusta, ME.

- USDA-FS (2000) Hemlock borer (Pest Alert) NA-PR-03-00: USDA Forest Service, Morgantown, WV.
- USDA-FS (2002) Elongated hemlock scale (Pest Alert) NA-PR-01-02: USDA Forest Service Northeastern Area Forest Health Protection, Morgantown, WV.
- USDA-FS (2005) Hemlock woolly adelgid (Pest Alert) NA-PR-09-05: USDA Forest Service, Northern Area State & Private Forestry, Newtown Square, PA.
- USDA-FS (2012) Counties with established HWA populations 2011: United States Forest Service - Northeastern Area.
- USDAFS (2002) Elongated hemlock scale (Pest Alert) NA-PR-01-02. USDA Forest Service Northeastern Area Forest Health Protection, Morgantown, WV.
- USGS (1999) Digital representations of tree species range maps from "Atlas of United States trees" by Elbert L. Little, Jr. (and other publications): U.S. Geological Survey Professional, Paper 1650.
- van Lenteren, J. C., Bale, J., Bigler, F., Hokkanen, H. M. T. and Loomans, A. J. M. (2006) Assessing risks of releasing exotic biological control agents of arthropod pests. *Annual Review of Entomology* 51: 609-634.
- Vidaković, M. (1991) Conifers : morphology and variation. Grafički zavod Hrvatske, Zagreb.
- Vieira, L. C., Lamb, A. B., Shiyake, S., Salom, S. M. and Kok, L. T. (in press) Seasonal abundance and synchrony between *Laricobius osakensis* (Coleoptera: Derodontidae) and its prey, *Adelges tsugae* (Hemiptera: Adelgidae), in Japan. *Annals of the Entomological Society of America*.

- Vieira, L. C., McAvoy, T. J., Chantos, J., Lamb, A. B., Salom, S. M. and Kok, L. T. (2011) Host range of *Laricobius osakensis* (Coleoptera: Derodontidae), a new biological control agent of hemlock woolly adelgid (Hemiptera: Adelgidae). Environmental Entomology 40: 324-332.
- Vieira, L. C., Salom, S. M. and Kok, L. T. (2012) Functional and numerical response of *Laricobius* spp. predators (Coleoptera: Derodontidae) on hemlock woolly adelgid, *Adelges tsugae* (Hemiptera: Adelgidae). Biological Control 61: 47-54.
- Wallace, M. S. and Hain, F. P. (2000) Field surveys and evaluation of native and established predators of the hemlock woolly adelgid (Homoptera: Adelgidae) in the southeastern United States. Environmental Entomology 29: 638-644.
- Wallin, K. F., Latty, T. M. and Ross, D. W. (2011) Orientation behavior of the predator *Laricobius nigrinus* (Coleoptera: Derodontidae) to hemlock woolly adelgid and host tree odors in a multi-chambered olfactometer. Environmental Entomology 40: 864-872.
- Ward, J. S., Montgomery, M. E., Cheah, C. A., Onken, B. P. and Cowles, R. S. (2004) Eastern hemlock forests: guidelines to minimize the impacts of hemlock woolly adelgid. NA-TP-03-04: USDA, Forest Service, Northeastern Area, State & Private Forestry, Morgantown, WV.
- Werner, S. M. and Cowles, R. A. (2007) Evaluation of imidacloprid treatment on hemlock woolly adelgid in Pennsylvania: Proceedings 18th U. S. Department of Agriculture interagency research forum on gypsy moth and other invasive species, January 9-12, 2007 (ed. by KW Gottschalk) USDA Forest Service, 2007GTR-NRS-P-28, Annapolis, MD, p. 85.

- Willacker, J. J., Sobczak, W. V. and Colburn, E. A. (2009) Stream macroinvertebrate communities in paired hemlock and deciduous watersheds. Northeastern Naturalist 16: 101-112.
- Young, J., Snyder, C., Akerson, J. and Hunt, G. (2000) Assessment of landscape correlates of eastern hemlock decline due to hemlock woolly adelgid: Proceedings: symposium on sustainable management of hemlock ecosystems in eastern North America (ed. by KA McManus, KS Shields and DR Souto) USDA - Forest Service, Durham, NH, p. 134.
- Young, R. F., Shields, K. S. and Berlyn, G. P. (1995) Hemlock woolly adelgid (Homoptera: Adelgidae): Stylet bundle insertion and feeding sites. Annals of the Entomological Society of America 88: 827-835.
- Yu, G., Montgomery, M. E. and Yao, D. (2000) Lady beetles (Coleoptera: Coccinellidae) from Chinese hemlocks infested with the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). The Coleopterists Bulletin 54: 154-199.
- Zilahi-Balogh, G. M. G. (2001) Biology of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and its potential as a biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae) in the eastern United States, Vol. Doctor of Philosophy in Entomology: Department of Entomology (ed. Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA, p. 110 pp.
- Zilahi-Balogh, G. M. G., Broeckling, C. D., Kok, L. T. and Salom, S. M. (2005) Comparison between a native and exotic adelgid as hosts for *Laricobius rubidus* (Coleoptera: Derodontidae). Biocontrol Science and Technology 15: 165-171.

Zilahi-Balogh, G. M. G., Humble, L. M., Lamb, A. B., Salom, S. M. and Kok, L. T.

(2003) Seasonal abundance and synchrony between *Laricobius nigrinus*

(Coleoptera: Derodontidae) and its prey, the hemlock woolly adelgid (Hemiptera:

Adelgidae). The Canadian Entomologist 135: 103 – 115.

Zilahi-Balogh, G. M. G., Kok, L. T. and Salom, S. M. (2002) Host specificity of

Laricobius nigrinus Fender (Coleoptera: Derodontidae), a potential biological

control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand

(Homoptera: Adelgidae). Biological Control 24: 192-198.

Appendix A **Species determination for host range studies (chapter 2) and functional and numerical response studies (chapter 3)**

A.1 Introduction

In 2010 it was discovered that there was a possibility that *Laricobius naganoensis* Leschen (Leschen 2011), a species only distinguishable morphologically from *Laricobius osakensis* Montgomery and Shiyake though the analysis of male genitalia, might have been among the specimens tested. To confirm that the specimens used in the experiments were *L. osakensis*, molecular analysis was used to identify the species of these specimens.

A.2 Methods

The specimens used in the studies described in chapter 2 and 3 were kept in dry storage. *Laricobius osakensis* adults were dissected under a microscope: the head, prothorax, elytra and genitalia were kept as voucher specimens, and the meso- and metathorax with membranous wings were used for the molecular analysis.

Genomic DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, California, USA). PCR was performed for the amplification of the partial cytochrome oxidase subunit I (COI) in 30 µL reactions containing: 15.5 µL sterile water, 3.0 µL 10× PCR Buffer, 2.4 µL dNTPs (10 mM), 4.8 µL MgCl₂ (25 mM), 1.0 µL BSA (10 mg/ml), 1.0 µl of forward primer LepF1 (10 mM), 1.0 µl of reverse primer LepR1 (10 mM), 0.3 µL Taq DNA polymerase, and 1.0 µL of DNA template. Thermal cycling

conditions were 95° C for 5 min. followed by 41 cycles of 45 s at 95° C, 45 s at 48° C, and 1 min at 72° C, with a final extension of 72° C for 5 min. Products of the PCR reaction were purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Sequencing reactions were performed with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems 3730 automated sequencer.

Edition and alignment of partial cytochrome oxidase subunit I (COI) sequences was performed in Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI).

A.3 Results

All voucher specimens analyzed for chapters 2 (Table A.1) and 3 (Table A.2) were found to be *L. osakensis*.

Table A.1 Sample name of voucher specimens used in host range studies (chapter 2) and the species determined by sequence analysis.

Sample name	Species Determined
08-LOSG1-02	<i>Laricobius osakensis</i>
08-LOSG1-05	<i>Laricobius osakensis</i>
08-LOSG1-08	<i>Laricobius osakensis</i>
08-LOSG1-09	<i>Laricobius osakensis</i>
08-LOSG1-10	<i>Laricobius osakensis</i>
08-LOSG1-11	<i>Laricobius osakensis</i>

Table A.2 Sample name of voucher specimens used in functional and numerical response studies (chapter 3), collection information, and the species determined by sequence analysis.

Sample name	Collection Information	Species
09-LONR-01, 02, 03	Japan; Nakayama; Nara Prefecture; 27 Nov 2009; Coll. A. B. Lamb	<i>Laricobius osakensis</i>
09-LOSG-02, 03, 04, 05, 07, 11, 12, 17, 18	Japan; Shiga Kogen; Nagano Prefecture; 28 Oct 2009; Coll. A. B. Lamb	<i>Laricobius osakensis</i>
09-LONK-01, 02, 03, 05, 09, 12, 14, 15, 17, 18, 19	Japan; Nikko-Yamato; Tochigi Prefecture; 28 Oct 2009; Coll. A. B. Lamb	<i>Laricobius osakensis</i>
09-LOSR-02, 03, 04, 09	Japan; Mount Nikko-Shirane; Gunma Prefecture; 28 Oct 2009; Coll. A. B. Lamb	<i>Laricobius osakensis</i>