Evaluation Of Hybridization Among Three *Laricobius* Species, Predators Of Hemlock Woolly Adelgid, (Adelgidae)

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ABSTRACT

Hybridization was evaluated among three *Laricobius* spp. involved in the biological control of hemlock woolly adelgid (*Adelges tsugae* Annand). Following lab mating studies, there was no evidence that *Laricobius osakensis* Montgomery and Yu could produce hybrid progeny with either *Laricobius nigrinus* Fender or *Laricobius rubidus* LeConte. Interaction between *L. osakensis* and *L. nigrinus* did not result in a lower production of progeny as a result of fitness costs associated with interspecific mating attempts. *Laricobius nigrinus* and *L. rubidus* hybrids were produced in the lab and collected in the field. Hybrid progeny showed very little evidence of decreased fitness. For example, there was no significant difference in the number of days it took for hybrids and pure parental species to develop from egg hatch to the prepupal stage, there was no difference among hybrids and pure parental species in the head capsule widths and larval lengths for the first through third instar, and there was evidence of an F_2 generation from field collected specimens. Hybrids produced in the lab had intermediate shaped genital paramere angles compared with parental species, and had elytra coloration similar to that of *L. rubidus*. Hybrids showed no host preference in the lab, but a preference for *Adelges tsugae* in the field. Of 12 site factors examined, only the number of years that *L. nigrinus* was present at the site was found to be associated with percent hybrids. Contamination of the *L. osakensis* colony with *Laricobius naganoensis* Leschen resulted in the need to develop molecular methods to differentiate *L. osakensis* from *L. naganoensis*. Three restriction enzymes were found that correctly differentiated the two species.

DEDICATION

This dissertation is dedicated to my daughter, Marilyn.

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ATTRIBUTION

One colleague aided in data collection for Chapter 5 and Appendix D of this dissertation. Two additional colleagues aided in research and writing for Chapter 6.

Gina A. Davis, PhD (Department of Entomology, Virginia Tech) is currently the Forest Health and Stewardship Program Manager with Idaho Department of Lands, Forest Health Program. Dr. Davis contributed data and methodology to Chapter 5. Field assessment of hybridization between *Laricobius nigrinus* and *L. rubidus*, predators of Adelgidae.

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Chapter 1. Introduction

1.1 The Invasive Hemlock Woolly Adelgid

In the early 1950's, the hemlock woolly adelgid (*Adelges tsugae*, Annand; hereafter HWA), a piercing/sucking insect from Japan, was found in Richmond, VA (Havill et al. 2006). HWA feeds on xylem ray parenchyma cells in young branches and twigs of hemlock causing needle loss, bud, branch, and often tree mortality within 4–20 years after infestation (McClure 1987, 1991, Young et al. 1995, Orwig et al. 2002). Tree damage associated with HWA is unlike that of other invasive adelgids, such as the balsam woolly adelgid (*Adelges piceae*, Ratzeburg), because HWA causes death of trees in all size and age classes, and because hemlock-hardwood stands are as susceptible as hemlock dominated stands (Orwig and Foster 1998, Orwig et al. 2002).

There are two hemlock species present in the eastern United States, eastern hemlock (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Engelman). Eastern hemlock can be found from Nova Scotia, south to Georgia, and west to Minnesota (Kincaid 2012), while Carolina hemlock is present only in small pockets in southern VA and western NC (Jetton et al. 2008). The two species are long-lived and shade-tolerant (Orwig and Foster 1998, Kincaid 2012). They create cool, dark, microenvironments with acidic soils that produce a unique habitat for a variety of understory herbs and shrubs (D'Amato et al. 2009), as well as invertebrate, fish, bird, and mammal species (Snyder et al. 2002, Tingley et al. 2002, Ross et al. 2003, Rohr et al. 2009).

Hemlock mortality results in reduced diversity of flora (Orwig and Foster 1998, Orwig et al. 2002, Sullivan and Ellison 2006) and fauna (Snyder et al. 2002, Tingley et al. 2002, Ross et al.

2003, Ellison et al. 2005, Allen et al. 2009, Rohr et al. 2009). Changes in microclimate, including changes in light, air and soil temperature, soil moisture, nutrient cycling, and hydraulic function (Jenkins et al. 1999, Kizlinski et al. 2002, Eschtruth et al. 2006, Stadler et al. 2006, Daley et al. 2007, Ford and Vose 2007, Orwig et al. 2008, Cobb 2010, Block et al. 2012, Lustenhouwer et al. 2012) result in the conversion of hemlock stands to hardwood stands (Orwig and Foster 1998, Kizlinski et al. 2002, Orwig et al. 2002, Small et al. 2005, Stadler et al. 2005, Eschtruth et al. 2006, D'Amato et al. 2009, Cobb 2010, Spaulding and Rieske 2010, Preisser et al. 2011, Orwig et al. 2012). These changes result in a decrease in the overall biodiversity of the fauna, as hardwood stands do not support as great a diversity of species (Snyder et al. 2002, Tingley et al. 2002, Ross et al. 2003, Ellison et al. 2005, Allen et al. 2009, Rohr et al. 2009, Kincaid 2012).

Eastern and Carolina hemlock have limited resistance to HWA (Bentz et al. 2002). In addition, they lack the ability to sprout or refoliate after defoliation. Consequently, reestablishment can only occur from seed transported from surviving trees or from the seedbank (Orwig and Foster 1998). Unfortunately, hemlock seeds are short-lived and do not persist in the seed bank (Marquis 1975, Sullivan and Ellison 2006).

Since 1990, HWA has been spreading at a rate of nearly 15 km per year in the South and eight km per year in the North (Evans and Gregoire 2007). Because it is an asexual insect with few natural enemies in the eastern U.S. (Montgomery and Lyon 1995, Wallace and Hain 2000), it is expected to spread in eastern North America until it occupies most of the range of eastern and Carolina hemlock (Cheah et al. 2004, Trotter and Shields 2009). Currently, HWA infests more

than 50% of the eastern hemlock range and 100% of the Carolina hemlock range in 18 states (Havill et al. 2011a). Hemlock woolly adelgid has caused severe mortality of both hemlock species (Orwig and Foster 1998) and over time we may see that the combined characteristics of both hemlock and HWA may result in the decimation of both eastern and Carolina hemlock throughout their range (Orwig and Foster 1998, Kincaid 2012).

1.2 Management of Hemlock Woolly Adelgid

Control of HWA has been a challenge because this insect is asexual; it has two generations per year, is active when fewer predators are present (fall, winter, and spring), its hosts lack resistance, and there are few natural enemies (Ward et al. 2004). Many methods to control HWA have been implemented, but none has been very successful on a forest scale (Ward et al. 2004, Orwig and Kittredge 2005). Insecticides such as imidacloprid can be effective, but due to high costs and the potential for negative impacts to the environment, this method is not sustainable in a forested setting (Ward et al. 2004). Soaps and oils have been found to be effective as well, but are also impractical for large areas (Ward et al. 2004). Preemptive and salvage logging has been attempted, but can induce even more profound ecosystem disruption than hemlock decline from HWA (Foster and Orwig 2006). In addition, preemptive and salvage logging reduce population-level genetic variability and may remove resistant individuals that could be used to develop resistant cultivars (Ingwell and Preisser 2011). A more promising method may be the development of resistant crosses between North American hemlock species and Asian species (Montgomery et al. 2009, Ingwell and Preisser 2011). Unfortunately, it will take years before this method can be implemented. Therefore, in the case of HWA, biological control may be the only large-scale management option at this time.

Classical biological control of insects involves the introduction of exotic natural enemies to control exotic pests (Lenteren et al. 2006). In the absence of parasitoids of Adelgidae, or host-specific pathogens (Reid et al. 2010), successful biological control of HWA in the eastern U.S. will likely require predatory insects (Montgomery and Lyon 1995). As of this time, several species of Coleoptera have been released including three Coccinellids: *Sasajiscymnus tsugae* Sasaji and McClure from Japan, *Scymnus sinuanodulus* Yu and Yao from China, and *Scymnus ningshanensis* Yu and Yao from China (Montgomery and Keena 2011), and two Derodontids: *Laricobius nigrinus* Fender from northwestern North America and *Laricobius osakensis* Shiyake and Montgomery from Japan. Other species currently under examination for potential release include two additional coccinellid species: *Scymnus* (P.) *coniferarum* Crotch and *Scymnus camptodromus* (Yu et Lui), *Leucopis* spp. in the family Chamaemyiidae (Diptera), and a generalist entomopathogenic fungus: *Lecanicillium muscarium* (Costa 2011, Montgomery et al. 2011a, Ross et al. 2011).

There is no evidence that either *S. sinuanodulus* or *S. ningshanensis* have established in the eastern U.S. (Montgomery and Keena 2011), but *S. tsugae* and *L. nigrinus* have successfully established (Elkinton et al. 2011). *Laricobius osakensis* was released in November 2012 for the first time but not enough time has passed to determine whether it has established. Although *S. tsugae* has been recovered, it is rarely if ever abundant enough to significantly affect HWA densities (Elkinton et al. 2011). *Laricobius nigrinus* on the other hand, has been recovered in large numbers at many release sites throughout the eastern U.S. (Mausel et al. 2008, Mausel et

al. 2010) and may be the most promising biological control agent released against HWA so far (Elkinton et al. 2011).

1.3 Laricobius spp.

Laricobius is one of four genera in the family Derodontidae. There are 20 described species of *Laricobius* known; 16 of these are native to Asia, three to North America, and one to Europe. *Laricobius* is an exclusively Holarctic genus and is believed to have derived from mycophagous ancestors (Leschen 2000, Leschen 2011).

Laricobius rubidus

Laricobius rubidus LeConte is the only *Laricobius* species native to eastern North America (Brown 1944, Lawrence 1989). Its distribution extends north to New Brunswick, west to Michigan, and as far south as North Carolina (Brown 1944, Wallace and Hain 2000). It is a small beetle (2-3 mm), with red striped black elytra (Leschen 2011). Its primary host is the pine bark adelgid (*Pineus strobi* Hartig; hereafter PBA) but it also feeds on *Adelges piceae* (Clark and Brown 1960) and HWA (Montgomery and Lyon 1995, Wallace and Hain 2000).

The life cycle of *L. rubidus* has been determined mainly through a study by Clark and Brown (1960) in New Brunswick, Canada. They found that the life cycle of *L. rubidus* was well synchronized with PBA. Adults appear in early spring and are active from March until June, with peak activity between mid-April to mid-May (Clark and Brown 1960, Montgomery and Lyon 1995, Zilahi-Balogh et al. 2005). Copulating adults were observed between March 31st and May 5th, and oviposition occurred from mid-April to the end of May (Clark and Brown 1960).

There are four larval instars, the first of which was observed in New Brunswick as early as April 20th, and the last instar observed as late as June 15th (Clark and Brown 1960). Mature larvae migrate to the soil to pupate (Zilahi-Balogh et al. 2005) from mid- to the end of June (Clark and Brown 1960). Adults then undergo an aestival diapause and become briefly active in October to early November in CT and VA, after which the adults disappear from the branches (Montgomery and Lyon 1995, Zilahi-Balogh et al. 2005). Adults have been recovered from duff samples in late January in Virginia and are thought to undergo a hibernal diapause at this time (Zilahi-Balogh et al. 2005).

Although PBA is the main host for *L. rubidus* and it has been found to have an ovipositional preference for PBA, *L. rubidus* can purportedly develop equally well on HWA (Zilahi-Balogh et al. 2005). Therefore, it has been concluded that HWA is a suitable host for *L. rubidus*.

Laricobius nigrinus

Laricobius nigrinus is a small (2-3 mm) black beetle, native to the western U.S. and Canada (Mausel et al. 2011). Its main host is HWA and its life cycle is well synchronized with that of its host (Zilahi-Balogh et al. 2002). *Laricobius nigrinus* has one generation per year (Zilahi-Balogh et al. 2003b). Both the beetles and HWA are active in fall, winter, and spring, and dormant in the summer (Mausel et al. 2011). There are four larval instars which are present in the spring (Mausel et al. 2011).

Laricobius nigrinus was imported into Virginia for evaluation as a biological control agent of HWA beginning in 1997 (Zilahi-Balogh et al. 2002) and field releases began in 2003 (Mausel et

al. 2011). As of this time, *L. nigrinus* has been released in 14 states and established successfully in isolated populations from the southern Appalachians to New England (Mausel et al. 2011). Population growth has been considerable and *L. nigrinus* has been shown to impact HWA populations (Mausel et al. 2008).

Although *L. nigrinus* prefers HWA for feeding and oviposition and only completed development on HWA in host range testing, it has been found to feed on other adelgids (Zilahi-Balogh et al. 2002, Mausel et al. 2011, Havill et al. 2012). While typically collected from HWA in the PNW and Canada, *L. nigrinus* from areas in the interior U.S. have been collected from *Pineus similis* Gillette on western white pine (*Pinus monticola* Douglas ex D. Don), *Adelges lariciatus* Patch on western larch (*Larix occidentalis* Nuttall), and *Adelges cooleyi* Gillette on Douglas-fir (*Pseudotsugae menziesii* (Mirb.) Franco) and Engelmann spruce (*Picea engelmannii* Parry ex Engelm.) (Mausel et al. 2011). As of this time, there has not been adequate sampling in the western U.S. to determine what it prefers in the field (Nathan Havill, personal communication). The interior strain of *L. nigrinus* is being evaluated as a potential biocontrol agent for the northern portion of the HWA range. The coastal and interior *L. nigrinus* populations cannot be differentiated via the partial cytochrome oxidase subunit I gene (COI gene) (Davis et al. 2011), but using six microsatellite loci, Havill et al. (2012) found a weak, but significant differentiation between the two populations.

Laricobius osakensis

Laricobius osakensis is a small (2-3 mm) beetle, native to Japan (Montgomery et al. 2011b). There are two color morphs, one with reddish colored elytra, and the other with nearly black

elytra (Lamb et al. 2011). Reddish colored specimens are typically female, while the dark brown are typically male (Lamb et al. 2011). The main host of *Laricobius osakensis* is HWA. This beetle is found in the same location as the source of the population of HWA that invaded the eastern U.S. (Havill et al. 2006), making *L. osakensis* a potential species of importance for biological control of HWA. *Laricobius osakensis* was discovered in 2005 (Montgomery et al. 2011b) and brought to the U.S. to study as a biological control agent of HWA in 2006. Its life cycle was found to be well synchronized with that of HWA in Japan (Vieira et al. 2013). It has also been found to be host-specific on HWA, only able to complete development to the adult stage on HWA and no threat to nontarget species populations (Vieira et al. 2011). Additionally, *L. osakensis* has also been found to have a higher predation rate, and a higher reproductive rate (Vieira et al. 2012), and may be more cold hardy than *L. nigrinus* (Ligia Cota Vieira, personal communication).

1.4 Hybridization

Using a section of the cytochrome C oxidase subunit I (COI) mitochondrial gene, Davis et al. (2011) found low sequence divergence between *L. nigrinus* and *L. rubidus*. An additional study by Montgomery et al. (2011b) confirmed that *L. nigrinus* and *L. rubidus* are sister species via a phylogeny of the genus *Laricobius*. These two studies, in addition to observations of *L. nigrinus* and *L. rubidus* mating in the field (Mausel et al. 2008) drew attention to the possibility that the two species could hybridize and produce viable progeny (Havill et al. 2012).

Hybridization between an introduced species and a native species is a potential unintended effect of biological control (Huxel 1999, Mooney and Cleland 2001, Hopper et al. 2006).

Hybridization in this context is the crossing of genetically distinguishable groups or taxa, leading to the production of viable hybrids (Mallet 2005). Species and/or populations that were formerly isolated geographically have been brought into contact with one another through many human activities including biological control (Huxel 1999, Lenteren et al. 2006). Species introduced for biological control can potentially spread throughout the region where they have been released, broadly overlap the distribution of a native species, and interbreed with the native species (Huxel 1999, Mooney and Cleland 2001, Hopper et al. 2006).

Hybridization is not often studied prior to the release of a non-native species due to the purported rarity of hybridization occurrences (Hopper et al. 2006). Additionally, it is thought that the risk of large impacts from interbreeding between native species and biological control agents is small. But data are lacking about both the likelihood and impact of interbreeding, and therefore more research is needed (Hopper et al. 2006).

1.5 Potential Outcomes of Hybridization

Introgression

Introgression is the transfer of genetic material from one species into another via hybridization (Arnold 1992). Fertile hybrid species may backcross to either parental species and introgress DNA from one species into another (Anderson 1953). This is true even if hybrid fitness is low and successful backcrosses are rare (Barton 2001). Introgression can be unidirectional; hybrids can fail to mate with individuals of one parental taxon, or such matings can be sterile. Even if hybridization is fertile in both directions, it is possible that such hybridization can produce fertile

offspring of only one sex (Rhymer and Simberloff 1996). Introgression can be highly selective, affecting only some parts of the genome, whereas other regions remain untouched (Mallet 2005). Introgressive hybridization can influence evolution in several ways: 1) reinforcement of reproductive isolation between incompletely diverged species; 2) genetic assimilation of one species by another; 3) outbreeding depression, a stable hybrid zone; 4) adaptive evolution; or even 5) the origin of a new species (Arnold 1997, Mallet 2007).

The outcome and impact of introgression depends on the selective advantage or disadvantage the introgressed genomic regions provide, as well as the frequency of introgression and the dispersal rate of the progeny (Barton 2001).

Reinforcement of Premating Isolation

Reinforcement is an increase in prezygotic isolation between hybridizing taxa in response to selection against interspecific mating (Servedio and Noor 2003). Prezygotic isolation can include differences in behavior, ecology, reproductive timing, gametic compatibility, and/or mechanical incompatibility of the genital organs (Edmands 2002, Xue et al. 2009). Evidence for reinforcement occurring in nature is mixed. Some consider reinforcement an unlikely outcome of secondary contact (Hewitt 1988, Liou and Price 1994), while others believe it is possible under particular circumstances (Noor 1999, Servedio and Noor 2003, Coyne and Orr 2004).

There are two key conditions thought to make reinforcement more likely: 1) When initial postzygotic isolation (reduction in fertility or viability in hybrid offspring) between the two species is strong enough to cause significant selection against hybridization (Liou and Price 1994); and 2) when there is sufficient initial divergence between the two populations in their

mate recognition systems (Liou and Price 1994). Under one or both of these conditions, assortative mating (Dobzhansky 1937, Servedio and Noor 2003), divergent reproductive traits (Howard 1993), and/or specialization on a host species (Xue et al. 2009) can evolve adaptively.

Complete speciation could occur if divergence between two species in both their premating and postzygotic isolating mechanisms has been sufficient (Liou and Price 1994). But if gene flow from outside of the hybrid zone occurs, premating adaptations that evolve in the zone are likely to be swamped out (Coyne and Orr 2004).

Genetic Assimilation/ Displacement

Genetic assimilation is the eventual extinction of a species as introgression occurs from a related species causing one to become genetically more like the other. Assimilation may occur if an introduced species genetically swamps the native species by producing a large number of progeny via increased fertility in the new range. Low population size can result in increased susceptibility of a native species to genetic assimilation and displacement due to hybridization (Huxel 1999). If the native species is rarer than the introduced species, it will tend to encounter and mate with the introduced species more frequently than its own species. The native species will become increasingly rare because of hybrid losses, whereas the biological control agent may become more common because it hybridizes less often (Huxel 1999). The result could be the rapid extinction of the native, rarer species (Liou and Price 1994).

Complete assimilation of both parental species is possible if hybrids have higher fitness than the parents in their own habitat. The hybrids may then replace the parental species due to

competition (Arnold 1997, Mallet 2007). There are several examples of species fusion, for example in Darwin's finches and cichlid fish (Mallet 2007).

Stable Hybrid Zone

A hybrid zone is a region where genetically distinct populations meet and interbreed, resulting in mixed ancestry. In a hybrid zone, introgression is not sufficient to merge the species, most likely due to outbreeding depression, but results in introgression of sequences into the gene pool of each (Lewontin and Birch 1966).

Outbreeding depression is a reduction in hybrid fitness due to intrinsic or extrinsic factors. Intrinsic outbreeding depression between hybridizing taxa is a result of the accumulation of genetic incompatibilities that developed over the time they were geographically isolated (Palmer and Feldman 2009). Recombinant genotypes produced through hybridization have not been previously subjected to selection and these genotypes will typically be less well adapted than those of their parents, resulting in selection against hybrids (Burke and Arnold 2001). Selection against hybrids is often exhibited as sterility or inviability (Haldane 1922). The production of sterile or inviable offspring could result in a decrease in fitness of the parental species due to an overall decrease in reproductive output (Allendorf et al. 2001).

Extrinsic outbreeding depression results from reduced adaptation to environmental conditions (Allendorf et al. 2001). Gene flow between two species may produce individuals with intermediate features that may not be well adapted to the environment due to the loss of important local adaptations that are necessary for viability (Allendorf et al. 2001).

Encounters between introduced and native insect species in biological control are contacts between newly sympatric species that were previously allopatric (Hopper et al. 2006). It is often expected that hybrids would be less fit than the parental species because they have not been subjected to natural selection, and should be less well adapted (Barton 2001).

Hybrid Vigor

Although hybrids tend to be less fit, this is not always the case (Rieseberg et al. 1996, Arnold 1997). Introgression can lead to adaptive genetic variation through several routes (Lewontin and Birch 1966). First, the genotypes of the hybrids may be quite diverse. Some genotypes may be more fit than the parents, even in the parental environment. Second, the ancestral genotype that connects the parental populations may be reestablished in the F_2 or later generations if the ancestral alleles have not been lost following divergence. It is expected that the ancestral lineage must have been reasonably fit in the past, and may even be more fit in the present environment. Third, if fitness is determined by interactions between different sets of genes, then recombinants which contain intact sets may have fitness as high or higher than either parent (Barton 2001). Fourth, elevated fitness may result due to specific ecological conditions within the hybrid zone, particularly if the zone is comprised of the conditions the interbreeding species are subjected to in their respective geographical ranges (Harrison 1990). Fifth, hybridization could give rise to genotypes that are more fit than their parents through the purging of mutational load. Mildly deleterious alleles can become fixed within lineages due to constraints of finite population size leading to gradual decrease in fitness. Hybrids can have increased vigor due to the masking of deleterious recessive alleles. In later generations, one possible outcome of hybrid vigor could be

the introgression of favorable alleles from one parental population into the other (Burke and Arnold 2001).

Hybrid vigor has been documented in previous studies (Arnold 1997, Burke and Arnold 2001). For instance, Lewontin and Birch (1966) found that introgression of genes from *Dacus neohumeralis* Hardy (Diptera: Tephritidae) into *Dacus tyroni* Frogg accelerated the genetic adaptation of the population to a high stress temperature.

Speciation

Homoploid hybrid speciation is the establishment of a reproductively isolated lineage through hybridization without a change in chromosome number (Coyne and Orr 2004). In order for homoploid hybrid speciation to occur, unique adaptations from combining divergent genomes must allow for the hybrids to become reproductively isolated from the parental species (Mooney and Cleland 2001). Homoploid hybridization can generate a new species when reproductive isolation occurs via routes such as: 1) availability of an empty niche; 2) strong ecological selection; 3) high hybrid fertility; 4) spatially separated habitats of parental and hybrid species; 5) hybrid founder events; 6) production of extreme phenotypes; and 7) sexual selection (Buerkle et al. 2000, Burke and Arnold 2001, Kirkpatrick and Ravigne 2002, Mavarez et al. 2006). For example, hybrids may occupy different spatial, temporal, or diet niches and/or assortative mating may develop leading to the reduction of gene flow between hybrids and parents, and eventually speciation (Seehausen 2004, Mavarez et al. 2006, Mallet 2007). Hybrid progeny may coexist with the parental taxa as distinct species or displace one or more parent species ecologically, eliminating evidence of their hybrid lineage over time (Mallet 2007).

It is usually argued that homoploid hybrid speciation is rare because hybrids must overcome chromosome and gene incompatibilities, and speciation must occur in sympatry, while at the same time hybrids must become reproductively isolated (Barton 2001, Mallet 2007). But there are several potential examples of homoploid hybrid speciation, including *Rhagoletis* flies (Schwarz et al. 2005), *Heliconius* butterflies (Mavarez et al. 2006), and African cichlids (Schelly et al. 2006). This may suggest that homoploid hybrid speciation is more common than previously thought (Gompert et al. 2006).

1.6 Hybridization in Biological Control

Data on hybridization as a result of biological control introductions or augmentations are rare (Hopper et al. 2006). There are only three systems besides the *Laricobius*/adelgid system where hybridization between an introduced biological control agent and a native species has been reported. These include laboratory experiments involving hybridization between *Chrysoperla carnea* (Chrysopidae) from Germany and *C. nipponensis* from Japan (Naka et al. 2005, Naka et al. 2006), *Diadegma semiclausum* (Ichneumonidae) from Asia, and Japanese *D. fenestrale* (Davies et al. 2009), and *Torymus sinensis* (Torymidae) from China and Japanese *T. beneficus* (Moriya 1992). Of the three examples, only hybridization between *T. sinensis* and *T. beneficus* was followed up with a field study and even then, the effects on biological control were not evaluated (Yara et al. 2010).

Recently it was discovered that *Laricobius rubidus* and *L. nigrinus* can hybridize and produce viable progeny (Havill et al. 2012). Due to the broad spectrum of potential outcomes that can

occur following hybridization and the lack of data on the subject, the outcome of this interaction needs to be addressed. The two main concerns regarding interbreeding between a biological control agent and native species are negative effects to the biological control program and/or loss of biodiversity.

1.7 Most Probable Outcome of Hybridization between L. rubidus *and* L. nigrinus

In order to predict what the outcome of hybridization between *L. nigrinus* and *L. rubidus* will be, it would be helpful to know several things: how closely related they are, the fitness and morphological characteristics of the hybrids, whether they diverged in allopatry, the structure of their hybrid zone, and what events are occurring within their zones of hybridization (Havill et al. 2012).

Laricobius nigrinus and *L. rubidus* have been found to be sister species. They are very closely related with a Kimura two-parameter distance (K2P) of 2.2% using DNA sequences from the 5' end of the rapidly evolving cytochrome oxidase subunit I (COI) gene. The genetic distance between the two species was only slightly higher than within each species (Davis et al. 2011), suggesting they diverged recently from a common ancestor (Havill et al. 2011b).

A 2009 preliminary laboratory study produced hybrid offspring from three interspecific pairs (G. Davis and T. Dellinger, unpublished). Many hybrids have also been collected from the field (Havill et al. 2012). The hybrids have variable elytra coloration and the male hybrids have variably shaped genital parameres compared with that of the parental species (Havill et al. 2012). The relative fitness of the hybrids to the parental species is not yet known. It is also unknown if

L. nigrinus and *L. rubidus* are in fact allopatric in their natural ranges, or have a zone of sympatry somewhere in central North America (Havill et al. 2012).

Most recent work involving hybridization between *L. nigrinus* and *L. rubidus* has focused on samples from eastern hemlock. Strong asymmetric genetic introgression on eastern hemlock towards *L. nigrinus* may suggest that *L. nigrinus* has more of a preference for this habitat than does *L. rubidus* (Havill et al. 2012). Collections from white pine may help determine whether there is a corresponding pattern of asymmetrical introgression in the preferred habitat of *L. rubidus* (Havill et al. 2012).

Stable Hybrid Zone

Currently, *L. nigrinus* and *L. rubidus* form several hybrid zones throughout the Appalachian Mountains where *L. nigrinus* has been released (Havill et al. 2012). The structure of these zones is very different from the usual model of a hybrid zone. Rather than a broad linear hybrid zone where both species have regions of allopatry with a more or less narrow region of sympatry where hybridization occurs, a patchy mosaic hybrid zone has been formed (Harrison and Rand 1989, Havill et al. 2012). A mosaic zone has been formed because *L. rubidus* can still be found in large regions of allopatry and because the preferred habitats of the different *Laricobius* species broadly overlap in the region where they are hybridizing (Havill et al. 2012).

Whether or not the two *Laricobius* spp. will maintain these hybrid zones in a stable form is unknown. The structure may change over time because the distribution of *L. nigrinus* may eventually overlap with *L. rubidus* completely. Moreover, each patch within the mosaic hybrid

zone could have a unique evolutionary trajectory. The outcome of any of the interactions within these patches may depend on the relative abundance of the two species, selection pressures within the patch, random drift, and the genetic composition at the boundary (Harrison and Rand 1989).

Reinforcement of Premating Isolation

There is typically a long period after speciation when hybridization can lead to genetic introgression. During introgression, important speciation processes such as reinforcement, an increase in prezygotic isolation between hybridizing taxa, can occur (Coyne and Orr 1997, Servedio and Noor 2003).

Three conditions favor reinforcement when hybrids are intermediate to parental species in their ecological niche: 1) large differences between the parental mean phenotypes; 2) strong stabilizing natural selection around a local ecological optimum; and 3) many loci contributing to the ecological adaptation (Kirkpatrick 2001).

The genital morphology of the two *Laricobius* species is not vastly different. It is unknown whether there is an ecological optimum pertaining to environmental characteristics such as climate, available food (particularly PBA), percent hemlock/white pine habitat, etc., within the shared habitat of the two *Laricobius* spp., therefore it is unknown if strong stabilizing natural selection is occurring around such an optimum. Finally, there is currently no information on which or how many loci may contribute to any ecological adaptation that may be occurring.

Cain et al. (1999) suggested reinforcement may occur more easily when hybrid zones exist in a mosaic structure than in a tension zone. This is because the formation of hybrids over a broader region can result in stronger selection for premating isolation. However, the increased opportunities for introgression in a mosaic zone may also have the tendency to homogenize the population (Servedio and Noor 2003).

Reinforcement would be more likely if this were a mechanism that helped drive the initial divergence (Havill et al. 2012). In the case of *L. nigrinus* and *L. rubidus* it is not clear what led to their divergence. Some possibilities are: 1) allopatric divergence due to historical changes in the distributions of the conifer hosts of their adelgid prey; 2) initial sympatric divergence associated with specialization on different adelgid prey (for example on hemlock adelgids versus pine adelgids in western North America) followed by allopatric divergence; or 3) isolation by distance, if they are still found in sympatry somewhere in central North America where adelgid prey such as larch adelgids bridge the known distribution of the two species (Havill et al. 2012). If *L. nigrinus* and *L. rubidus* did diverge in allopatry, adaptations that increase reproductive isolation are unnecessary and therefore one of the last features to differentiate (Hoskin et al. 2005). If the two species still meet somewhere between their current known distributions, they may form a natural hybrid zone where gene flow between the two species is presently occurring (Havill et al. 2012).

If reinforcement is occurring between the two *Laricobius* spp., over time we will see a decrease in hybridization and eventually a termination of hybridization as reinforcement leads to permanent species boundaries. As of this time, the increasing proportion of hybrids recovered at

several *L. nigrinus* release sites (Davis et al. 2011, Havill et al. 2012) suggest that reinforcement is not occurring.

Genetic Assimilation/ Displacement

Within a mosaic hybrid zone, hybridization and recombination will lead to gene flow across the species boundary, but chromosomal segments surrounding genes affecting fitness or preference in different habitats may remain differentiated (Harrison and Rand 1989). Factors that promote the persistence of two distinct species reduce the probability of assimilation and extinction (Harrison and Rand 1989). *Laricobius nigrinus* prefers HWA on hemlock (Zilahi-Balogh et al. 2002) and *L. rubidus* prefers PBA on white pine (Zilahi-Balogh et al. 2005). Additionally, because proportions of the two species vary from site to site (e.g. due to the number of *L. nigrinus* released) and local selection pressures also vary (e.g. proportion of hemlock and/or white pine present), extinction of either species is unlikely.

Current evidence suggests that assimilation is not occurring because individuals are still being identified as pure species and percent hybridization across the landscape is low (7%) (Havill et al. 2012). These factors and the host preferences of *L. nigrinus* and *L. rubidus* suggest that species separation will be maintained with some gene flow between the two species (Havill et al. 2012).

Hybrid Vigor

When HWA was introduced into the eastern U.S. from Japan, a new ecological niche was created. *Laricobius* hybrids could be better adapted to this niche than their parents if they

receive a preference for HWA from their *L. nigrinus* parents, and hardiness in eastern climates from *L. rubidus* (Havill et al. 2012).

Hybrid diets have been found to be intermediate to that of the parental species in past studies (Schluter 1993, Weider 1993, Schluter 1995). But asymmetrical introgression towards *L. nigrinus* found on eastern hemlock (Havill et al. 2012) suggests that *L. nigrinus* has a predilection for this habitat over *L. rubidus*.

Even if the hybrids themselves are not better adapted to this new niche, hybridization could accelerate adaptation of *L. nigrinus* to the new and unoccupied niche of HWA on eastern hemlock through introgression of adaptive genes from *L. rubidus* (Havill et al. 2012). It has been found that a species that is rare in its native habitat may become more common with increasing amount of favorable habitat in its new range (Huxel 1999).

Speciation

Hybrids tend to be best adapted to intermediate optima, and so are typically poorly adapted to either parental environment (Barton 2001). Extreme habitats relative to the requirements of parents, rather than habitats with characteristics intermediate between the parental niches are typically necessary for hybrid speciation to occur (Buerkle et al. 2000). In the case of *L. nigrinus*, *L. rubidus,* and their hybrids, no extreme habitat is known to exist. Additionally, when divergence is low, there may be little chance of major novelties arising in hybrids (Abbott et al. 2013), another characteristic that would make it more likely for hybrid speciation to occur.

Without the major traits deemed necessary for speciation to occur, extreme habitat and major novelties, it is unlikely that *L. nigrinus* x *L. rubidus* hybrids will become a separate species.

1.8 Hybridization among L. osakensis, L. nigrinus, *and* L. rubidus

Species that are phylogenetically close are more likely to interbreed than species that are phylogenetically distant (Coyne and Orr 1997). While *L. nigrinus* and *L. rubidus* are closely related sister species, *L. osakensis* is in a separate clad with other Asian species (Montgomery et al. 2011b). *Laricobius osakensis* can be distinguished from *L. nigrinus* and *L. rubidus* morphologically via a lack of ocelli, and differences in the parameres and median lobe of the male genitalia (Montgomery et al. 2011b). Using the partial cytochrome oxidase subunit I (COI) gene, the mean genetic distance (K2P) between *L. osakensis* and *L. nigrinus,* and *L. osakensis* and *L. rubidus* was found to be approximately 15.0%, whereas the distance between *L. nigrinus* and *L. rubidus* is only 2.2% (Montgomery et al. 2011b). Almost all hybridizing geographic races within *Heliconius* butterfly species have mtDNA sequence differences of less than 2%; species that hybridize occasionally in the wild are at most 2-6% divergent; whereas no hybrids are found between species >10% divergent (Mallet et al. 1998). Given this information, it is unlikely that *L. osakensis* will be able to hybridize with *L. nigrinus* and *L. rubidus*, but it is still important to know how the species will interact prior to introduction of *L. osakensis* (Story et al. 2012). Even if they do not hybridize and produce viable progeny, attempts at interbreeding can lead to a substantial reduction in the potential population growth rate via incompatible interspecific crosses such as was found between *Trichogramma platneri* Nagarkatti (Trichogrammatidae) and *T. minutum* Riley (Stouthamer et al. 2000).

Interspecific Mating with no hybridization

Attempting to mate interspecifically could lower the fitness of the *Laricobius* spp. via lower reproductive output even if hybridization does not occur. For example, two species may recognize one another as mates and attempt copulation (Arnqvist 1998). When there is a large difference in genital morphology between hybridizing species, interspecific copulation can result in genital injuries that in turn cause mortality of the copulating individuals and reduce the fitness of the hybridizing population (Kubota and Sota 1998). There are differences between *L. osakensis* and the two North American species in the characteristics of the male genitalia, and it is possible that injury may occur. *Laricobius osakensis* has slender parameres and a median lobe that tapers after the orifice, whereas *L. nigrinus* and *L. rubidus* have parameres that are more stout in the apical half and a median lobe that begins to taper before the orifice (Montgomery et al. 2011b).

Another issue that may occur during attempted interspecific copulation involves the reception of interspecific male seminal products. When receiving interspecific male seminal products, these foreign products often fail to stimulate the female, resulting in a decrease in oviposition and/or a decrease in the desire to remate (Eberhard 1996).

Even if there are no issues with the genitalia, the seminal products, or sperm transport and/or storage, infertile eggs could result from developmental failures in embryos between species that have diverged sufficiently, potentially lowering the overall reproductive output of the parental species (Eberhard 1996). Additionally, males of some insect species have sperm removal/ displacement devices (Waage 1986, Ono et al. 1989) and/or sperm plugs (Aiken 1992) which

could result in the removal or denial of viable sperm from intraspecific mating before or following interspecific mating (Eberhard 1996). Females of some species can negate these effects through avenues such as premature interruption of copulations; lack of sperm transport to storage and/or fertilization sites within the female; discharge or digestion of the current male's sperm or those of previous or subsequent males; lack of ovulation; abortion; lack of oviposition; rejection or removal of mating plugs; prevention of removal of plugs by subsequent males; selective use of stored sperm; selective fusion with sperm that have reached the egg; lack of sperm activation and/or lack of sperm nourishment (Eberhard 1996). Any of these traits could result in lower reproductive output.

1.9 Conclusion

More evidence is needed, but given the information we have it is most likely that hybridization between *L. nigrinus* and *L. rubidus* will result in a mosaic hybrid zone, which may or may not incur either hybrid vigor or outbreeding depression. Determining the final outcome of hybridization between *L. nigrinus* and *L. rubidus* is important for the HWA biological control program because, as noted above, hybridization has the potential to have negative consequences. It could lead to adaptations broadening the host range or changing the host preference of the parental species and/or hybrids (Huxel 1999). In addition, hybridization could lead to the local extinction of one or both of the parental species (Lenteren et al. 2006, Seehausen et al. 2008), decreasing biodiversity in the eastern U.S.

Although current evidence suggests that hybridization between *L. osakensis* and the two North American *Laricobius* species will not occur, past history suggests that these interactions should

be studied. Even if interspecific crosses among these species do not produce viable progeny, the risk of injury to the adults and the risk of lower fitness via lower reproductive output warrant closer examination.

Chapter 2. Assessment of the potential for hybridization between Laricobius nigrinus *and* Laricobius osakensis*, predators of the hemlock woolly adelgid*

2.1 Introduction

Laricobius nigrinus and Laricobius rubidus

Laricobius nigrinus Fender is a predatory beetle native to the Pacific Northwest and Canada where it feeds on the invasive hemlock woolly adelgid (*Adelges tsugae*, Annand; hereafter HWA). In 2003, *L. nigrinus* was released at 22 sites in the eastern U.S. as a biological control agent of HWA. The predator has established at 13 of the 22 release sites and has been found to have an impact on HWA (Mausel et al. 2010, Mausel et al. 2011). Recently, it was discovered that *L. nigrinus* can hybridize with *Laricobius rubidus* LeConte (Havill et al. 2012), a native eastern U.S. predator of the pine bark adelgid (*Pineus strobi* Hartig). *Laricobius nigrinus* and *L. rubidus* are sister species (Davis et al. 2011, Montgomery et al. 2011b), which have been shown to produce viable hybrid progeny (Havill et al. 2012).

Hybridization between *L. nigrinus* and *L. rubidus* is a concern because of potential impacts to the biological control of HWA, as well as concerns regarding biodiversity (Hopper et al. 2006, Havill et al. 2012). For example, if hybrids have reduced fitness, they may have less of an impact on HWA than their parents (Arnold 1997, Goldson et al. 2003). Alternatively, hybridization could result in recombination that introduces advantageous alleles or new combinations that contribute to adaptive evolution (Lewontin and Birch 1966, Arnold 1997). This could result in hybrid progeny that are more fit than the parental species (Arnold 1997) and potentially capable of having a greater impact on HWA. Either scenario could result in reducing

the distinctiveness of *L. nigrinus* and/or *L. rubidus* through genetic introgression at field sites where they are both present (Arnold 1997).

The effects of hybridization between the two species are currently being studied. So far, introgression has been asymmetrical and many individuals are still being identified as pure species (Havill et al. 2012). It is possible that a patchy distribution (a mosaic hybrid zone) of parentals and hybrids (Harrison and Rand 1989) will result, since the preferred habitats of each *Laricobius* species [*Tsuga canadensis* (L.) Carrière/ *Tsuga caroliniana* Englem. and *Pinus strobus* L.] overlap throughout much of eastern North America (Havill et al. 2012).

Laricobius osakensis

In 2006, *Laricobius osakensis* Shiyake and Montgomery was imported from Japan into the U.S. as another potential biological control agent of HWA. *Laricobius osakensis* is native to the same region of Japan as the HWA strain found in the eastern U.S.; therefore, *L. osakensis* likely evolved with the HWA strain established in the eastern U.S. (Havill et al. 2006, Lamb et al. 2011). In a study of comparative interactions between *Laricobius* spp., Story et al. (2012) found that *L. osakensis* feeds more and has a higher fecundity than *L. nigrinus*. Additionally, *L. osakensis* may be better suited to the southern U.S. than *L. nigrinus* based on the wide range of elevations (80–1850 m) at which it has been collected in Japan (Lamb et al. 2011). For these reasons, the evaluation of *L. osakensis* for release into the field has been a priority in the biological control of HWA.

In contrast to *L. nigrinus* and *L. rubidus* which are closely related sister species, *L. osakensis* is in a separate clade, together with other Asian species (Montgomery et al. 2011b). The mean genetic distance (K2P) between *L. osakensis* and *L. nigrinus* was found to be 15.2%, whereas the distance between *L. nigrinus* and *L. rubidus* is 2.2% (Montgomery et al. 2011b). In addition, there are major morphological differences between *L. osakensis* and the North American species such as the presence of ocelli in *L. osakensis* but not in *L. nigrinus* or *L. rubidus* (Leschen 2011). Evolutionary divergence is likely to impact the propensity to hybridize. For example, *Heliconius* butterfly species that hybridize occasionally in the wild had mitochondrial DNA sequences that were at most 2–6% divergent; whereas no hybrids are found between species with >10% divergence (Mallet et al. 1998). We therefore expect that it is unlikely that *L. osakensis* will be able to hybridize with *L. nigrinus*. Nevertheless, it is important to determine prior to its introduction how *L. osakensis* will interact with the other *Laricobius* species.

Even if *L. osakensis* and *L. nigrinus* do not hybridize and produce viable progeny, it is possible that attempts at interbreeding could lead to substantial reduction in the population growth rate via incompatible interspecific crosses such as was found between *Trichogramma platneri* Nagarkatti (Trichogrammatidae) and *T. minutum* Riley (Stouthamer et al. 2000). For example, different species may recognize one another as mates and attempt copulation (Arnqvist 1998). When there is a large difference in genital morphology between species, interspecific copulation can result in genital injuries which in turn can result in mortality of the copulating individuals and a reduction in the fitness of the population (Kubota and Sota 1998). There are differences in the male genitalia between *L. osakensis* and the two North American species, so it is possible that injury may occur. *L. osakensis* has slender parameres and a median lobe that tapers after the

orifice, whereas *L. nigrinus* and *L. rubidus* have parameres that are much more stout in the apical half and a median lobe that begins to taper before the orifice (Montgomery et al. 2011b).

Another potential issue regarding interspecific copulation involves the reception of interspecific male seminal products. When receiving interspecific male seminal products, it is possible for the male seminal products to fail to stimulate the female, resulting in a decrease in oviposition and/or a decrease in the desire to remate (Eberhard 1996). In addition, males of some insect species have sperm removal/displacement devices (Waage 1986, Ono et al. 1989) and/or sperm plugs (Aiken 1992), which could result in the removal or denial of viable sperm from intraspecific mating before or following interspecific mating (Eberhard 1996). Females of some species can negate these effects through premature interruption of copulations; lack of sperm transport to storage and/or fertilization sites within the female; discharge or digestion of the current male's sperm or those of previous or subsequent males; lack of ovulation or oviposition; abortion; rejection or removal of mating plugs; prevention of removal of plugs by subsequent males; selective use of stored sperm; selective fusion with sperm that have reached the egg; lack of sperm activation and/or lack of sperm nourishment (Eberhard 1996). Any of these traits could result in lower reproductive output. Even if there are no issues with physical damage to the genitalia, the seminal products, or sperm transport and/or storage, infertile eggs could result from developmental failures in embryos between species that have diverged sufficiently, potentially lowering the overall reproductive output of the parental species (Eberhard 1996).

The purpose of this study, therefore, was to determine if *L. osakensis* could mate with *L. nigrinus*, if they can produce hybrid progeny, and whether mating interferes with reproductive

output. To this end, fitness components such as fecundity, fertility, viability, and mate choice were compared among intraspecific and interspecific mating pairs.

2.2 Methods

No-choice lab mating trials

No-choice lab mating experiments were conducted in 2010 and 2011 to determine whether *L. osakensis* could mate with *L. nigrinus* and produce viable eggs. Treatments consisted of pairing *L. osakensis* with *L. osakensis* (Lo x Lo), *L. nigrinus* with *L. nigrinus* (Ln x Ln), and *L. nigrinus* with *L. osakensis* (Ln x Lo).

All beetles used in the experiment were (assumed) virgin, lab-reared, F_1 adults. Gender was not determined prior to pairing of adults to avoid injuring the beetles. Removing individuals from the soil following pupation when the genitalia are visible (Zilahi-Balogh et al. 2006) results in high mortality (Salom et al. 2009) and squeezing the abdomen of adults so that the genitalia are extruded (Shepherd et al. 2011) could damage the reproductive system and/or sexual organs.

The number of potential mating pairs in each treatment was based on the availability of adult beetles. Because of low emergence of *L. osakensis* at the Virginia Tech Insectary in fall 2009, 23 Lo x Lo, 31 Ln x Ln, and 54 Ln x Lo crosses were made. The beetles were paired in December 2009 and egg production was followed into spring 2010. In the fall 2010, increased emergence of *L. osakensis* permitted pairing of 50 Lo x Lo, 52 Ln x Ln, and 100 Ln x Lo. Pairings were made in December 2010 and data collection proceeded into spring 2011. The number of Ln x Lo mating pairs in each year was approximately doubled in order to increase the

statistical power to determine significant differences in fitness when the female of the pair was either *L. nigrinus* or *L. osakensis*.

The paired beetles were kept in 50 x 9 mm polystyrene Petri dishes with ventilation holes cut into the top and covered with polyester mesh. The dishes were filled with HWA-infested hemlock branches and kept in a growth chamber under the conditions shown in Table 2.1. Beetles were fed once each week and placed in clean dishes. If a beetle from one of the pairs died, both individuals were removed from the study and placed in separate microcentrifuge tubes with 95–100% ethanol.

The study allowed for the measurement of three fitness components: fecundity (the number of eggs produced per cross), fertility (the number of prepupae produced per cross), and viability (the number of adults produced per cross). Fecundity was determined by counting eggs weekly, on the branches in each of the dishes. In 2010, the lengths of all eggs that could be extracted from within HWA ovisacs without sustaining damage were also measured; in 2011, the length of one egg from each dish was measured each week.

Gender was unknown at the outset of the study; therefore individuals were sexed at the conclusion of the study and pairs included ♂♀, ♀♀, and ♂♂. This allowed us to compare the number of eggs produced by ♂♀ mating pairs and unmated ♀♀ pairs. In 2010, a large number of eggs were observed in the dishes of the Ln x Ln and Ln x Lo crosses with two females (a total of 367 eggs from 20 ♀♀ pairs) compared with the number of eggs found within the dishes containing two males (total of 11 eggs from 11 ♂♂ pairs). The eggs in the ♂♂ dishes were

probably wild *L. rubidus* eggs brought in on the HWA-infested hemlock branches supplied to beetles. The eggs in the ♀♀ dishes could also include wild *L. rubidus* eggs, or sterile eggs oviposited by unfertilized females, or both. For this reason, the number of eggs produced by ♂♀ and ♀♀ pairs in 2011 was compared among the three crosses. This comparison was made because if *Laricobius* females are capable of producing sterile eggs in the absence of mating, then the eggs found in the dishes of the ♂♀ Ln x Lo mating pairs could also be sterile and not the result of successful interspecific crossing. If ♂♀ Ln x Lo mating pairs produce sterile eggs, we might expect ♀♀ Ln x Lo mating pairs to produce a greater number of eggs than the ♂♀ Ln x Lo mating pairs because of the presence of two females. We would expect the opposite result for the Lo x Lo and Ln x Ln mating pairs, because many insects that produce sterile eggs oviposit fewer eggs prior to copulation and oviposition of fertile eggs (Ridley 1988, Wheeler 2001, Brent et al. 2011). No comparison was made for the 2010 data because there was only one ♀♀ Lo x Lo pair and the pair died during the second week of the experiment without producing eggs.

In 2010, eggs from the Ln x Lo treatment were often found outside of the HWA ovisacs (e.g., on a needle) and would appear to shrivel over time. For this reason, in 2011, the number of eggs oviposited inside and outside of HWA ovisacs and the number of non-shriveled and shriveled eggs were compared within ♂♀ and ♀♀ pairs of Lo x Lo and Ln x Ln and among the ♂♀ crosses. If eggs oviposited outside of the ovisacs and shriveled eggs characterize sterile eggs, we would expect to find more of these in the dishes of ♀♀ pairs, than in those of ♂♀ pairs. Additionally, we would expect to find more of these eggs within the dishes of ♂♀ Ln x Lo pairs, than ♂♀ Lo x Lo or Ln x Ln pairs if the eggs oviposited by Ln x Lo pairs are indeed sterile.

Fertility was assessed by placing the eggs collectively in rearing funnels by cross treatment (≈250 eggs per funnel in 2010; ≈150 eggs per funnel in 2011) and rearing the larvae that emerge to the prepupal stage [for specifics on rearing methods see (Salom et al. 2012)]. Funnels were checked daily and all prepupae were placed into soil containers (50 per container) by cross to rear to adulthood. Viability was determined by counting all adults that emerged from the soil containers the following fall. The dates of emergence were recorded and the adults were placed into individual microcentrifuge tubes with 95–100% ETOH.

All adults used in the no-choice lab mating experiment, and all adult progeny that emerged from soil containers were dissected. The genitalia were extracted and mounted on slides and the heads and elytra were saved as vouchers and deposited at the Virginia Tech Insect Museum or the Yale Peabody Museum of Natural History. The thoraces were used to extract DNA, and the identity of beetles was determined using genetic analyses (described below).

Development Study

Experiments were conducted in 2010, 2011, and 2012 to determine the average duration of development from egg hatch to the prepupal stage for progeny from the Lo x Lo, Ln x Ln, and Ln x Lo crosses. Eggs used for this study were obtained from the mating pairs in the no-choice lab mating experiments in 2010 and 2011, and from the mate change experiment (see below) in 2012.

In 2010, eggs were collected once per week from March through April if available. Eggs were collected from 11 Lo x Lo (25 eggs), 16 Ln x Ln (24 eggs), and 25 Ln x Lo (47 eggs) mating pairs. In 2011, eggs were collected from 24 Lo x Lo (24 eggs), 26 Ln x Ln (26 eggs), and 48 Ln x Lo (48 eggs) mating pairs with no replication from March 8-19. In 2012, eggs were collected from 9 Lo x Lo (51 eggs), 8 Ln x Ln (36 eggs), and 12 Ln x Lo (36 eggs) mating pairs between February 22 and March 28.

The length of each egg was measured and the egg was placed on a ~2 cm long HWA-infested hemlock branch. The branches were placed individually in 50 x 9 mm polystyrene Petri dishes with ventilation holes and kept in a growth chamber at 13˚C, 12:12 (L:D) h. The eggs were checked daily for hatch and if hatching occurred, additional HWA-infested branches of hemlock were added to the dish as needed to provide food for the developing larva. Prepupae were defined as large larvae wandering the bottom of the dish (Zilahi-Balogh et al. 2003a). Once a larva developed into a prepupa, the date was recorded and the individual was collected and placed in a microcentrifuge tube with 95-100% ETOH for genetic determination.

Fluorescence study

In 2011 and 2012, fluorescent dye was used to determine whether eggs produced by crosses between *L. nigrinus* and *L. osakensis* had been fertilized. If *L. osakensis* were to be released and the beetles do not have a species preference, and the eggs are fertilized but not viable, this may lower the fitness of *Laricobius* spp. through wasted reproductive effort.

In March 2011, a single egg was chosen from four Ln x Lo and four Ln x Ln mating pairs (8 eggs total). Additionally, five eggs were chosen from five *L. osakensis* virgins and three eggs from three *L. nigrinus* virgins. Virgins were female beetles that had been kept individually in dishes from the time of emergence so that they did not have the opportunity to mate. Virgin eggs were used as sterile (unfertilized) controls. All eggs used in this experiment were approximately one week old. In March 2012, 11 eggs were collected from Lo x Lo pairs, 10 eggs were collected from Ln x Ln pairs, and 11 eggs were collected from Ln x Lo pairs.

Eggs were placed individually onto a slide and five µl of Prolong Gold with DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) were dropped onto the egg. DAPI fluoresces in contact with DNA (nuclei). A coverslip was placed onto the egg/dye and pressed to break open the egg and release its contents. The slide was then placed in the dark to incubate for 20 min, after which the egg was viewed using a Zeiss Axiovert 200 confocal microscope. We hypothesized that fertilized eggs would exhibit fluorescing nuclei from either sperm or the dividing cells of the growing zygote, while sterile eggs would most likely not.

Mate Choice

A mate choice experiment was conducted to determine whether encounters between *L. nigrinus* and *L. osakensis* would lower their fitness via a decrease in the production of progeny as a result of inviable interspecific mating.

On March 17, 2011, approximately 120 (assumed) virgin *L. nigrinus* were placed together into a feeding container and 120 (assumed) virgin *L. osakensis* beetles were placed into another feeding

container to allow the beetles to mate. The elytra of *L. osakensis* were marked red using non-toxic markers (Opaque Stix) so they could be distinguished later from *L. nigrinus*. On March 24, the beetles were separated into individual dishes and a week later the dishes were checked for eggs in order to distinguish males from females. On April 12, the elytra of *L. nigrinus* were marked with blue so that differential mate choice was not affected by the presence or absence of marker. By this time, it was hoped that females would have oviposited all fertile eggs from intraspecific mating that occurred in the feeding containers. The beetles were then separated into groups of four. Eight groups contained four *L. osakensis* (two ♂ and two ♀), eight contained four *L. nigrinus* (two ♂ and two ♀), and sixteen groups contained two *L. nigrinus* (♂ and ♀) and two *L. osakensis* (♂ and ♀). Four days later, each group of four was placed into a separate 50.8 cm x 76.2 cm sleeve cage enclosing HWA-infested hemlock branches in Blacksburg, VA. The beetles remained in these cages for a week, after which the branches were cut and brought back to the lab. The adults were collected and placed into ETOH for dissection to confirm sex and for genetic analysis to confirm species. The number of HWA ovisacs on each branch was counted and the branches were placed into separate funnels with approximately 25 additional HWA-infested hemlock branches to rear progeny to the prepupal stage. The progeny were collected, counted, and placed into 95-100% ETOH for genetic analysis.

Mate Change

The purpose of this experiment was to determine whether interspecific mating before intraspecific mating negatively affects the genitalia and/or the production of eggs. Sixty F_1 *L. osakensis* were marked with red and 60 F_1 *L. nigrinus* were marked with blue (Sharpie water-based poster paint marker) on January 2, 2012. Two days later, 42 *L. nigrinus* were paired with

42 *L. osakensis*. Sixteen Ln x Ln and 20 Lo x Lo pairs were used as controls. The beetles were kept in 50 x 9 mm Petri dishes with foliage infested with *A. tsugae* adults in a growth chamber at the temperatures and photoperiods shown in Table 2.1. On March 28, 2012, the interspecific pairs (Ln x Lo) were randomly re-mated, intraspecifically, as were the controls (Lo x Lo and Ln x Ln). Each week thereafter, the number of eggs oviposited per mating pair was counted.

All adults used in this experiment were dissected to confirm sex and species and were kept as voucher specimens. The genitalia were removed and mounted on slides to determine whether there was damage. The numbers of eggs produced from the Ln x Ln and Lo x Lo and the re-mated intraspecific crosses (RmLn x Ln and RmLo x Lo) were compared.

Genetic Analysis

After completion of the no-choice lab mating, the mate choice, and the mate change experiments, all adults were identified to species using the partial cytochrome oxidase subunit I (COI) gene (Davis et al. 2011). Adults were identified to species because the *L. osakensis* and *L. nigrinus* colonies are reared in the same facility; therefore it is possible that accidental mixing of species could occur. Additionally, because field collected branches with HWA are used as food; it is possible that wild *L. rubidus* may be present within the colonies.

DNA was extracted using the DNAeasy kit (Qiagen Inc., Valencia, CA). Partial cytochrome oxidase subunit I (COI) was amplified using primers LepF1 and LepR1 (Herbert et al. 2004). PCR was performed in 30 µl reactions containing 3.0 µl 10X PCR Buffer, 2.4 µl dNTPs (10 mM), 4.8 µl $MgCl_2$ (25 mM), 1.0 µl BSA (10 mg/ml), 1.0 µl of each primer (10 mM), 0.3 µl Taq

DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µl DNA template. Thermocycling conditions 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 kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied BioSystems 3730xl automated sequencer. Sequences were aligned using the SeqMan Pro program of Lasergene 8.0 software (DNASTAR; http://www.dnastar.com).

All progeny from the no-choice lab mating, the development, and mate choice experiments were genetically identified using the partial *wingless* gene (Montgomery et al. 2011b). This nuclear gene was used in the place of the mitochondrial COI gene for progeny because it would be able to detect F_1 hybrids, while COI only determines the maternal identity. DNA was extracted using the DNAeasy kit (Qiagen Inc., Valencia, CA). Partial *wingless* was amplified using the primers Wg578 and WgAbR (Wild and Maddison 2008). PCR was performed in 30 µl reactions containing 3.0 µl 5X PCR Buffer, 2.4 µl dNTPs (10 mM), 2.4 µl $MgCl_2$ (25 mM), 1.0µl BSA (10 mg/ml), 1.0 µl of each primer (10 mM), 0.3 µl Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µl DNA template. Thermocycling conditions were 95°C for 5 min followed by 35 cycles of 45 s at 95°C, 45 s at 54°C, and 1 min at 72°C, with a final extension of 72°C for 5 min. Sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied BioSystems 3730xl automated sequencer (Applied Biosystems). Sequences were edited using Sequencer 4.2.2 (Gene Codes Corporation, Ann Arbor, MI) and aligned using MUSCLE 3.6 (Edgar 2004).

Statistical Analysis

No-choice lab mating trials: Fecundity

The mean number of eggs produced per week was calculated for the beetle pairs that survived to the end of the observation period (13 weeks in 2010 and 19 weeks in 2011). The data (mean eggs/week) for the two years were then combined, tested for normality, and analyzed using a 1-way ANOVA followed by multiple comparison test with Fisher's LSD (Zar 2010) to test for significant differences in overall fecundity among the three crosses (Lo x Lo, Ln x Ln, Ln x Lo). JMP Pro 10 and significance level of $\alpha = 0.05$ were used for this and other analyses that follow.

The patterns of oviposition over time for the three crosses (Lo x Lo, Ln x Ln, Ln x Lo) were examined using only the 2011 data because three weeks of data are missing in March 2010 during peak oviposition. The mean number of eggs per pair (Petri dish) was calculated and plotted against time (19 weeks). Differences in the patterns of oviposition were then assessed by fitting the relationship of percent cumulative mean egg production at each week to a Weibull function (Wagner et al. 1984, Dodson 2006).

$$f(x) = 100\left(1 - e^{-(x/\alpha)^{\beta}}\right) \qquad (1)$$

where *f(x)* is the percent cumulative mean number of eggs at each week (x) , α can be interpreted as a rate parameter, which represents the time at which 63.2% of cumulative mean egg production occurred (Dodson 2006), and β describes the shape of the curve. The fit of the data to the Weibull function was carried out by nonlinear least squares regression in TableCurve 5.01 (SYSTAT Software 2002).

The mean length (mm) of eggs per cross was determined from measurements taken in 2010 and 2011. In 2010, more than one egg was measured per dish per week; these measurements were averaged each week for each dish. A Wilcoxon/ Kruskal-Wallace test (Rank Sums) was used to determine if there were significant differences in the length of eggs produced by ♂♀ Lo x Lo, Ln x Ln, and Ln x Lo pairs and ♂♀ and ♀♀ pairs within each cross. If significant, this was followed by multiple comparisons using the Wilcoxon Method.

Chi-square analysis was used to compare the total weekly average number of eggs per dish in 2011 for all ♂♀ and ♀♀ pairs within the three crosses (i.e., ♂♀ vs. ♀♀: Lo x Lo, Ln x Ln, and Ln x Lo) and to compare the total weekly average number of shriveled eggs and non-shriveled eggs, as well as the total weekly average number of eggs found inside and outside of ovisacs per dish between ♂♀ vs. ♀♀ pairs and among the three crosses for all pairs that survived for the entire 19 weeks of the study.

No-choice lab mating trials: Fertility and Viability

Significant differences among the three crosses in the number of eggs that developed and did not develop to prepupae (fertility) were determined using a chi-square analysis. A similar analysis was used to test for significant differences in the number of prepupae from each of the crosses that developed to the adult stage (viability).

Mate Choice

The ratio of the number of larvae per sleeve cage to the number of HWA ovisacs per branch was calculated, and an ANOVA was used to determine if this ratio differed among the three crosses

(Lo x Lo, Ln x Ln, and Ln x Lo). The ratio of larvae to ovisacs was used as the response variable because *Laricobius* spp. appears to vary the number of eggs they oviposit based on the number of HWA ovisacs available (Vieira et al. 2012).

Mate Change

Chi-square analysis was used to compare the total weekly average number of eggs per dish between the remated intra-intraspecific pairs (Lo x Lo and Ln x Ln) and re-mated inter-intraspecific pairs (RmLo x Lo and RmLn x Ln).

2.3 Results

Laricobius osakensis and *L. nigrinus* were observed attempting to mate with each other immediately following pairing. Overall, *Laricobius* spp. did not appear to have any preference when choosing a mate. Not only did different species of *Laricobius* attempt to mate, but males also attempted to mount and copulate with other males.

In 2010, eggs were found in the dishes of mating pairs of Ln x Lo as early as the third week after pairing. In 2011, oviposition began in the first week of observation (December 28, 2010–January 4, 2011).

No-Choice Lab mating trials

Several dishes with beetle pairs from each of the three crosses (Lo x Lo, Ln x Ln, and Ln x Lo) were eliminated from the study in each year. The reasons for removal included death of or accidental loss of one or both adults in the pair, the presence of a ♂♂ pair in the dish as

determined by dissection, the presence of the same species in what was supposed to be an interspecific Ln x Lo cross, or the presence of *L. rubidus*. As a result, 5 Lo x Lo, 11 Ln x Ln, and 8 Ln x Lo pairs were available for analysis in 2010, and 22 Lo x Lo, 18 Ln x Ln, and 24 Ln x Lo pairs in 2011. The dishes from each of the crosses for the two years (2010 and 2011), which were not eliminated, were combined for analysis. There was a significant difference among all three cross treatments, in the mean number of eggs produced per week ($F = 24.63$; $df = 2, 89$; $P < 0.0001$). Lo x Lo pairs produced the most eggs/week with 7.62 (± 0.54 SE), followed by Ln x Ln pairs with 5.03 (± 0.51). The interspecific cross, Ln x Lo, produced the fewest eggs/week with 2.48 (± 0.49). The pattern of egg production over time in 2011 is shown in Figure 2.1A.

Maximum egg production for all three crosses occurred between observation weeks 13 and 15 and was greater for Ln x Ln than Lo x Lo. The overall average egg production per week was significantly greater for Lo x Lo than Ln x Ln or Ln x Lo (Figure 2.1A). The fit of the data to the Weibull function showed that egg production by Lo x Lo occurred at a significantly greater rate than for Ln x Ln and Ln x Lo pairs (Figure 2.1B). Based on the non-overlapping 95% confidence intervals, Lo x Lo pairs produced 63.2% of the cumulative mean number of eggs significantly earlier ($\alpha = 11.77$; 95% CI = 11.47–12.07 weeks) than Ln x Ln pairs ($\alpha = 12.91$; 95% CI = 12.67–13.15 weeks) and Ln x Lo pairs ($\alpha = 13.52$; 95% CI = 13.33–13.72 weeks).

Egg Length

Egg length was significantly different among the three ♂♀ crosses ($\chi^2 = 45.1388$; $df = 2$; $P < 0.0001$). All three crosses were significantly different from one another. The Ln x Ln cross produced the longest eggs, followed by Lo x Lo, then Ln x Lo (Table 2.2). Comparing opposite-

sex to same-sex pairs within a cross type, Lo x Lo ♂♀ had significantly longer eggs than ♀♀ (p=0.0154), while there was no difference in the lengths of eggs produced by Ln x Ln and Ln x Lo ♂♀ and ♀♀ pairs (p = 0.9930 and p = 0.9162, respectively).

♂♀ vs. ♀♀ Pairs

There was a significant difference among the crosses in the total weekly average number of eggs per dish in 2011 (χ^2 = 30.995; df = 2; P < 0.0001). The Lo x Lo and Ln x Ln ♂♀ pairs produced a greater percentage of eggs than their ♀♀ counterparts (56.50: 43.50 and 72.80: 27.20, respectively), while the opposite was true of Ln x Lo (40.75: 59.25) (Figure 2.2).

Shriveled Eggs and Eggs Produced Out of Ovisacs

There was a significant difference in the percent of shriveled vs. non-shriveled eggs found in the intraspecific ♂♀ and ♀♀ dishes (χ^2 = 64.184; df = 1; P < 0.0001, Figure 2.3A). For both ♂♀ and ♀♀ intraspecific pairs, the percent of non-shriveled eggs was greater than that of shriveled eggs (98.35: 1.65 and 92.58: 7.42, respectively), but the ♀♀ pairs did have a greater proportion of shriveled eggs compared with non-shriveled eggs than did the ♂♀ pairs (Figure 2.3A).

There was a significant difference among the three crosses (Lo x Lo, Ln x Ln, and Ln x Lo) in the percent of shriveled eggs and non-shriveled eggs found within the dishes (χ^2 = 101.289, df = 2, P < 0.0001, Figure 2.3B.). The Ln x Ln cross produced the smallest percent of shriveled eggs (10.78) and Ln x Lo the largest (46.71, Figure 2.3B). The Ln x Lo cross produced the smallest percent of non-shriveled eggs (16.53), while Ln x Ln and Lo x Lo pairs produced a similar percent of non-shriveled eggs (36.71 and 46.77, respectively, Figure 2.3B).

There was a significant difference in the percent of eggs found inside vs. outside of the wool in intraspecific ♂♀ and ♀♀ dishes ($\chi^2 = 122.457$; df = 1; $P < 0.0001$, Figure 2.3A). For both ♂♀ and ♀♀ intraspecific pairs, the percent of eggs found inside the wool was greater than that found outside the wool (99.18: 0.82 and 91.93: 8.07, respectively), but a greater proportion of eggs were found outside the wool vs. inside the wool for ♀♀ pairs compared with ♂♀ pairs (Figure 2.3A).

There was a significant difference among the three crosses (Lo x Lo, Ln x Ln, and Ln x Lo) in the percent of eggs found inside vs. outside the woolly HWA ovisacs ($\chi^2 = 259.204$, df = 2, $P < 0.0001$, Figure 2.4B). The Ln x Lo cross oviposited a larger percent of eggs outside of the woolly ovisacs compared with the other two crosses (73.33, Figure 2.4B). The Ln x Lo cross oviposited the lowest percent of eggs inside the ovisacs (16.53), compared with the Ln x Ln and Lo x Lo crosses which oviposited a similar percent of eggs inside the ovisacs (36.71 and 46.77, respectively, Figure 2.4B).

Fertility and Viability

In both 2010 and 2011, prepupae began dropping from rearing funnels in March. Prepupae continued to drop through June for the Lo x Lo and Ln x Ln cross in 2010 and 2011, but stopped at the end of April for the Ln x Lo cross in 2010 and at the end of May in 2011. In 2011, two prepupae that developed from the Ln x Lo cross were placed in ETOH for genetic analysis and were determined to be pure *L. osakensis*.

There was a significant difference among the three crosses in the proportion of prepupae that emerged from eggs ($\chi^2 = 2685.592$, df = 2, P< 0.0001; Figure 2.5A). Lo x Lo and Ln x Ln had a significantly greater proportion of prepupae emerge from eggs than Ln x Lo (51.54, 45.71, and 2.75, respectively). The percent of prepupae that did not develop was similar for the Ln x Lo and Lo x Lo cross, while the Ln x Ln cross had the lowest percent of undeveloped prepupae (41.48, 34.38, and 24.14, respectively, Figure 2.5A).

In 2010, adult emergence began with the emergence of one adult beetle from the Ln x Lo cross on September 13. Emergence of adults continued through January 2011. In 2011, emergence of adult beetles began with the emergence of two adults from the Ln x Ln cross and one adult from the Lo x Lo cross on October 24. Emergence of adults continued through December 2011.

There was a significant difference among the three crosses in the percent of adults that developed/ did not develop from prepupae ($\chi^2 = 8.340$, df = 2, P = 0.0155; Figure 2.5B). Lo x Lo and Ln x Ln had a greater emergence of adults from prepupae than Ln x Lo (50.79: 47.54: 1.67, respectively), but also had the greater percent of undeveloped adults compared with Ln x Ln and Ln x Lo (52.42: 44.52: 3.06, respectively, Figure 2.5B).

Fifteen of the 16 adult beetles that emerged from the Ln x Lo cross were genetically analyzed; eight were found to be pure *L. osakensis* and seven pure *L. nigrinus.* No hybrids were found.

Development Study

None of the eggs (59 total) from the Ln x Lo cross hatched in 2010, 2011, or 2012 (Table 2.3). It was observed that eggs that did not hatch shriveled over time. Sixty- four percent of Lo x Lo and 75% of Ln x Ln eggs hatched in 2010, 2011, and 2012 and 29% of eggs from both crosses developed to the prepupal stage (Table 2.3).

Fluorescence Study

Nine out of 11 Lo x Lo eggs were from ♂♀ pairs; 3 were replications from the same dish, all nine fluoresced nuclei. The other two eggs were ♀♀ pairs and did not fluoresce nuclei.

Eleven of the 14 Ln x Ln eggs were from ♂♀ pairs; three were replications from the same dish, all 11 fluoresced nuclei (example, Figure 2.6A). The other three eggs were from ♀♀ pairs, one fluoresced nuclei and the other two did not.

Of the 15 Ln x Lo eggs, two adults from two separate dishes could not be identified; so 3 eggs, 2 from one dish, and another from a separate dish, were dropped from the analysis. Four eggs were from ♂♀ pairs, 2 of which were replications from the same dish and 8 eggs were from ♀♀ pairs. None of these eggs fluoresced nuclei (example, Figure 2.6B).

None of the virgin eggs from *L. nigrinus* (3 eggs) or *L. osakensis* (5 eggs) fluoresced nuclei.

Mate choice

No significant difference was found in the ratio of larvae to ovisacs among the three crosses ($F = 0.9325$, $df = 2$, $P = 0.4051$). A sample of 118 larvae from the Ln x Lo cross were identified

using genetic analysis; none were found to be hybrids. Forty-three were identified as *L. nigrinus*, and 75 as *L. osakensis*.

Mate Change

By the end of this experiment, six Lo x Lo (intra-intraspecific), four Ln x Ln (intra-intraspecific) four RmLo x Lo (inter-intraspecific), and three RmLn x Ln (inter-intraspecific) were included in statistical analysis due to mortality. Three weeks (March 28 through April 18) of egg count data were available for analysis for the same reason. There was a significant difference between the intraspecific and remated crosses ($\chi^2 = 4.570$, df = 1, P = 0.0325) in the total weekly average number of eggs per dish. The Lo x Lo remated cross produced a greater percent of eggs than the intraspecific cross (64.06: 35.94, Figure 2.7). The Ln x Ln cross showed the opposite trend, with the intraspecific cross producing a much greater percent of eggs than the inter-intraspecific remated cross (87.1: 12.9, Figure 2.7). There was no evidence of broken or torn genitalia in the male specimens that had been remated. Damage was observed in the female samples, but this probably occurred during dissection, rather than as a result of mating.

2.4 Discussion

Fitness

The interspecific ♂♀ cross produced fewer eggs, prepupae, and adults, and produced the smallest eggs compared with the intraspecific ♂♀ crosses. It is likely that the eggs produced by interspecific ♂♀ mating pairs were not fertilized, and the prepupae and adults were not hybrid offspring, but were the result of contamination. This is based on several observations. First, interspecific ♀♀ pairs produced more eggs than interspecific ♂♀ pairs, while the opposite is true

of the intraspecific pairs (Figure 2.2). More eggs would be produced by interspecific ♀♀ pairs than the ♂♀ interspecific pairs because there are two females producing sterile eggs, instead of one. For the intraspecific pairs, there would be more eggs in the ♂♀ dishes than in the ♀♀ dishes, because the ♂♀ mating pairs are producing fertile eggs. Females of some insect species that produce sterile eggs, greatly reduce their oviposition rate until after they have copulated (Ridley 1988). Once copulated, products from male accessory glands and testes can act as allohormones, increasing female oviposition rates (Brent et al. 2011).

Second, the presence of eggs oviposited outside the ovisacs that shriveled over time led to the suspicion that these eggs were probably sterile. The fact that a greater proportion of shriveled to non-shriveled eggs and eggs oviposited outside versus inside of ovisacs was produced by ♀♀ pairs compared with ♂♀ pairs may support this hypothesis. Interspecific ♂♀ pairs produced the lowest percent of non-shriveled eggs and a greater percent of eggs oviposited outside of ovisacs than the intraspecific ♂♀ pairs (Figures 2.3B and 2.4B).

Third, none of the prepupae or adults produced by the interspecific cross was found to be hybrids. This raises the question: why were non-hybrid prepupae and adults found in the Ln x Lo crosses? Initially, it was thought that these prepupae were wild *L. rubidus* that had been brought in on HWA-infested branches, but none of the progeny was identified as *L. rubidus*. In 2010, there were two accidental intraspecific crosses among the interspecific pairs identified with genetic analysis: one with two *L. osakensis,* and one with two *L. nigrinus*. It is possible that the prepupae that developed from the Ln x Lo cross in 2010 were from the accidental Lo x Lo cross, since the prepupae turned out to be *L. osakensis*. However, a greater number of prepupae

dropped from the Ln x Lo funnels in 2011 and there were no accidental intraspecific crosses found within the interspecific crosses that year. Although we did not analyze any of the prepupae from 2011 (these went directly into the soil container to rear to the adult stage and the adults that emerged were then analyzed), we suspect that *Laricobius* spp. mate as soon as they make contact with one another following emergence from the soil. On one occasion, two *L. nigrinus* were observed mating in a soil container directly following emergence. Although these two beetles were not used in our mating experiments, other pre-mated adults may have inadvertently been included. Because *Laricobius* females can store sperm following copulation, the offspring resulting from interspecific crosses may be from intraspecific mating that occurred in the soil containers before being used in experiments. If this is the case, it suggests that *Laricobius* spp. females can hold sperm in their spermatheca for at least two months, since the first prepupae from these crosses were collected in March 2011.

Finally, no eggs from interspecific pairs in the development study hatched in 2010, 2011, or 2012, while those from the intraspecific crosses did.

The reason why *Laricobius* spp. produce sterile eggs is unknown. It is possible that sterile eggs are oviposited after a certain amount of time has passed without fertilization. Or perhaps sterile eggs are produced as an extra food source for fertile eggs that hatch into larvae (Downes 1988) or for other predators in order to protect the fertile eggs hidden within the ovisacs.

Although ♂♀ Lo x Lo mating pairs produced the most eggs, the Ln x Ln cross had the highest percent development from egg to prepupae and lowest percent of undeveloped eggs (Figure

2.5A). Additionally, Ln x Ln ♀♀ pairs produced the lowest percent of non-fertile eggs in relation to their ♂♀ counterparts than did the Lo x Lo and Ln x Lo pairs (Figure 2.2). Finally, ♂♀ Ln x Ln produced the lowest percent of shriveled eggs and eggs oviposited outside of the HWA ovisacs than did the ♂♀ Lo x Lo and Ln x Lo pairs (Figure 2.3B and 2.4B). These results suggest that *L. osakensis* produced a greater proportion of sterile eggs to fertile eggs than *L. nigrinus*. It is unknown whether this result is due to lab conditions or is universally true of the species, but could be determined using field studies.

Fluorescence

The lack of stained nuclei in the virgin eggs and the presence of nuclei fluorescing in mated *Laricobius* spp. eggs suggest that these nuclei are from the dividing cells of zygotes. We found that all 11 eggs from Ln x Ln, and all 9 eggs from Lo x Lo fluoresced nuclei, while the 4 eggs from Ln x Lo did not. There was one ♀♀ Ln x Ln egg that fluoresced nuclei. It is likely that this egg was either from *L. rubidus* brought in on the hemlock branches, or a fertilized egg that occurred as a result of mating inside of the soil containers. These data provide further evidence that the eggs found in the Ln x Lo dishes are sterile and have not been fertilized.

Mate Choice

The results of the mate choice test suggest that there is no fitness cost as a result of a lower production of progeny when *L. osakensis* and *L. nigrinus* occur together. However, there were a number of problems with this experiment particular to this study system that may render these results preliminary.

For the experiment, it was necessary to determine which beetles were male and female prior to placement of two males and two females together in the sleeve cage. The probability of choosing two males and females correctly, however, was much less than for the no-choice test. Because sex of the adults could not be determined *a priori* without potentially causing harm to their reproductive organs, the beetles were mated before the experiment and separated into individual dishes. They were then checked for eggs to determine which were males and females. Female *Laricobius* spp., however, can store sperm. Three weeks was probably not long enough for females to rid themselves of sperm in the spermatheca from previous intraspecific mating. Therefore, the progeny from these crosses could have come from intraspecific mating that took place prior to the experiment. However, it is likely that interspecific copulation took place in the sleeve cages. What these data suggest is that no fitness costs are likely from interspecific copulation due to sperm replacement (sperm from a different *Laricobius* spp. replacing sperm from a prior intraspecific encounter) or through damage to the reproductive organs.

Mate Change

The mean number of eggs produced by RmLn x Ln was relatively low compared with the intraspecific Ln x Ln control (87.1: 12.9, Figure 2.7). This may suggest a fitness cost to *L. nigrinus* following copulation with *L. osakensis*.

Conclusions

We found no evidence that *L. osakensis* and *L. nigrinus* can hybridize. This is consistent with genetic evidence suggesting that *L. osakensis* is in a separate clad with other Asian *Laricobius* species and is not closely related to either *L. nigrinus* or *L. rubidus* (Montgomery et al. 2011b).

Whether there are fitness costs when *L. osakensis* and *L. nigrinus* mate interspecifically is not completely clear and may need to be investigated further.

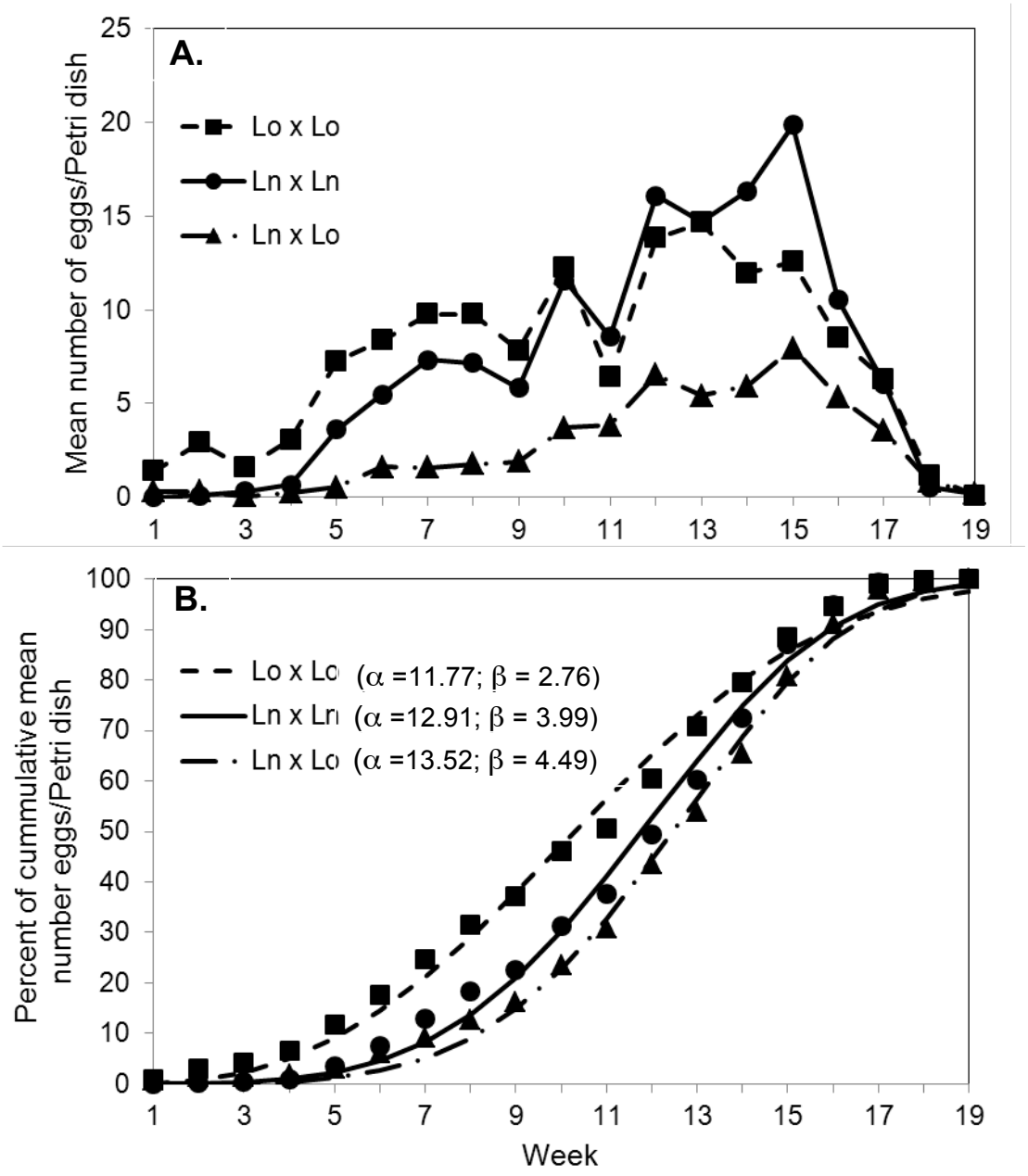

Figure 2. 1. Oviposition patterns in a no-choice lab mating study for three crosses of *Laricobius spp.* during 19 weeks of observation in 2011. (**A.**) Mean number of eggs per dish (per pair) over time; (**B.**) Relationship between percent cumulative mean number of eggs per dish and week (symbols) and fit of the data to the Weibull function, $f(x) = 100\left(1 - e^{-(x/\alpha)^{\beta}}\right)$ (lines). Lo is *Laricobius osakensis*; Ln is *L. nigrinus*.

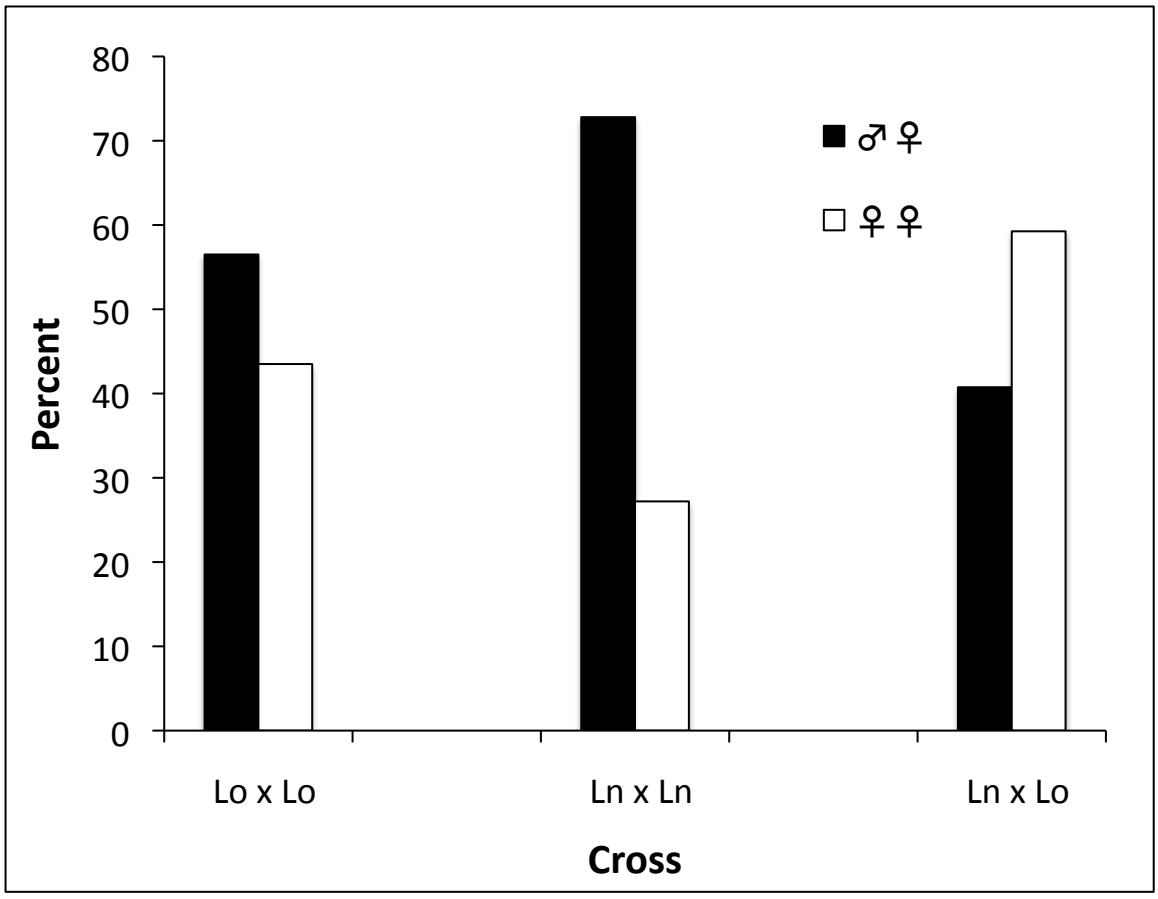

Figure 2. 2. Results of contingency analysis showing the percent of the total weekly average number of eggs produced per Petri dish between ♂♀ and ♀♀ pairs of the following three crosses: *L. osakensis* x *L. osakensis* (Lo x Lo), *L. nigrinus* x *L. nigrinus* (Ln x Ln), and *L. nigrinus* x *L. osakensis* (Ln x Lo).

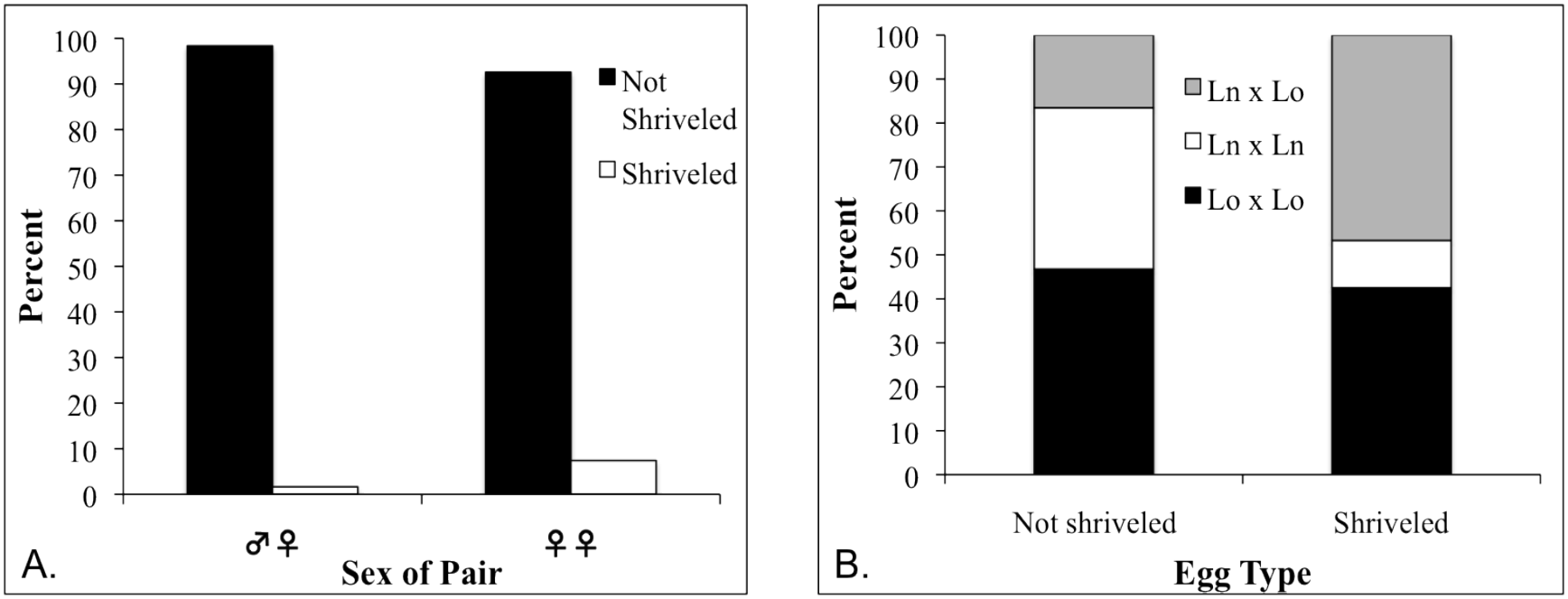

Figure 2. 3. Results of contingency analysis showing the percent of **(A.)** shriveled eggs between ♂♀ and ♀♀ Lo x Lo and Ln x Ln pairs and **(B.)** the percent of non-shriveled and shriveled eggs produced among three crosses; *L. osakensis* x *L. osakensis* (Lo x Lo), *L. nigrinus* x *L. nigrinus* (Ln x Ln), and *L. nigrinus* x *L. osakensis* (Ln x Lo).

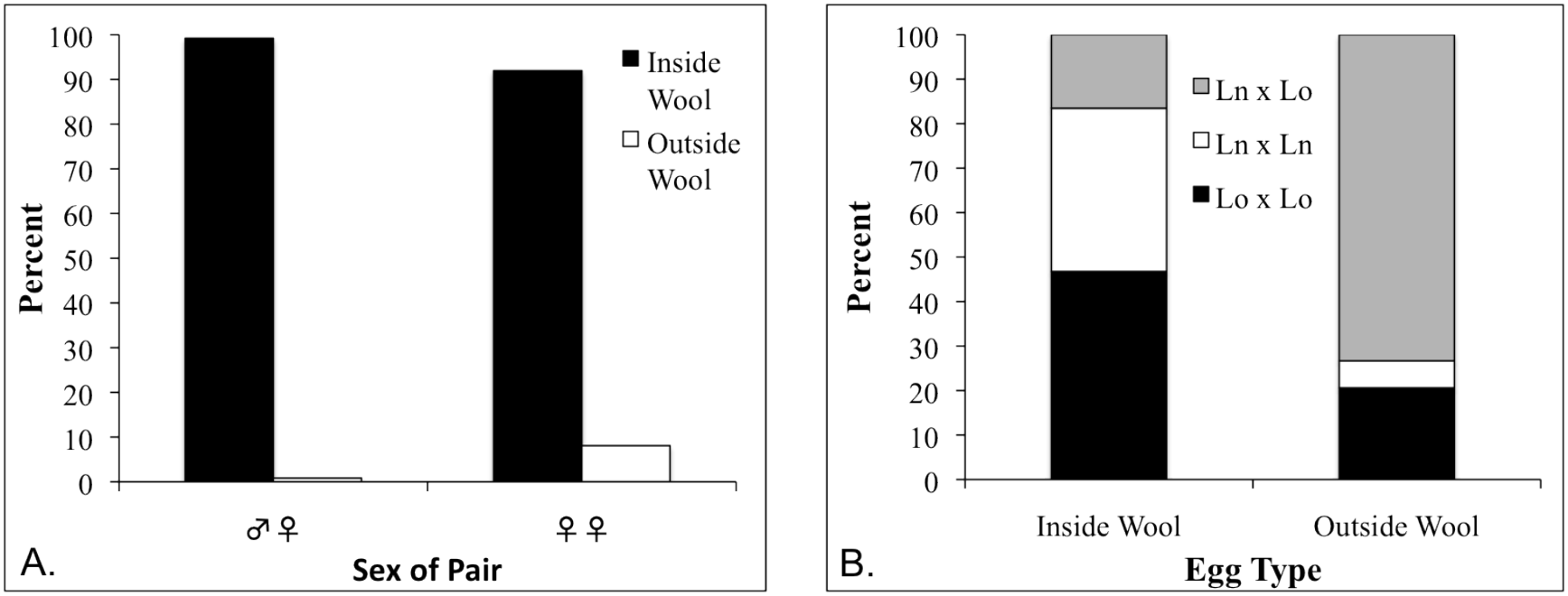

Figure 2. 4. Results of contingency analysis showing the percent of eggs found within and outside of hemlock woolly adelgid ovisacs **(A.)** between ♂♀ and ♀♀ Lo x Lo and Ln x Ln pairs and **(B.)** among three ♂♀ crosses; *L. osakensis* x *L. osakensis* (Lo x Lo), *L. nigrinus* x *L. nigrinus* (Ln x Ln), and *L. nigrinus* x *L. osakensis* (Ln x Lo).

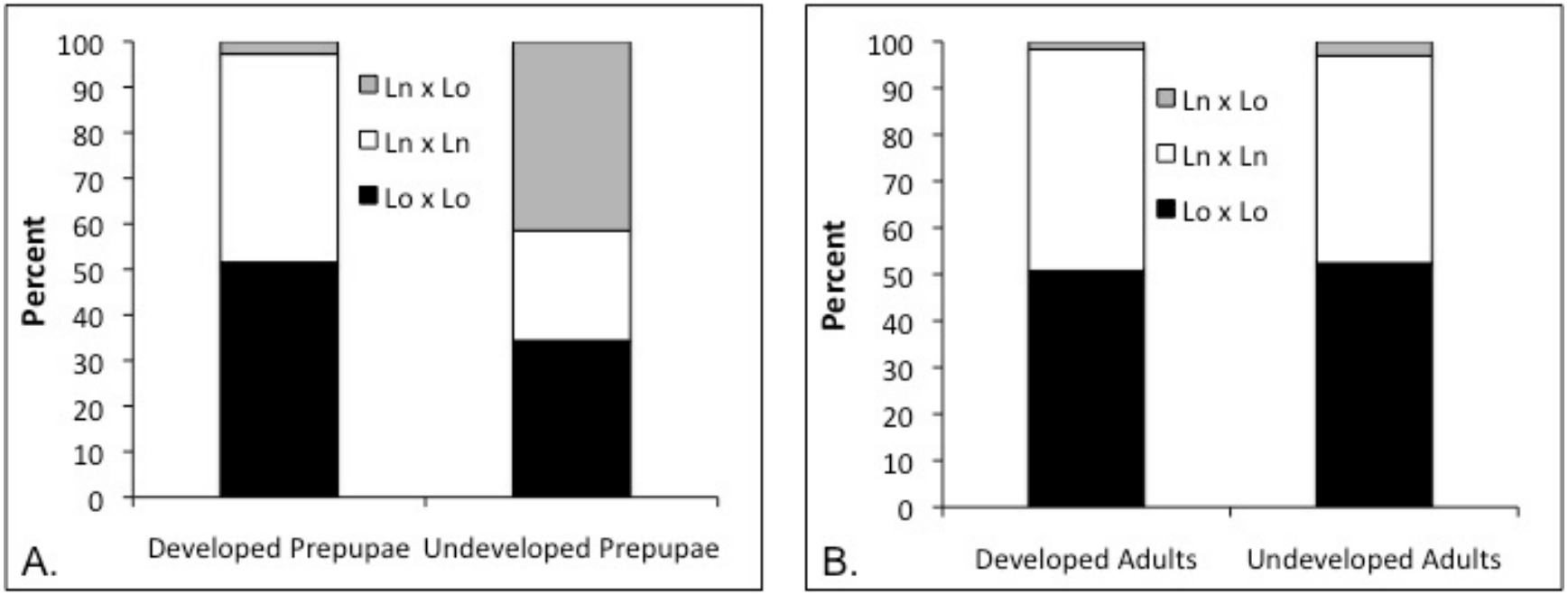

Figure 2. 5. **(A.)** Percent development of prepupae from eggs and **(B.)** the percent development of adults from prepupae among three crosses: *L. osakensis* x *L. osakensis* (Lo x Lo), *L. nigrinus* x *L. nigrinus* (Ln x Ln), and *L. nigrinus* x *L. osakensis* (Ln x Lo).

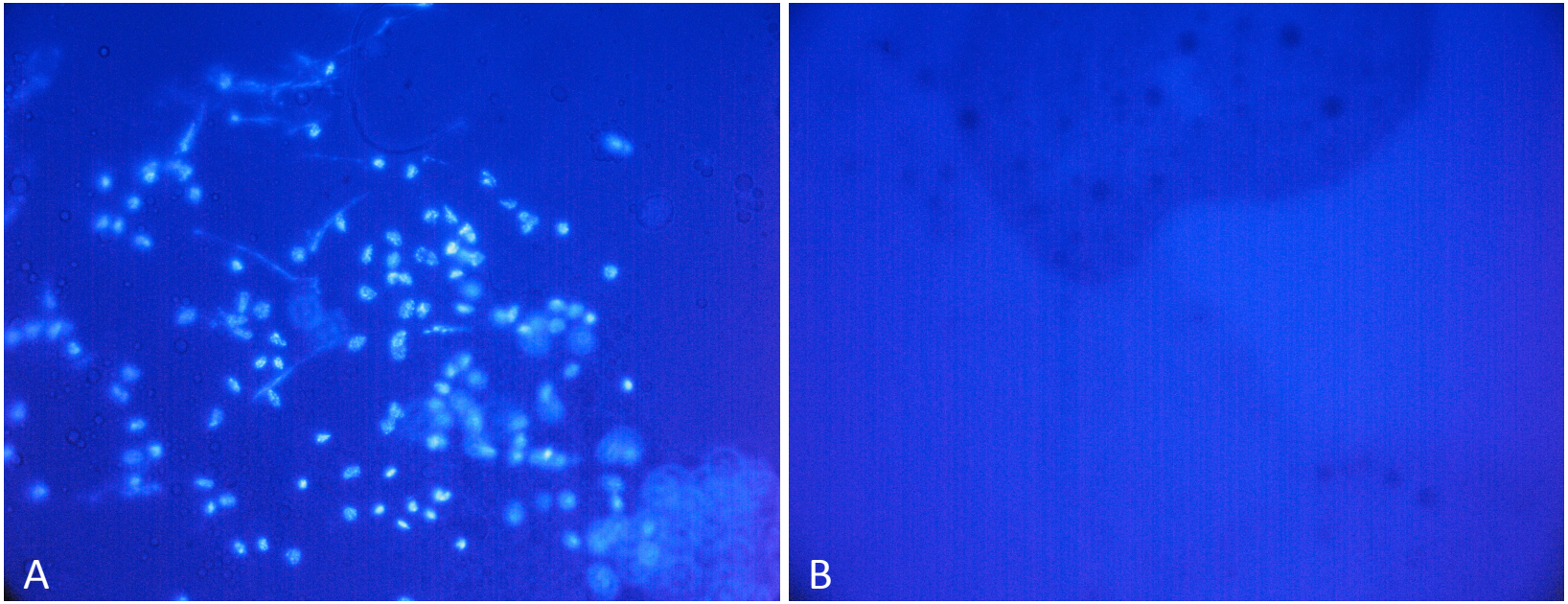

Figure 2. 6. Content of an egg produced by **(A.)** a mating pair of *L.* nigrinus and **(B.)** a mating pair of *L. nigrinus* and *L. osakensis* Eggs were stained with Prolong Gold with DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) to fluoresce nuclei.

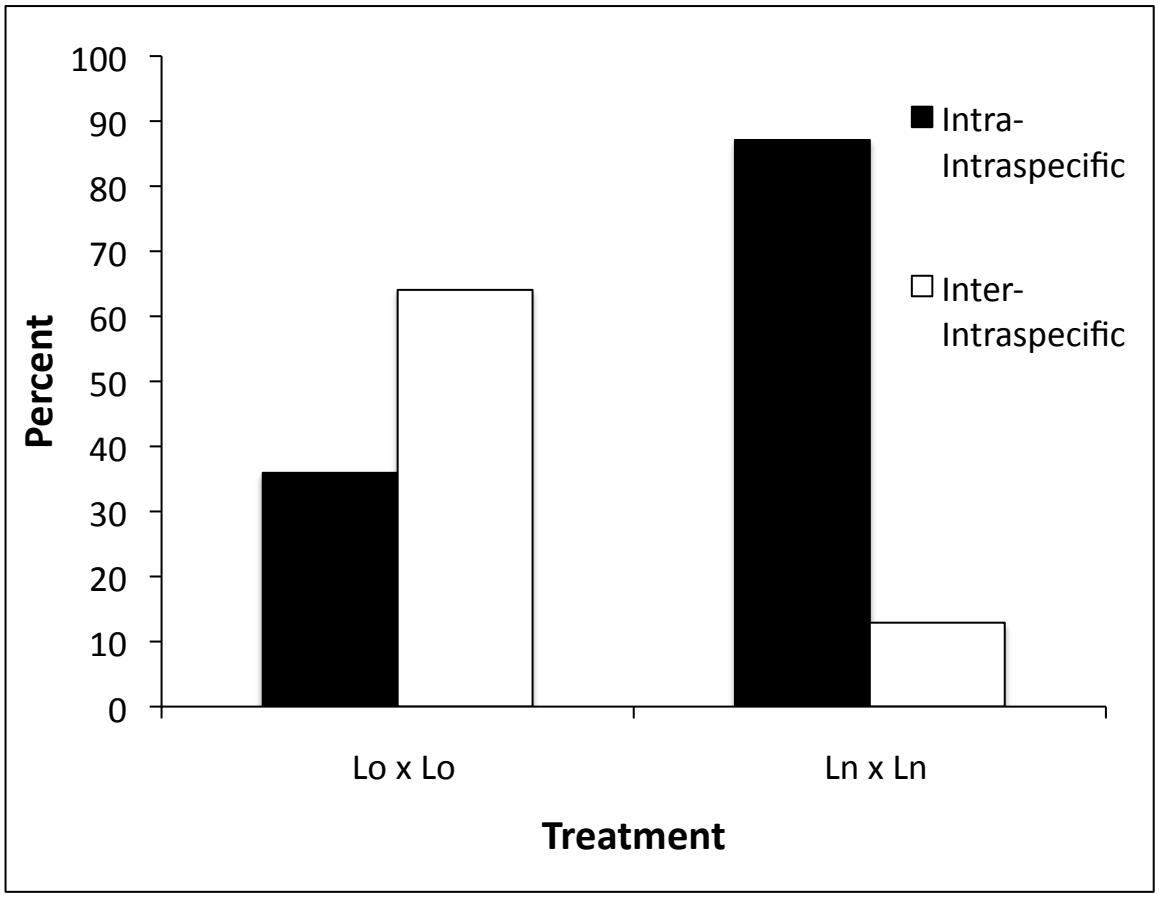

Figure 2. 7. Results of contingency analysis showing percent of eggs produced by *L. osakensis* x *L. osakensis* (Lo x Lo) and *L. nigrinus* x *L. nigrinus* (Ln x Ln) mating pairs that had previously been mated interspecifically (Inter- to Intraspecific) vs. the percent of eggs produced by Lo x Lo and Ln x Ln pairs that had been previously mated intraspecifically (Intraspecific).

Table 2. 1. Temperature and photoperiod used in the 2010 and 2011 no-choice mating studies using *Laricobius* spp. (Salom et al. 2012).

	Adults	
	Temp ˚C (D/N)	Photoperiod (L: D) h
October	6/4	10.5: 13.5
December	4/3	9.5: 14.5
January	4/3	10:14
February	4/3	11:13
March 1st	6/4	12:12
March 15th	8/5	12:12
April 1st	10/6	12:12
May 1st	10/8	12:12
	Larvae/ Prepupae	
March	13/13	12:12
May	19/19	12:12
October	13/13	12:12

Table 2. 2. Average length of eggs (±SE, mm) of different sex pairs (♂♀) and same sex pairs (♀♀) showing significant differences among the crosses (♂♀ pairs only) in lowercase and significant differences between ♂♀ and ♀♀ pairs within each cross in uppercase (Wilcoxon Method of Multiple Comparisons). Lo is *L. osakensis*; Ln is *L. nigrinus*.

	♂♀ (mm)	♀♀ (mm)
Lo x Lo	0.4503 ± 0.0018 Aa	0.4420 ± 0.0057 B
Ln x Ln	0.4578 ± 0.0022 Ab	0.4606 ± 0.0031 A
Ln x Lo	0.4387 ± 0.0019 Ac	0.4399 ± 0.0022 A

Table 2. 3. The number of eggs and the percent that developed into larvae and prepupae for three crosses: *L. osakensis* x *L. osakensis* (Lo x Lo), *L. nigrinus* x *L. nigrinus* (Ln x Ln), and *L. nigrinus* x *L. osakensis* (Ln x Lo) in 2010, 2011, and 2012. *n* = the number of ♂♀ mating pairs that provided eggs for the development study in 2010, 2011, and 2012 for each cross.

Year	Cross	n	No. of Eggs	No. of Larvae (%)	No. of Prepupae (%)
2010	Lo x Lo	8	20	17 (85)	12 (60)
	Ln x Ln	7	8	5 (60)	3 (40)
	Ln x Lo	8	20	0 (0)	0 (0)
2011	Lo x Lo	17	17	6 (35)	4 (24)
	Ln x Ln	18	18	13 (72)	7 (39)
	Ln x Lo	27	27	0 (0)	0 (0)
2012	Lo x Lo	7	36	24 (67)	5 (14)
	Ln x Ln	4	23	19 (83)	4 (17)
	Ln x Lo	4	12	0 (0)	0 (0)
Total	Lo x Lo	32	73	47 (64)	21 (29)
	Ln x Ln	29	49	37 (75)	14 (29)
	Ln x Lo	39	59	0 (0)	0 (0)

Chapter 3. Assessment of hybridization between Laricobius nigrinus *and* Laricobius rubidus *in the lab.*

3.1 Introduction

Laricobius nigrinus Fender, a predatory beetle native to western North America, was released as a biological control agent of the hemlock woolly adelgid (*Adelges tsugae* Annand; hereafter HWA) in the eastern United States. *Laricobius rubidus* LeConte is a native predator of the pine bark adelgid (*Pineus strobi* Hartig; hereafter PBA). Recently it was discovered that *L. nigrinus* and *L. rubidus* could mate and produce hybrid progeny (Havill et al. 2012).

Sometimes progeny from crosses between two species are less fit (Burke and Arnold 2001). If the hybrid progeny of *L. nigrinus* x *L. rubidus* are less fit, biological control of HWA using *L. nigrinus* could be affected through lower reproductive potential. It is important for a biological control agent to be fit and capable of reaching its reproductive potential. Especially in the case of *Laricobius* spp., since HWA is an asexual insect with two generations per year and as such, has a very high reproductive rate.

Laricobius nigrinus prefers HWA and the main host of *L. rubidus* is PBA; therefore the hybrid progeny may have a preference for one of these two adelgid species, or perhaps no preference. If the hybrids were to prefer HWA, this would be advantageous for biological control, but could increase populations of PBA and therefore increase damage to eastern white pine. Changes in PBA populations would depend on whether *L. rubidus* maintains its genetic identity as a pure species and/or the efficacy of other natural enemies in controlling PBA populations, such as

Leucopis spp. (Diptera: Chamaemyiidae). If hybrids prefer PBA, this could have an effect on biological control of HWA and/or an effect on other native enemies of PBA, depending upon the extent of hybridization. If the hybrids have no preference, this would be advantageous for biological control since the beetles would most likely exploit the vast HWA resource available.

The purpose of this study was to determine the fitness and host preference of *L. nigrinus* x *L. rubidus* hybrid progeny. Fitness comparisons between intraspecific and interspecific crosses included number of eggs, prepupae, and adults produced per cross. Additionally, the development and morphology of parental species and their hybrids was compared. This information may help determine how hybridization will affect the biological control of HWA using *L. nigrinus*, the future of *L. nigrinus* as a biological control agent, and also, the potential effects to PBA, its native enemies, and eastern white pine.

3.2 Methods

To determine the fitness of *L. nigrinus* x *L. rubidus* hybrids, no-choice lab mating experiments were carried out in 2011 and 2012. Treatments consisted of pairing: *L. nigrinus* x *L. nigrinus* (Ln x Ln), *L. rubidus* x *L. rubidus* (Lr x Lr), and *L. nigrinus* x *L. rubidus* (Ln x Lr). The fitness components measured were fecundity: number of eggs produced per cross, fertility: the number of prepupae produced per cross, and viability: the number of adults produced per cross.

Spring 2011

Laricobius nigrinus were collected from Seattle, WA and *L. rubidus* were collected from Dismal Falls, VA. The sex of adult beetles can be determined by either squeezing their abdomen so that

the genitals are extruded (Shepherd et al. 2011) or by removing prepupae from the soil when the genitalia are visible. It has not been determined whether the former method affects the reproductive system negatively therefore this method was not used, nor was the second due to the potential for high mortality (Salom and Lamb 2009). For this reason, the beetles used in this experiment were not sexed prior to pairing.

In February 2011, 15 Ln x Ln, 15 Lr x Lr, and 30 Ln x Lr pairs were formed. Pairs were kept in 50 x 9 mm polystyrene Petri dishes with two-cm ventilation holes cut into the top and covered with polyester mesh. The dishes were kept in a growth chamber at the temperatures and photoperiod shown in Table 2.1. *Laricobius nigrinus* pairs were placed into dishes with fresh HWA on hemlock collected weekly, and mating pairs of *L. rubidus*, and *L. nigrinus* x *L. rubidus* were placed into dishes with fresh HWA on hemlock and fresh PBA on eastern white pine collected weekly.

Data collection began two weeks after pairing (March 2011). This delay was necessary to allow *Laricobius* females to oviposit some or all of their fertilized eggs prior to data collection in case they had any sperm stored.

Food was replaced weekly and the number of *Laricobius* eggs in each dish was counted. The length of one egg per dish was measured each week using a dissecting microscope with an ocular scale. All eggs were reared in funnels to the prepupal stage (150 eggs per funnel) [for rearing methods see Salom et al. (2012)]. Funnels for the Ln x Ln cross contained approximately 100 thirty-cm long HWA-infested hemlock branches, while funnels for the Lr x Lr and Ln x Lr cross

contained approximately 50 thirty-cm long HWA-infested hemlock branches and 50 thirty-cm long PBA-infested white pine branches. Prepupae that developed were counted and placed in soil containers to rear to the adult stage (50 prepupae per soil container). Adults that emerged the following fall were counted and their date of emergence recorded. Adults that emerged were placed into individual dishes so that they would remain virgins for backcrossing experiments.

At the end of the experiments, all adults were placed into separate microcentrifuge tubes with 95-100% ETOH. If a beetle died during the experiment, the beetle and its mate were placed into separate microcentrifuge tubes with 95-100% ETOH. Adults were dissected; the genitalia were mounted onto slides, heads and elytra were kept as voucher specimens, and the remaining parts were used for genetic analysis to confirm species.

Spring 2012

Wild-caught *L. nigrinus* and *L. rubidus* were again used in the 2012 no-choice lab mating experiments. By the end of the spring 2011 season it was determined that *Laricobius* spp. did indeed have a spermatheca (personal observation) and could store sperm for at least 1.5 months (Ligia Cota Vieira, personal communication). For this reason, beetles used for this experiment were collected and paired earlier in the year. The hope was that copulation would not occur until spring, but if it did occur, females would have time to oviposit any fertilized eggs from these interactions prior to the start of data collection.

Fifty-seven *L. rubidus* were collected from Dismal Falls, VA and 108 *L. nigrinus* were collected from Seattle, WA in December 2011. Pairing began in January 2012, resulting in 10 Ln x Ln, 10

Lr x Lr, and 23 Ln x Lr pairs. Fitness measurements began a week after pairing. Beetles in this experiment were reared and fitness measurements were collected as in the previous year.

Development Test

To determine whether there was a difference in the development time of hybrids compared with the parental species, individual eggs were followed from egg hatch until the prepupal stage. Eggs were collected from pairs in the no-choice lab mating experiments in 2011 and 2012. In 2011, 8 eggs were collected from eight different Ln x Ln pairs, nine eggs were collected from nine different Lr x Lr pairs, and 11 eggs were collected from 11 different Ln x Lr pairs. In 2012, 14 eggs from three Ln x Ln pairs, three eggs from one Lr x Lr pair, and 14 eggs from four Ln x Lr pairs were collected.

All eggs were placed individually on top of an HWA ovisac located on a 2-cm hemlock branch in 50 x 9 mm polystyrene Petri dishes with ventilation holes cut into the top and covered with polyester mesh. The dishes were kept in a growth chamber at 13°C, 12:12 L: D h and checked daily for hatch. The date of hatch was recorded and additional HWA and PBA were added as needed for food. The prepupal stage was determined when the larvae began searching the bottom of the dish for a pupation site (Zilahi-Balogh et al. 2003a); this date was also recorded. All larvae and prepupae that developed were placed into separate microcentrifuge tubes with 95-100% ETOH to determine species and/or hybrid through genetic analysis.

To measure and compare head capsule widths and larval lengths, additional eggs were collected from the mating pairs in the no-choice mating experiments. Larvae that hatched from the eggs

used in the development study were not used for this measurement because prior experimentation found that the larvae do not survive well when manipulated (Heather Story, personal communication). The head capsule widths and lengths of 20 larvae from each instar per cross were measured if available. All larvae were placed in 95-100% ETOH for genetic analysis following measurement.

Host Preference

To determine the host preference of *L. nigrinus* x *L. rubidus* hybrid progeny, a choice host preference test was attempted in the spring of 2011. F_1 adults that emerged from collections of wild-caught *Laricobius* larvae the previous spring at Middle Creek, NC were used for this experiment. Beetles from NC were used because there was a large emergence (203 adults) and hybridization was known to already be occurring at this site. These beetles were kept in feeding containers (50 beetles per container) with a mixture of approximately 25 HWA-infested hemlock and 25 PBA-infested white pine branches.

The beetles were separated into individual dishes with both HWA-infested hemlock and PBA-infested white pine on March 23, 2011. On March 30th, the branches within the individual dishes were removed and the sex of the beetles was determined based upon the presence or absence of eggs in the ovisacs of HWA and/or PBA. The elytra of all beetles identified as females were painted with a non-toxic marker (Opaque Stix). All beetles were placed back together into feeding containers to ensure that the females were mated and ovipositing during the host preference test.

On April 13th, 65 females were placed into individual dishes for choice tests (HWA and PBA). Each dish contained a sprig of hemlock with approximately 20 HWA ovisacs and a square of white pine bark containing approximately 20 PBA ovisacs. White pine tips infested with PBA were not used for this experiment since tips rarely contained more than five PBA individuals. The females were placed in the center of the dish, between the HWA-infested hemlock and the PBA-infested white pine. Beetles were kept in a growth chamber at 13°C, 12:12 L: D h for one week. After a week, the dishes were opened and the location of the beetle was recorded (found on hemlock, white pine, or Petri dish). Beetles were placed into 95-100% ETOH for dissection to confirm sex and to determine species/hybrid through genetic analysis. The number of eggs oviposited on or within the HWA and PBA ovisacs was counted in each dish.

Morphological Characteristics

Progeny from the no-choice lab mating experiment in 2011 were used to determine if lab-reared *L. nigrinus* x *L. rubidus* hybrids would have intermediate features (paramere angles and elytra coloration) as was found for wild-caught hybrids in a previous study (Havill et al. 2012). All adult progeny were dissected, the elytra were kept in separate glass vials with 95-100% ETOH and the genitals were mounted onto slides. A DinoXcope microscope was used to view the genitalia and photographed. The photographs were opened in the program Image J (Abramoff et al. 2004) and the angles of the parameres were measured on each side using the angle tool. The two measurements were averaged for each sample.

Photographs were taken of one elytron per sample using a DinoXcope microscope. The pictures were opened in Image J and the proportion of black on each elytron was determined as described in Havill et al. (2012).

Genetic Analysis

The identities of the *L. rubidus* adults used in experimentation and larvae produced by Ln x Lr crosses were determined using six nuclear microsatellite loci (LaGT01, LaCA04, LaGT07, LaGT13, LaCA14, LaCA16) (Klein et al. 2010; Havill et al. 2012). Microsatellite loci were amplified using the conditions described in (Klein et al. 2010). Fragment analysis was completed at the DNA Analysis Facility on Science Hill at Yale University in New Haven, CT using a 3730xl 96-capillary genetic analyzer. Genotypes were scored using Genemapper 4.0 (Applied Biosystems). The programs Structure 2.3.2 (Pritchard et al. 2000) and NewHybrids 1.1 (Anderson and Thompson 2002) were used to distinguish hybrids from parent species. Structure runs used 20,000 burn-in iterations followed by 100,000 sample iterations and NewHybrid runs were completed with 10,000 burn-in iterations followed by 100,000 sample iterations (Havill et al. 2012). For NewHybrids, the results of all four potential classes of hybrid were summed to obtain the probability of assignment as a hybrid. The results of five independent runs were averaged for both Structure and NewHybrids. Each larva was assigned to a parental species if Structure analysis resulted in $q > 0.80$, or as a hybrid if $0.20 < q < 0.80$. If the category with the highest probability of assignment from NewHybrids agreed with Structure, that assignment was retained. If they did not agree, the specimen was assigned to the category with the higher probability of the two analyses (Havill et al. 2012).

Laricobius nigrinus adults used in experimentation were identified using the partial cytochrome oxidase subunit I (COI) gene (Davis et al. 2011). DNA was extracted using the DNAeasy kit (Qiagen Inc., Valencia, CA). Partial cytochrome oxidase subunit I (COI) was amplified using primers LepF1 and LepR1 (Herbert et al. 2004). PCR was performed in 30 µl reactions containing 3.0 µl 10X PCR Buffer, 2.4 µl dNTPs (10 mM), 4.8 µl $MgCl_2$ (25 mM), 1.0 µl BSA (10 mg/ml), 1.0 µl of each primer (10 mM), 0.3 µl Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µl DNA template. Thermocycling conditions 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 kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied BioSystems 3730xl automated sequencer. Sequences were aligned using the SeqMan Pro program of Lasergene 8.0 software (DNASTAR; http://www.dnastar.com).

Statistics

For the following analyses, JMP Pro 10 and an alpha of 0.05 were used when relevant.

No-choice lab mating trials: Fecundity, Fertility, and Viability

The length (mm) of eggs was compared among the crosses using the Wilcoxon/ Kruskal Wallace test (Rank Sums) (Ott and Longnecker 2001). If significant differences were found, the Wilcoxon Method of multiple comparisons was used to determine if the length of eggs was significantly different among all three crosses.

Development Study

The length of time (days) it took for progeny to develop from egg to the prepupal stage was compared among the crosses using ANOVA. To compare head capsule widths and larval lengths, a Hierarchical cluster analysis using the Ward method was run to separate the measurements into four categories (representing the four instars). The number of clusters was not set prior to the analysis. A Wilcoxon/ Kruskal Wallace test (Rank Sums) was run to compare the measurements among the crosses for each instar. If significant differences were found, the Wilcoxon Method of multiple comparisons was run to determine where the differences lie.

Morphological Characteristics

The angles of the parameres were compared using ANOVA. If significant differences were found, a student's T test was run to determine where the significant difference lies. The proportion of black coloration in the elytra was compared among the crosses using Wilcoxon/ Kruskal Wallace test (Rank Sums). If significant differences were found, the Wilcoxon Method of multiple comparisons was run to determine where the differences lie.

Host Preference

A Wilcoxon/ Kruskal Wallace test (Rank Sums) was run to determine if there was a significant difference in the number of eggs oviposited on HWA vs. PBA for the hybrids and *L. nigrinus*. Chi-square was used to determine if there was a significant difference in locations where the beetles were found when the dish was opened at the end of the experiment.

3.3 Results

Fitness

Following data collection in 2011, it was discovered that the number of prepupae that developed was much greater than the number of eggs produced by ♂♀ mating pairs for the Ln x Ln and Ln x Lr crosses (Table 3.1). This suggests that females were still ovipositing fertile eggs from mating in the field. Adding together the total number of eggs produced by both ♂♀ and ♀♀ mating pairs, the number of prepupae produced is more logical (i.e. 2011: Ln x Ln: 844 eggs: 672 prepupae). Because of these results, the fitness measurements among the crosses cannot be statistically compared since we do not know which of the progeny produced were from mating in the lab or in the field.

Although the adult beetles in the no-choice lab mating experiment had mated in the field and were producing progeny from these interactions, it was clear that they were mating and producing progeny from interactions in the lab as well. This can be determined from the 217 adults that emerged from the 2011 Ln x Lr cross, 119 were identified; 58 were found to be *L. nigrinus*, 5 *L. rubidus*, and 56 hybrids (Figure 3.1). Beetles used in this experiment could not have produced hybrids from mating in the wild because the *L. nigrinus* used in this experiment were collected in Seattle, WA where *L. rubidus* is not present and the *L. rubidus* used in this experiment were collected in an area of VA where the closest *L. nigrinus* release site is 32 km away. The hybrids produced by the Ln x Lr cross show asymmetric introgression towards *L. nigrinus* (Figure 3.2). Of the five progeny produced by the Ln x Lr cross in 2012, all were found to be *L. nigrinus*.

The length of eggs (mm) was not compared among the crosses since it was unknown whether the eggs measured were produced from mating in the lab or the field. Since the data were available, the length of eggs (mm) produced by *L. nigrinus* and *L. rubidus* was compared and was not found to be statistically different ($p = 0.1554$; *L. nigrinus*: 0.4511 mm and *L. rubidus*: 0.4455 mm).

Development

Although the fitness data could not be compared, the data on development were valid. The progeny used in this experiment were genetically analyzed following experimentation; therefore species was determined and any progeny from the Ln x Lr cross that were not hybrids were dropped from analysis within this category.

The percent of eggs that developed to the prepupal stage was similar for the Ln x Lr and Ln x Ln crosses (Table 3.2). No significant difference was found among crosses in the number of days it took for the progeny to develop from egg hatch to the prepupal stage ($F = 0.0816$, $df = 2$, $P = 0.9222$). The Ln x Ln cross took 22.9 days to develop on average, the Lr x Lr cross; 23 days, and the Ln x Lr cross; 22 days.

First instar measurements could only be compared between *L. nigrinus* and the hybrids as data were not available for *L. rubidus*. The only data available for the 4th instar were from *L. nigrinus*; therefore 4th instar measurements were not included in the analysis. There was no significant difference among crosses in their head capsule widths or larval lengths for any of the instars where data were available (Table 3.3).

Host Preference

Sixty-one beetles were confirmed to be females and identified to species. Four of these were hybrids; the remainder was identified as *L. nigrinus*. There was no significant difference in the number of eggs oviposited in the HWA ovisacs vs. the PBA ovisacs (χ^2 = 1.0331, df = 1, P = 0.3094) for the hybrids. *Laricobius nigrinus* oviposited significantly more eggs in the HWA ovisacs than the PBA ovisacs (χ^2 = 36.1986, df = 1, P < 0.0001). *Laricobius nigrinus* oviposited an average of 7.37 eggs in the HWA ovisacs and 1.7 eggs in the PBA ovisacs. There was no significant difference in where the beetles were found when the dishes were opened for either the hybrids or *L. nigrinus* (χ^2 = 3.082, df = 2, P = 0.2141). The hybrids were found 1:1 on hemlock vs. white pine (Table 3.4).

Morphological Measurements

The angles of the parameres were found to be significantly different among pure species and hybrids (F = 14.4210, df = 2, P < 0.0001). The mean paramere angle for *L. nigrinus* was 49.07, *L. rubidus*; 70.17, and the hybrids; 58.59 and were significantly different among all three comparisons (*L. rubidus* vs. *L. nigrinus*: P < 0.0001, *L. rubidus* vs. hybrid: P = 0.0084, *L. nigrinus* vs. hybrid: P = 0.0009) (Student's T-test).

The proportion of black found in the elytra was significantly different among the crosses (χ^2 = 96.1277, df = 2, P < 0.0001). The difference was observed between *L. nigrinus* and the hybrids (P < 0.0001) and *L. nigrinus* and *L. rubidus* (P < 0.0001), but not between *L. rubidus* and the

hybrids (P = 0.8684). The mean proportion of black found in *L. nigrinus* was 0.9409, *L. rubidus*; 0.5632, and the hybrids; 0.5966.

3.4 Discussion

Little conclusive information was collected regarding the fitness of Ln x Lr hybrids. We found that hybrids can be produced in the lab and introgression was skewed more towards *L. nigrinus* than *L. rubidus* (Figure 3.2). There was no significant difference between the parental species and the hybrids in the length of time (days) it took for development from egg hatch to the prepupal stage, nor were there significant differences in the head capsule widths and larval lengths. None of these data suggest that hybrids are less fit than the parental species.

If hybrids are as fit as their parental counterparts, there is less probability of negative effects to the biological control of HWA as a result of lower reproductive potential. It is possible that fit hybrids may increase the chance of either: 1.) The displacement of *L. rubidus* through competition, or 2.) The assimilation of *L. rubidus* through introgression. The probability of either of these outcomes depends on several factors, including the proportion of *L. nigrinus*, *L. rubidus*, and hybrids in the population, the host preference of hybrids, and whether the hybrid population continues to increase, as was found by Havill et al. (2012).

The host preference test suggests that the hybrids have no preference between hemlock and white pine. This result is similar to other studies that have shown that hybrid diets are generally intermediate between the diets of the parental species (Weider 1993, Schluter 1995, Grant and Grant 1996). Having no preference should not lower the fitness of Ln x Lr hybrids since they are

intermixed with the parental species and well-distributed in the environment (Grant and Grant 1996) forming a mosaic hybrid zone (Havill et al. 2012), rather than situated in a narrow hybrid zone between two vastly different ecological regions. Since HWA is incredibly abundant in the eastern U.S., it is likely that the hybrids will exploit this resource, which would be beneficial for biological control of HWA. How a lack of host preference will affect PBA populations is less obvious. It is possible that the result will be a decrease in PBA populations, but this is only likely to occur if percent hybridization continues to increase over time.

As in Havill et al. (2012), the angles of the parameres were significantly different among the parental species and the hybrids, with the hybrids being variable with an intermediate mean. The parameres are projections of the male genitalia that fit into the grooves and pits of of the female pygidium. The size and shape of these organs fit together like puzzle pieces (Macagno et al. 2011). Although it is not known whether the pygidial flap acts as a lock that is able to exclude heterospecific matings, Macagno et al. (2011) state that it is likely that proper coupling of the pygidium and the parameres favor efficient sperm transer, making heterospecific matings less effective. For this reason, intermediate genital features may aid in limiting gene flow through reproductive isolation (Sota et al. 2000, Gompert et al. 2006). But given that *L. rubidus* and *L. nigrinus* readily hybridize, it is unlikely that hybrids will become reproductively isolated from the parental species.

In this study, F_1 hybrid elytra coloration was similar to *L. rubidus*. Havill et al. (2012) found that hybrids had variable and intermediate elytra coloration to that of the parental species, but their field collected hybrids were probably a mix of different hybrid classes. Differences in the

coloration of the elytra could affect mate choice (Gompert et al. 2006). If *Laricobius* exhibit assortative mating based on the color of the elytra, this could lead to a lower reproductive potential of *Laricobius* spp. through fewer encounters with potential mates of their own type in the field. Given the fact that *L. nigrinus* and *L. rubidus* readily hybridize, and *L. osakensis* and *L. nigrinus* have been found to readily attempt copulation (Chapter 2), it is unlikely that assortative mating based on differences in elytra coloration occurs among *Laricobius* spp.

Conclusion

There are many potential outcomes of this interaction that could have effects on both the biological control of HWA and biodiversity within the PBA guild. The fitness and host preference of *L. nigrinus* x *L. rubidus* hybrids should continue to be assessed in the lab and especially in the field.

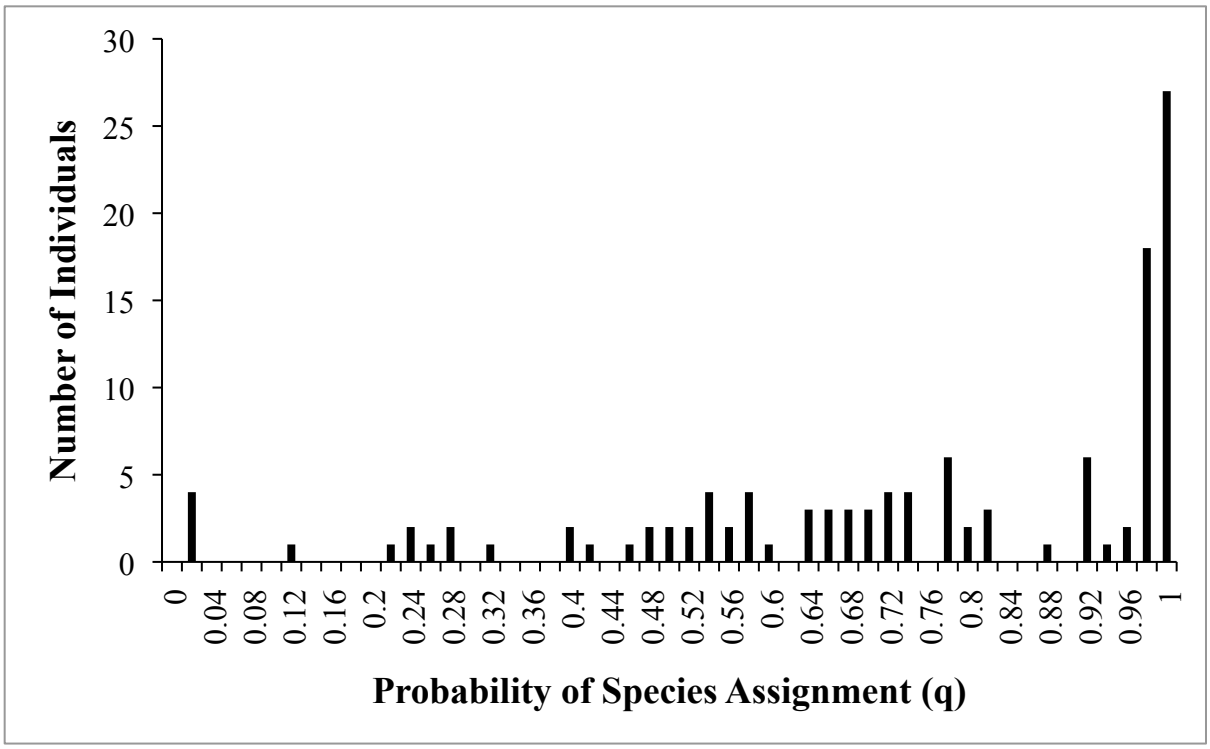

Figure 3. 1. Histogram of the probability of species assignment (q) for progeny produced by wild-caught *Laricobius nigrinus* and *L. rubidus*. A value of zero represents pure *L. rubidus*; a value of one represents pure *L. nigrinus*; .50 represents an F_1 hybrid.

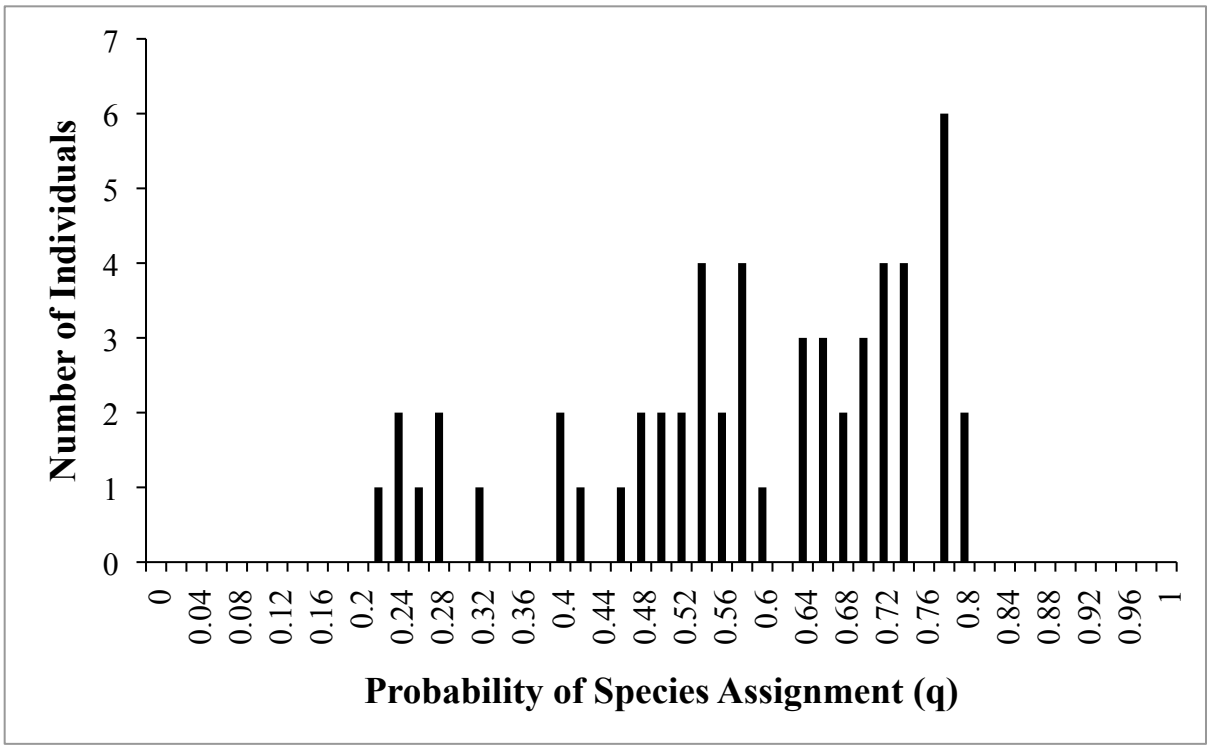

Figure 3. 2. Histogram of the probability of species assignment (q) for only the progeny labeled as hybrids produced by wild-caught *Laricobius nigrinus* and *L. rubidus*. A value of zero represents pure *L. rubidus*; a value of one represents pure *L. nigrinus*; .50 represents an F_1 hybrid.

Table 3. 1. The total number of eggs produced and placed into funnels for ♂♀ and ♀♀ pairs of *L. nigrinus* x *L. nigrinus* (Ln x Ln), *L. rubidus* x *L. rubidus* (Lr x Lr) and *L. nigrinus* x *L. rubidus* (Ln x Lr) and the total number of prepupae and adults that developed from these crosses in 2011 and 2012.

		Eggs			Prepupae	Adults
		♂♀	♀♀	Total		
Ln x Ln	2011	200	644	844	672	173
Lr x Lr	2011	339	293	632	41	5
Ln x Lr	2011	490	13	503	505	217
Ln x Ln	2012	72	96	168	25	6
Lr x Lr	2012	11	n/a	11	1	0
Ln x Lr	2012	68	78	146	7	5

Table 3. 2. The percent of larvae and prepupae that developed from eggs produced by mating pairs of *L. nigrinus* x *L. nigrinus* (Ln x Ln), *L. rubidus* x *L. rubidus* (Lr x Lr) and *L. nigrinus* x *L. rubidus* (Ln x Lr). n = the number of mating pairs.

	n	Eggs	Larvae	Prepupae
Ln x Ln	11	22	73%	27%
Lr x Lr	10	12	42%	17%
Ln x Lr	15	25	60%	28%

Table 3. 3. 1st through 3rd instar head capsule widths (±SE, mm) and larval lengths (mm) for *L. nigrinus*, *L. rubidus*, and *L. nigrinus* x *L. rubidus* hybrids.

	Head Capsule Width (mm)			Larval Length (mm)		
	1st	2nd	3rd	1st	2nd	3rd
L. nigrinus	0.1250 ± 0.0072	0.1897 ± 0.0043	0.4314 ± 0.0104	0.7656 ± 0.0180	0.8685 ± 0.0553	2.7857 ± 0.1608
L. rubidus	n/a	0.1906 ± 0.0041	0.4800 ± 0.0000	n/a	0.7479 ± 0.0251	2.4400 ± 0.0000
Hybrid	0.1313 ± 0.0000	0.1875 ± 0.0000	0.4000 ± 0.0231	0.8500 ± 0.0284	0.8167 ± 0.1105	2.6700 ± 0.3492

Table 3. 4. Results of Chi-square analysis showing the percent of hybrids and *L. nigrinus* found on hemlock, white pine, or the base of the Petri dish in which they were enclosed, following a host preference experiment.

	Hybrid	*L. nigrinus*
Hemlock	50.00	52.63
White Pine	50.00	22.95
Petri Dish	0.00	24.59

Chapter 4. Assessment of hybridization between L. osakensis *and* L. rubidus *in the lab.*

4.1 Introduction

Laricobius nigrinus Fender, a predatory beetle native to western North America, was released as a biological control agent of the hemlock woolly adelgid (*Adelges tsugae* Annand; hereafter HWA) in the eastern U.S. *Laricobius rubidus* LeConte is a native predator of the pine bark adelgid (*Pineus strobi* Hartig; hereafter PBA). Following release, it was discovered that *L. nigrinus* and *L. rubidus* could mate and produce viable progeny. This interaction is currently being studied in the lab and field. Hybridization between the two species may affect biological control efforts using *Laricobius* spp. Additionally, *L. rubidus* may be displaced by *L. nigrinus* or be assimilated through hybridization at sites where the two species are present. This could result in changes in populations PBA, the main host of *L. rubidus*, which in turn could affect other natural enemies of PBA and/or eastern white pine (*Pinus strobus* L.).

Laricobius osakensis Shiyake and Montgomery is a predator of HWA native to Japan. This predatory beetle was recently imported into the U.S. to study as a biological control agent of HWA. As a result of the interaction between *L. nigrinus* and *L. rubidus*, it was considered important to determine if *L. osakensis* and *L. rubidus* could mate and/or produce progeny prior to releasing *L. osakensis* in the eastern U.S.

Laricobius osakensis and *L. rubidus* are not as closely related (Montgomery et al. 2011b), as are *L. nigrinus* and *L. rubidus* (Davis et al. 2011, Montgomery et al. 2011b). For this reason, it is

unlikely that the two species will be capable of producing viable progeny. Even if they are not capable of producing progeny, attempts to copulate could result in a range of effects such as injured genitalia, as was found with carabid beetles (Sota and Kubota 1998, Sota et al. 2000), and an overall decrease in the production of progeny as a result of differences in the characteristics of the reproductive systems (e.g. male seminal products) and/or developmental failures in embryos (Eberhard 1996).

The purpose of this study was to determine if *L. osakensis* could mate and/or produce viable progeny with *L. rubidus* and if so, determine if there were fitness costs associated with the interaction.

4.2 Methods

To determine if *L. osakensis* and *L. rubidus* could mate and produce progeny, a no-choice lab mating experiment was completed in 2011 and 2012. The experiment consisted of three treatment crosses: *L. osakensis* x *L. osakensis* (Lo x Lo), *L. rubidus* x *L. rubidus* (Lr x Lr), and *L. osakensis* x *L. rubidus* (Lo x Lr). Fitness measurements included: fecundity; the number of eggs produced per cross, fertility; the number of progeny that developed into prepupae per cross, and viability; the number of progeny that developed to the adult stage per cross.

In 2011, wild-caught adults were used because F_1 *L. rubidus* were not available (see Appendix 1). In February 2011, 15 wild-caught *L. osakensis* (WLo x WLo), 15 wild-caught *L. rubidus* (WLr x WLr), and 15 wild-caught *L. osakensis* x 15 wild-caught *L. rubidus* (WLo x WLr) pairs were formed. In January 2012, F_1 and wild-caught (W) adults were used and these treatments

consisted of 7 F_1Lo x F_1Lo pairs, 12 F_1Lo x F_1Lr pairs, and 9 F_1Lo x WLr pairs. Since so few F_1 *L. rubidus* individuals were available, an Lr x Lr control was not included.

Mating pairs were kept in a 50 x 9 mm polystrene Petri dishes with a 2-cm hole cut in the top and covered with polyester mesh. *L. osakensis* mating pairs were placed in dishes with HWA-infested hemlock, while crosses containing *L. rubidus* were placed in dishes with both HWA-infested hemlock and PBA-infested white pine. PBA-infested white pine was included in these dishes because PBA is the main host of *L. rubidus*. HWA was added because few PBA are typically located at the tips of white pine branches, and since *L. rubidus* has been found to be capable of feeding and developing on HWA (Zilahi-Balogh et al. 2005), it was thought that HWA would be a good supplemental food source.

Dishes were kept in a growth chamber under the temperatures and photoperiods shown for adults in October through May in Table 2.1. In 2011, data collection was delayed two weeks in 2011 following the formation of treatment pairs, because it was not known at the time if *Laricobius* spp. had a spermatheca capable of storing sperm. If *Laricobius* spp. were capable of storing sperm from previous interactions in the wild, the hope was that they would oviposit all fertilized eggs before data collection began. Rather than delaying data collection in 2012, wild-caught beetles were collected earlier in the season (December 2011) and kept in individual dishes until they were mated in January 2012. This method was attempted in the hope that *Laricobius* did not mate until spring.

Once data collection began, beetles were placed in clean dishes with fresh adelgid-infested branch sprigs once a week. The branches from the previous week were collected and the number of eggs located on these branches was counted. The length of one egg per dish was measured each week.

All eggs that were not used for the development study (described below) were placed into funnels by cross to rear the eggs to the prepupal stage [rearing: see Salom et al. (2012)]. Funnels containing eggs from the Lo x Lo crosses contained approximately 100 HWA-infested hemlock branches (30-cm long), while funnels containing eggs from the Lr x Lr and Lo x Lr cross contained approximately 50 HWA-infested hemlock branches (30-cm long), as well as 50 PBA-infested white pine branches. All prepupae that developed were counted and placed into soil containers (50 per container) to rear to the adult stage. All adults that emerged were counted and placed into separate microcentrifuge tubes with 95-100% ETOH for genetic analysis.

If a beetle from one of the mating pairs died during the experiment, the beetle and its mate were placed into separate microcentrifuge tubes with 95-100% ETOH for dissection to determine sex and to confirm species following genetic analysis. All beetles that were still alive at the end of the experiment were also placed into individual tubes with ETOH for dissection. The heads and elytra were kept as voucher specimens, the genitalia were mounted onto slides, and the remaining parts were placed back into microcentrifuge tubes and used for genetic analysis.

Development

Experiments were completed in 2011 and 2012 to determine if there were differences in the time (days) it took for progeny from the three crosses to develop from egg hatch to the prepupal stage. The eggs used in this study were collected from mating pairs in the no-choice lab mating experiment. In 2011, six eggs were collected from six individual WLo x WLo pairs, nine were collected from nine WLr x WLr mating pairs, and 15 eggs were collected from 15 WLo x WLr mating pairs. In 2012, five eggs were collected from one F_1Lo x F_1Lo mating pair and six eggs were collected from one F_1Lo x WLr mating pair. The eggs were placed individually onto an HWA ovisac located on a two-cm-long sprig of hemlock. The sprigs were placed into 50 x 9 mm polystrene Petri dishes with a two-cm hole cut into the top and covered with polyester mesh. The dishes were placed into a growth chamber at 13°C, 12:12 L: D h.

The eggs were checked daily for hatch and the date of hatch was recorded. Additional HWA and PBA were added to the dish as needed. The prepupal stage was determined as the point when the larvae began wandering around the base of the dish looking for a pupation site (Zilahi-Balogh et al. 2003a) and the date this occurred was recorded. All larvae and prepupae were placed into separate microcentrifuge tubes with 95-100% ETOH for genetic analysis following experimentation.

To determine if there were differences in the size of the larvae among the crosses, the head capsule widths and larval lengths were measured using an ocular microscope with a calibrated scale. A previous study found that manipulation of larvae predisposed them to mortality (Heather Story, personal communication); therefore 80 additional eggs were collected from each cross, and 20 of these were reared to each instar (4 instars) for measurements.

Genetic Analysis

The F_1 adults and wild-caught beetles from Japan that were used in this study were identified to species using the partial cyctochrome oxidase subunit I (COI) gene. DNA was extracted using the DNAeasy kit (Qiagen Inc., Valencia, CA). Partial cytochrome oxidase subunit I (COI) was amplified using forward primer LepF1 and reverse primer LepR1 (Herbert et al. 2004). PCR was performed in 30 µl reactions containing 3.0 µl 10X PCR Buffer, 2.4 µl dNTPs (10mM), 4.8 µl $MgCl_2$ (25 mM), 1.0 µl BSA (10mg/ml), 1.0 µl of each primer (10 mM), 0.3 µl Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µl DNA template. Thermocycling conditions 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 kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied BioSystems 3730xl automated sequencer. Sequences were aligned using the SeqMan Pro program of Lasergene 8.0 software (DNASTAR; http://www.dnastar.com).

Wild-caught *L. rubidus* were identified using six nuclear microsatellite loci (LaGT01, LaCA04, LaGT07, LaGT13, LaCA14, LaCA16) (Klein et al. 2010; Havill et al. 2012). Microsatellites were used to confirm the absence of *L. nigrinus* in the area where *L. rubidus* was collected (Dismal Falls, VA).

Microsatellite loci were amplified using the conditions described in Klein et al. (2010). Fragment analysis was completed at the DNA Analysis Facility on Science Hill at Yale

University in New Haven, CT using a 3730xl 96-capillary genetic analyzer. Genotypes were scored using Genemapper 4.0 (Applied Biosystems). The programs Structure 2.3.2 (Pritchard et al. 2000) and NewHybrids 1.1 (Anderson and Thompson 2002) were used to confirm identity. Structure runs used 20,000 burn-in iterations followed by 100,000 sample iterations and NewHybrid runs were completed with 10,000 burn-in iterations followed by 100,000 sample iterations (Havill et al. 2012). For NewHybrids, the results of all four potential classes of hybrid were summed to obtain the probability of assignment as a hybrid. The results of five independent runs were averaged for both Structure and NewHybrids. Each specimen was assigned to a parental species if Structure analysis resulted in $q > 0.80$, or as a hybrid if $0.20 < q < 0.80$. If the category with the highest probability of assignment from Newhybrids agreed with Structure, that assignment was retained. If they did not agree, the specimen was assigned to the category with the higher probability of the two analyses (Havill et al. 2012).

Progeny from the Lo x Lr cross were identified using the partial *wingless* gene (Montgomery et al. 2011b). DNA was extracted using the DNAeasy kit (Qiagen Inc., Valencia, CA). Partial *wingless* was amplified using the primers Wg578 and WgAbR (Wild and Maddison 2008). PCR was performed in 30 µl reactions containing 3.0 µl 5X PCR Buffer, 2.4 µl dNTPs (10 mM), 2.4 µl $MgCl_2$ (25 mM), 1.0µl BSA (10 mg/ml), 1.0 µl of each primer (10 mM), 0.3 µl Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µl DNA template. Thermocycling conditions were 95°C for 5 min followed by 35 cycles of 45 s at 95°C, 45 s at 54°C, and 1 min at 72°C, with a final extension of 72°C for 5 min. Sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied BioSystems 3730xl automated sequencer (Applied Biosystems). Sequences were edited using

Sequencer 4.2.2 (Gene Codes Corporation, Ann Arbor, MI) and aligned using MUSCLE 3.6 (Edgar 2004).

Statistics

The percent of eggs that developed from hatch to the larval and prepupal stage were calculated per cross. The number of days it took for larvae to develop from egg hatch to the prepupal stage per cross was analyzed using the Wilcoxon/ Kruskal Wallace test (Rank Sums). If significant differences were found, the Wilcoxon Method of multiple comparisons was used to determine where the differences lie. JMP Pro 10 and an alpha of 0.05 were used for this analysis.

4.3 Results

It was clear when looking at the 2011 data that the beetles had mated previously in the wild. Eggs produced by the ♀♀ pairs should have been sterile if the females were virgin or if enough time had elapsed for the females to rid themselves of sperm stored in the spermatheca. Yet, the ♂♀ WLo x WLo pairs produced 410 eggs, and 453 prepupae emerged (Table 4.1). For this reason, the fitness data were not statistically analyzed since there was no way to determine if the eggs, prepupae, and adults produced were from mating in the lab or the field.

Of the eggs produced by the 2011 WLo x WLr pairs, 358 developed to the adult stage. Thirty-seven were genetically identified; 24 were found to be *L. osakensis* and 13 to be *L. rubidus*; none was found to be hybrids.

In 2012, the adult beetles used in the no-choice lab mating study exhibited high mortality. Eight of the nine F_1Lo x F_1Lr pairs died by January 31, 2012, just five weeks after being paired. The last living pair died on March 6, before peak oviposition began. Although the F_1Lo x F_1Lo and F_1Lo x WLr crosses produced eggs, only eggs from the F_1Lo x F_1Lo cross developed past the egg stage (Table 4.1).

Development

Only one larva could be identified from the WLo x WLr cross. This was found to be a pure *L. osakensis* and not a hybrid. The eggs produced from WLo x WLo mating pairs had the greatest percent development from the egg to the prepupal stage, while eggs from WLr x WLr mating pairs had the least (Table 4.2). There was no significant difference in the duration for larvae to develop among the crosses ($\chi^2 = 5.3601$, df = 2, P = 0.0686). The WLo x WLo cross took an average of 21.67 days to develop from egg hatch to the prepupal stage (n = 3), WLr x WLr; 23 days (n = 2), and WLo x WLr; 18.63 days (n = 8).

4.4 Discussion

The experiments described here were unsuccessful as a result of the use of wild-caught beetles. F_1 adults, rather than wild-caught adults, should be used for lab mating experiments since *Laricobius* spp. females have a spermatheca and can store sperm. Although the experiments were unsuccessful, evidence suggests that it is unlikely that *L. osakensis* and *L. rubidus* can mate and produce viable progeny because the two species are not closely related (Montgomery et al. 2011b). Additionally, pre-mated, wild-caught beetles used for the Ln x Lr no-choice lab mating

study produced hybrids (see Chapter 3). If Lo x Lr were capable of producing hybrids, some of the progeny analyzed should have been identified as hybrids, as in the case of Ln x Lr.

Conclusion

Due to the fact that lab experimentation was largely unsuccessful and *L. osakensis* has been released in the field, populations of *L. rubidus*, *L. nigrinus*, and *L. osakensis* should be monitored in and around release areas. The outcome of the interaction among these three species may have effects on the biological control of HWA and may guide future decisions in biological control releases.

Table 4. 1. The total number of eggs put into funnels to rear to the prepupal stage for ♂♀ and ♀♀ pairs. The total column includes eggs from all pairs: ♂♀, ♀♀, unidentified pairs, and accidental single species crosses in the case of WLo x WLr in 2011. n = the number of mating pairs.

		♂♀		♀♀		Total		
		n	Eggs	n	Eggs	n	Eggs	Prepupae	Adults
WLo x WLo	2011	3	410	2	355	15	812	453	137
WLr x WLr	2011	9	339	3	293	15	633	41	5
WLo x WLr	2011	15	1100	4	564	30	2000	896	358
F_1Lo x F_1Lo	2012	1	0	0	0	7	32	7	1
F_1Lo x F_1Lr	2012	9	0	1	0	12	0	0	0
F_1Lo x WLr	2012	3	2	5	61	9	63	0	0

Table 4. 2. The percent of larvae and prepupae that developed from eggs produced by mating pairs of wild-caught: *Laricobius osakensis* x *L. osakensis* (WLo x WLo), *L. rubidus* x *L. rubidus* (WLr x WLr), and *L. osakensis* x *L. rubidus* (WLo x WLr). n = the number of mating pairs.

	n	Eggs	Larvae	Prepupae
WLo x WLo	3	3	100.00%	66.67%
WLr x WLr	9	9	66.67%	33.33%
WLo x WLr	9	9	77.78%	55.56%

Chapter 5. Field assessment of hybridization between Laricobius nigrinus *and* L. rubidus*, predators of Adelgidae*

5.1 Introduction

Laricobius nigrinus Fender and *Laricobius rubidus* LeConte are predatory beetles in the family Derodontidae that feed exclusively on adelgids. *Laricobius rubidus* is native to the eastern United States and primarily feeds on pine bark adelgid (*Pineus strobi* Hartig; hereafter PBA), while *L. nigrinus* is native to western North America and has been found to feed on several species of adelgid, including hemlock woolly adelgid (*Adelges tsugae* Annand; hereafter HWA). Beginning in 2003, *L. nigrinus* was released in the eastern United States as a biological control agent of HWA. Several years after releases began, it was discovered that *L. nigrinus* was hybridizing with *L. rubidus* (Havill et al. 2012).

Laricobius nigrinus and *L. rubidus* are sister species that recently diverged from a common ancestor, as evidenced by a Kimura two-parameter (K2P) DNA sequence distance of 2.2% in the mitochondrial COI gene (Davis et al. 2011, Montgomery et al. 2011b). Hybridization between the species could impact the efficacy of biological control of HWA using *L. nigrinus*. Additionally, hybridization could cause displacement of *L. rubidus* at sites where *L. nigrinus* has been released.

Currently, *L. nigrinus* and *L. rubidus* form a mosaic of hybrid zones throughout the eastern U.S. (Havill et al. 2012). A mosaic pattern formed, rather than a broad linear hybrid zone where both species have regions of allopatry and a narrow region of sympatry where hybridization occurs,

likely because: 1) *L. nigrinus* has been released in a patchy pattern; 2) the preferred habitats (*Tsuga canadensis* (L.) Carrière, *Tsuga caroliniana* Engelmann and white pine; *Pinus strobus* L.) of the two *Laricobius* species broadly overlap in the region where they are hybridizing; and 3) *L. rubidus* can still be found in large regions of allopatry (Havill et al. 2012). Whether or not the two *Laricobius* spp. will maintain these hybrid zones in a stable form is unknown. The distribution of *L. nigrinus* and/or hybrids may eventually overlap with *L. rubidus* completely. Additionally, each individual patch within the mosaic hybrid zone could have a unique evolutionary trajectory resulting in several different outcomes (Harrison and Rand 1989). Potential outcomes include genetic assimilation or displacement of *L. rubidus* and/or *L. nigrinus*, reinforcement of premating isolation resulting in permanent speciation of *L. nigrinus* and *L. rubidus*, hybrid vigor, creation of a third hybrid species, or maintenance of a stable hybrid zone.

Objectives

Not much is known about hybridization of introduced biological control agents (Hopper et al. 2006). Due to the broad spectrum of potential outcomes that can occur following hybridization and the lack of data on the subject, it is important to address the outcome of this interaction.

The objectives of this study were to use genetic markers to: 1.) Determine if hybridization is occurring at ten study sites where *L. nigrinus* has been released, 2.) Document how hybridization is changing over time, 3.) Infer host preference of hybrids in a natural setting, and 4.) Identify factors that may drive hybridization. This information may help determine the evolutionary trajectory of the interaction between *L. nigrinus* and *L. rubidus*.

5.2 Methods

Ten sites where *L. nigrinus* had been previously released were chosen for this study (Table 5.1). *L. rubidus* was known to be present at all sites but Burns Creek and Devil's Fork, VA. Three of the sites (TN, NC, and Rothrock, PA) were already known to have hybridization occurring. All sites were visited once a year, when *Laricobius* larvae were most numerous. This was determined by redbud (*Cercus canadensis* L.) burst, which has been found to be synchronous with peak larval production (Mausel 2007).

At each site, sixteen points were chosen based upon the presence of an adelgid infestation on both hemlock and white pine. Adelgid infestations were specifically chosen because *Laricobius* larvae are found within the woolly ovisacs of HWA and PBA where they feed on the adelgid eggs. Approximately twenty-five 30-cm long adelgid-infested branches were collected from each point. These branches were placed in plastic bags that were filled with air and made air tight to keep from crushing any larvae present during transport.

At each point, basal area per acre (BA/acre) and trees per acre (TPA) were measured within 40 m^2 ($1/100^{th}$ acre) fixed-area plots (Avery and Burkhart 2002). All trees with a DBH $\geq$ 2.54 cm were included in the BA/acre measurements and all trees present were included in the TPA measurements.

The population of adelgids at the points where branches were collected was qualitatively assessed. For HWA, a low population consisted of approximately 10 or fewer adelgids, a medium population consisted of between 11 and 50 adelgids, and a high population consisted of

at least 50 adelgids on a 30-cm long portion of branch. For PBA, a low population consisted of ≤ 5 adelgids, a medium population consisted of approximately 6 to 10 adelgids, and a high population consisted of > 11 adelgids on a 30-cm long portion of branch.

The adelgid-infested hemlock and white pine branches were brought back to the Virginia Tech Insectary and placed in funnels to rear larvae to the prepupal stage [for rearing see (Salom et al. 2012)]. Branches from two points were placed in one funnel, resulting in a total of eight hemlock funnels and eight white pine funnels per site. All prepupae were collected and placed in separate ETOH vials by funnel. Ten prepupae were randomly chosen from each vial for genetic analysis.

Genetic Analysis

The following analysis was used to determine species of all *Laricobius* larvae in order to calculate the percent of *L. nigrinus*, *L. rubidus* and hybrids found at each site.

The identities of the larvae were determined using six nuclear microsatellite loci (LaGT01, LaCA04, LaGT07, LaGT13, LaCA14, LaCA16) (Klein et al. 2010; Havill et al. 2012). Microsatellite loci were amplified using the conditions described in Klein et al. (2010). Fragment analysis was completed at the DNA Analysis Facility on Science Hill at Yale University in New Haven, CT using a 3730xl 96-capillary genetic analyzer. Genotypes were scored using Genemapper 4.0 (Applied Biosystems). The programs Structure 2.3.2 (Pritchard et al. 2000) and NewHybrids 1.1 (Anderson and Thompson 2002) were used to distinguish hybrids from parent species using the criteria described in Havill et al. (2012). Briefly, Structure runs used 20,000 burn-in iterations followed by 100,000 sample iterations and NewHybrid runs were

completed with 10,000 burn-in iterations followed by 100,000 sample iterations (Havill et al. 2012). For NewHybrids, the results of all four potential classes of hybrid were used to determine the probability that hybrids found on hemlock and white pine were F_1's, F_2's, *L. rubidus* backcrosses, or *L. nigrinus* backcrosses. These four classes were then summed to obtain the probability of assignment as a hybrid regardless of class. The results of five independent runs were averaged for Structure and NewHybrids. Each larva was assigned to a parental species if Structure analysis resulted in $q > 0.80$, or as a hybrid if $0.20 < q < 0.80$. If the category with the highest probability of assignment from NewHybrids agreed with Structure, that assignment was retained. If they did not agree, the larvae were assigned to the category with the higher probability of the two analyses.

Statistics

All statistical analysis used an alpha of 0.05 in the program JMP Pro 10 when relevant.

Laricobius Populations

To determine how hybridization changed from 2010-2012, a Chi-square analysis was performed using the total count of *L. nigrinus*, *L. rubidus*, and hybrids (Appendix C) collected from hemlock and white pine at all 10 sites in the study. The distribution of *L. nigrinus*, *L. rubidus*, and hybrids collected from hemlock and white pine at the 10 sites was compared each year.

Additional data from the TN, NC, and Rothrock, PA sites were available from a previous study that took place from 2007-2009 (Davis et al. 2011). This study used the same methodology to collect *Laricobius* larvae, but from hemlock only. Using these data, as well as the data collected

at these sites from 2010-2012 from hemlock only (Appendix D), changes in hybridization, as well as the change in the parental species composition, were analyzed using regression analysis.

Hemlock vs. White pine

The difference in the distribution of *L. nigrinus*, *L. rubidus* and hybrids on hemlock and white pine was analyzed using Chi-square analysis. The total number of *L. nigrinus*, *L. rubidus*, and hybrids on white pine vs. hemlock at all 10 sites for 2010-2012 (Appendix C) was used for this analysis.

Site Factors Affecting Hybridization

Several factors were used to determine if a specific characteristic or characteristics of the sites where *L. nigrinus* was released had an effect on the percent of hybrids found at the sites. These factors included: latitude/longitude, elevation, hemlock (HEM) and white pine (WP) basal area per acre (BA/acre), HEM and WP trees per acre (TPA), the number of *L. nigrinus* released at each site, and the number of years *L. nigrinus* was present at the site (Appendix E). Because the BA/acre and TPA measurements were biased (measured specifically at hemlock and white pine points), all 10 sites were revisited in the fall of 2012 and an unbiased measurement of HEM and WP BA/acre and TPA was taken using fixed area plots (Avery and Burkhart 2002). These measurements were included in the following analysis as well as the factors listed above (Appendix E).

A principal components analysis was run to test for multicollinearity among the site factor variables and to determine the principal components. The variables included in this analysis

were: latitude, longitude, elevation, unbiased HEM and WP BA/Acre, unbiased HEM and WP TPA, biased HEM and WP BA/Acre, biased HEM and WP TPA, the number of *L. nigrinus* released at the sites, and the number of years *L. nigrinus* was present at the sites. The analysis included only data from 2010-2012. The principle components selected (above 0.7), included: latitude, longitude, biased WP TPA, biased HEM BA/Acre, number of *L. nigrinus* released at the sites and the number of years *L. nigrinus* was present at the sites. These components were used in a stepwise regression analysis to determine if any showed a relationship with the percent of hybrids found at the sites.

5.3 Results

By 2011, hybridization was occurring at all sites except for Devil's Fork, VA. Neither pure *L. rubidus* larvae nor hybrids were ever found at this site. This is the only site in this study where white pine was not present.

HWA populations were elevated relative to PBA populations, which remained low throughout the three years of the study (Figure 5.1A and B).

Laricobius Populations

The distribution of collections among the three *Laricobius* spp. each year (2010-2012) was significant ($\chi^2 = 80.040$, df = 4, $p < 0.0001$). The average percent of *L. nigrinus* was greater than that of *L. rubidus* and the hybrids in 2010- 2012, peaking in 2012. Percent *L. rubidus* was greater than that of the hybrids from 2010-2012, with peak percent in 2011. The hybrid population remained steady over time with a slight increase in 2011 (Figure 5.2).

At the three sites where *Laricobius* population data were available from 2007-2012, populations of *L. nigrinus* show a clear increase over time, *L. rubidus* a decrease, and there was a relatively steady population of hybrids after they appear in 2008 (Figure 5.3). Regression analysis showed that the increase in the proportion of *L. nigrinus* over time was significant ($F = 22.8398$, $df = 1$, $P = 0.0088$, $r^2 = 0.85$), as was the decrease in the proportion of *L. rubidus* ($F = 15.6436$, $df = 1$, $P = 0.0167$, $r^2 = 0.80$).

Hemlock vs. White pine

Laricobius nigrinus was found more often on hemlock than white pine from 2010-2012 (Figure 5.4). *Laricobius rubidus* was found on both hemlock and white pine, while the hybrids were found on both species in 2011, but almost solely on hemlock in 2010 and 2012 (Figure 5.4). Contingency analysis showed that there was a significant difference in the distribution of the *Laricobius* species on hemlock vs. white pine from 2010-2012 (Figure 5.5; $\chi^2 = 550.306$, $df = 2$, $P < 0.0001$). Percent *L. nigrinus* was greater on hemlock (95.69), while percent *L. rubidus* was similar on hemlock (43.72) vs. white pine (56.28). The distribution of hybrids was greater on hemlock than on white pine (76.10, Figure 5.5).

All hybrids found from 2010-2012 were identified as F_2's using the program NewHybrids. Of the *Laricobius* spp. collected on hemlock, there was a clear pattern of asymmetrical introgression towards *L. nigrinus* (Figure 5.6). Of the *Laricobius* spp. found on white pine, there was a less discernable pattern towards *L. rubidus* (Figure 5.7).

Site factors affecting hybridization

Mixed regression analysis showed that the number of years that *L. nigrinus* was present at the site was the only factor related to percent of hybrids (Figure 5.8; $P = 0.0025$, $r^2 = 0.30$). The other factors (number of *L. nigrinus* released at the sites, WP TPA, HEM BA/Acre, Latitude, and Longitude) did not significantly affect the percent hybrids found at these sites (Table 5.2).

5.4 Discussion

Potential Outcomes of Hybridization

Genetic Assimilation/ Displacement

Genetic assimilation is the loss of a species as introgression occurs from a related species causing one to become genetically more like the other. If *L. nigrinus* and/or *L. rubidus* were to become assimilated through hybridization this could result in the loss of *L. rubidus* and/or *L. nigrinus* as distinct species at sites where *L. nigrinus* has been released. It is possible that *L. rubidus* could be completely lost throughout its range, depending on *L. nigrinus* dispersal. The loss of either species may be less likely though, because proportions of the two species vary from site to site (e.g. number of *L. nigrinus* released) and local selection pressures also vary (e.g. percent of hemlock and/or white pine present) (Harrison and Rand 1989). Factors such as these may promote the persistence of the two distinct species, reducing the probability of assimilation and extinction (Harrison and Rand 1989).

Assimilation could have other effects, such as introgression; gene flow from one species to another. Introgression could result in hybrids that have no host preference, or preference for HWA or PBA. If the hybrids choose to feed on HWA rather than PBA, populations of PBA may

increase, potentially resulting in increased damage to eastern white pine. This result may depend upon whether or not *L. rubidus* maintains its genetic integrity as a pure species and/or the importance of other natural enemies, such as *Leucopis* spp., in the maintenance of PBA populations. If the hybrids feed on PBA instead of HWA, this could result in a decrease in the efficacy of *L. nigrinus* as a biological control agent and/or affect other native enemies of PBA. The increase in *L. nigrinus* populations over time suggests that this species is not becoming assimilated through hybridization, but the decrease in populations of *L. rubidus* could potentially be due to assimilation through hybridization/ introgression.

The decrease in *L. rubidus* populations over time could also be due to another factor; displacement. Displacement could occur if *L. nigrinus* outcompetes *L. rubidus*. This is thought to have happened in another system following release of a biological control agent of the chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu). In this case, the early spring strain of *Torymus beneficus* Yasumatsu et Kamijo was displaced by *Torymus sinensis* Kamijo (Yara et al. 2007). Using COI and ITS2 sequences, the two species (including both the early and late spring strains of *T. beneficus* were found to have very little genetic variation, and were found to be capable of mating and producing hybrid progeny (Yara 2004, 2006). Evidence suggested that hybridization was not a main factor in the displacement of *T. beneficus* by *T. sinensis*, but instead, competition in relation to reproductive potential (Yara et al. 2007). As with assimilation, displacement of *L. rubidus* by *L. nigrinus* could have similar effects on PBA and in turn, eastern white pine.

Reinforcement of Premating Isolation

Reinforcement is an increase in premating isolation between species in response to any type of selection against hybridization, including; habitat, temporal, behavioral, mechanical, and/or gametic differences (Servedio and Noor 2003). Cain et al. (1999) suggested reinforcement may occur more easily when hybrid zones exist in a mosaic structure rather than in a tension zone. This is because the formation of hybrids over a broader region can result in stronger selection for premating isolation. However, the increased opportunities for introgression in a mosaic zone may also have the tendency to homogenize the population (Servedio and Noor 2003).

Reinforcement would be more likely if this were a mechanism that helped drive the initial divergence (Havill et al. 2012). In the case of *L. nigrinus* and *L. rubidus* it is not clear what led to their divergence. Some possibilities are: 1) allopatric divergence due to historical changes in the distributions of the conifer hosts of their adelgid prey; 2) initial sympatric divergence associated with specialization on different adelgid prey (for example on hemlock adelgids versus pine adelgids in western North America) followed by allopatric divergence; or 3) isolation by distance, if they are still found in sympatry somewhere in central North America where adelgid prey such as larch adelgids bridge the known distribution of the two species (Havill et al. 2012). If *L. nigrinus* and *L. rubidus* did diverge in allopatry, adaptations that increase reproductive isolation are unnecessary and therefore one of the last features to differentiate (Hoskin et al. 2005). If the two species still meet somewhere between their current known distributions, they may form a natural hybrid zone where gene flow between the two species is presently occurring (Havill et al. 2012).

If reinforcement is occurring between the two *Laricobius* spp., over time we will see a decrease in hybridization and eventually a termination of hybridization as reinforcement leads to permanent speciation. In this study, percent hybrids remained relatively stable; therefore we do not have evidence that reinforcement is occurring.

Hybrid Vigor

When HWA was introduced into the eastern U.S. from Japan, a new niche was created. *Laricobius* hybrids could be better adapted to this niche than their parents if they receive a preference for HWA from their *L. nigrinus* parents, and hardiness in eastern climates from *L. rubidus* (Havill et al. 2012). As stated above, hybridization was relatively stable through the three years of this study, which may suggest that the hybrids are not better adapted than their parents.

Even if the hybrids themselves are not better adapted to this new niche, hybridization could accelerate adaptation of *L. nigrinus* to the new and unoccupied niche of HWA on eastern hemlock through introgression (Havill et al. 2012). It has also been found that a species that is rare in its native habitat may become more common with increasing amount of favorable habitat in its new range (Huxel 1999). The increase in *L. nigrinus* populations over time could be due to accelerated adaptation as a result of introgression of favorable genes, or simply a numerical response to the abundance of food/ favorable habitat.

Speciation

Hybrids tend to be best adapted to conditions intermediate to that of the parental optima (Barton 2001). Extreme habitats relative to the requirements of parents, rather than habitats with characteristics intermediate between the parental niches are typically necessary for speciation to occur (Buerkle et al. 2000). In the case of *L. nigrinus*, *L. rubidus,* and their hybrids, no extreme habitat is known to exist. Additionally, because divergence between the two species is low, there may be little chance of major novelties arising in their hybrids (Abbott et al. 2013). Given these factors, it is unlikely that *L. nigrinus* x *L. rubidus* hybrids will form a new species distinct from their parent species.

Possible outcome of hybridization based on current evidence

Based on the data presented here, two possible evolutionary outcomes of the interaction between *L. nigrinus* and *L. rubidus* may be: 1) *L. nigrinus* and *L. rubidus* will maintain stable hybrid zones with occasional hybridization between the two species; or 2) *L. nigrinus* may displace *L. rubidus* on hemlock at sites where they are both present.

These two scenarios seem possible, because there has been an increase in *L. nigrinus* populations, a decrease in *L. rubidus* populations, and steady levels of hybridization on hemlock, while the data from both hemlock and white pine do not necessarily show a decrease in *L. rubidus* populations. If assimilation or hybrid vigor were occurring, we would expect to see an increase in the number of hybrids. If reinforcement were occurring we would expect to see a decrease in hybridization. Whether *L. nigrinus* has become more abundant due to accelerated adaptation as a result of introgression or simply due to the abundance of available food (numerical response) is unknown.

It is clear from the analysis of *Laricobius* spp. distribution on hemlock vs. white pine (Figure 5.5) and from the histograms showing species assignment for *Laricobius* collected on hemlock and white pine (Figures 5.6 and 5.7) that *L. nigrinus* prefers hemlock habitat while *L. rubidus* may not have a preference. This suggests that isolation between the two species due to habitat preference may not occur, but stable hybrid zones may still persist if *L. nigrinus* displaces *L. rubidus* on hemlock and occasional gene flow continues between the species.

The only site factor that had any relationship with percent of hybrids found at these sites was the number of years *L. nigrinus* was present at the site. This asserts that if you release *L. nigrinus*, hybridization will occur and it will occur at an increasing rate, perhaps to a plateau, as *L. nigrinus* becomes more abundant and contacts between *L. nigrinus* and *L. rubidus* become more frequent.

While the evidence collected here may point to the potential outcomes of hybridization listed above, there may be several other factors affecting the results of this study: 1) the analysis used to determine the identities of the *Laricobius* spp. is coarse, and it is possible that later generation backcross hybrids were not detected. Therefore it is possible that some beetles identified as pure parental species were in fact hybrids (Havill et al. 2012); 2) changes in weather conditions (eg. precipitation and temperature) from year to year during collection could have had an effect on the number of *L. nigrinus*, *L. rubidus*, and hybrids collected. This would depend upon the climatic preference of each species; an attribute that is not well known in relation to *L. rubidus* or the hybrids; 3) it is possible that the decrease in *L. rubidus* populations from 2010-2012 was due

to a decrease in PBA (Figure 5.3). It is unknown whether PBA showed a similar decrease from 2007-2009 to explain the decrease in population levels of *L. rubidus* during this time as well; and 4) it is likely that not enough time has passed since the two *Laricobius* spp. were placed back into secondary contact to determine what the final evolutionary outcome will be.

Conclusion

It is clear from the interaction between *L. nigrinus* and *L. rubidus* that these closely related species can and will hybridize. Future biological control projects should consider the potential of hybridization between the biological control agent under assessment and any related native species. Continual monitoring of hybridization between *L. nigrinus*, *L. rubidus,* and their hybrids will be necessary to determine if any of the probable evolutionary outcomes listed above are indeed occurring, or if new data suggests an alternative outcome.

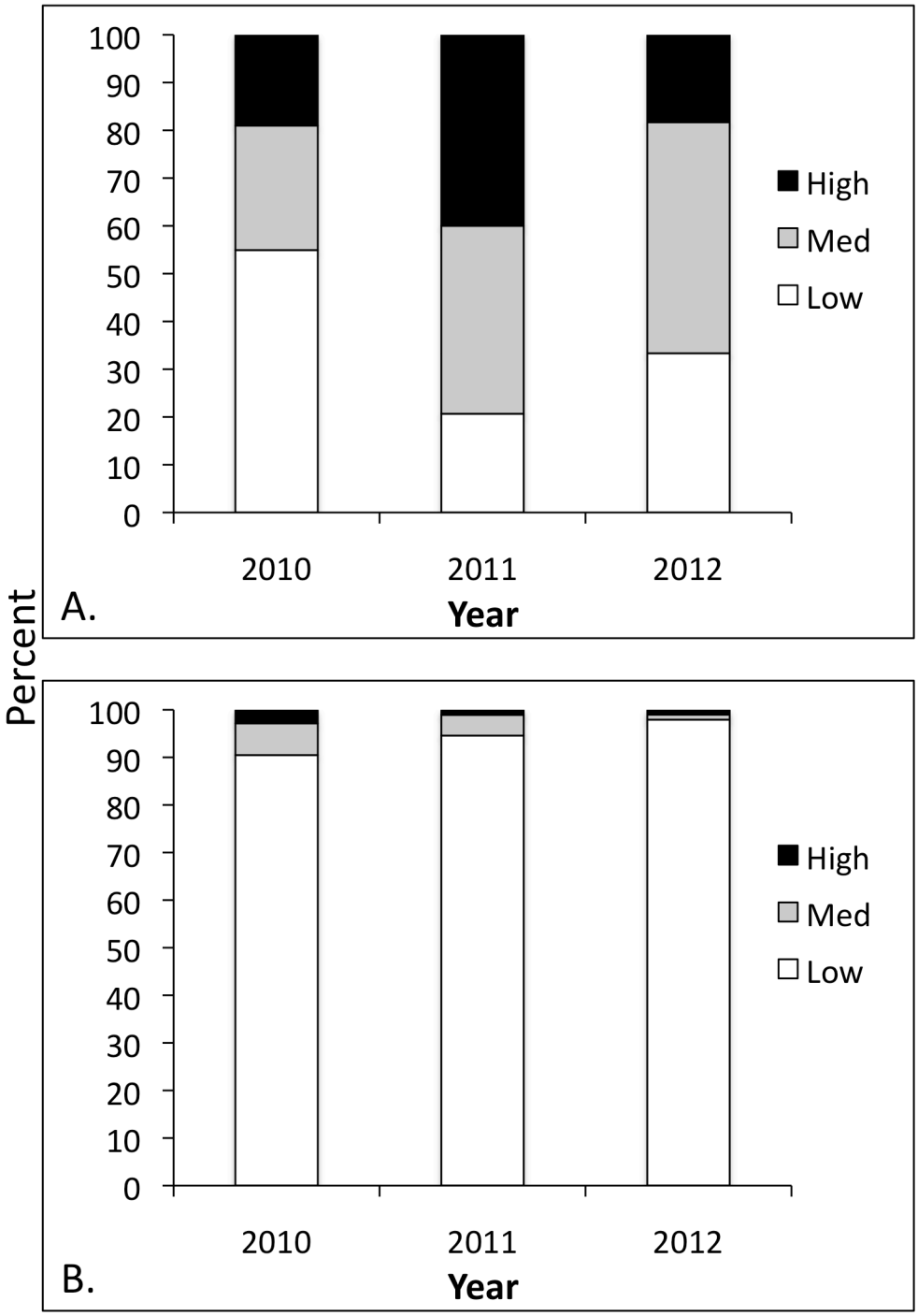

Figure 5. 1. (A.) Percent of hemlock trees/ points sampled in 2010-2012 that had a low, medium, or high infestation of hemlock woolly adelgid (low: <10 adelgids, medium: 11-50 adelgids, high: > 50 adelgids on a 30-cm long portion of branch) and (B.) Percent of eastern white pine trees/ points sampled in 2010-2012 that had a low, medium, or high infestation of pine bark adelgid (low: ≤ 5 adelgids, medium: 6-10 adelgids, high: > 11 adelgids on a 30-cm long portion of branch).

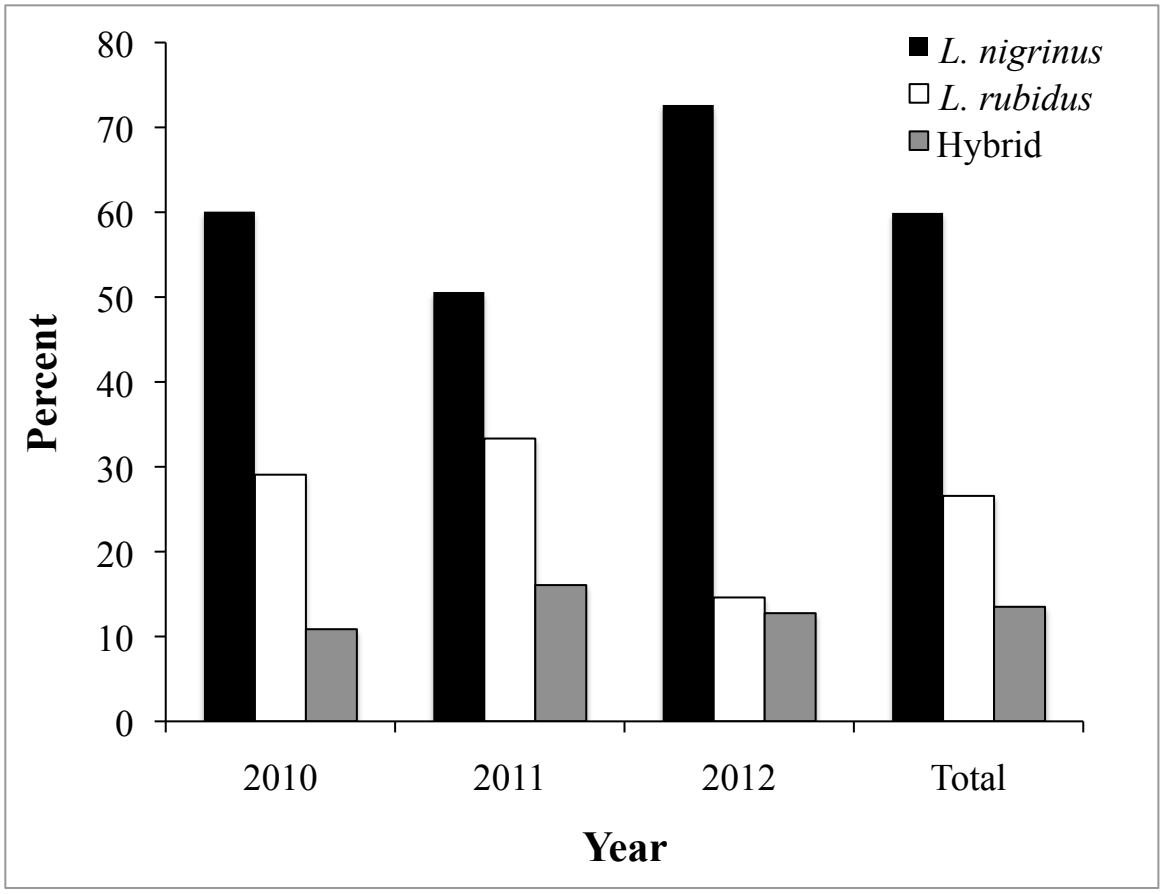

Figure 5. 2. Distribution of *Laricobius* beetles (*L. nigrinus*, *L. rubidus*, and their hybrids) found on hemlock and white pine for 2010, 2011, 2012 and all three years (Total). The distribution of collections among the three *Laricobius* spp. each year (2010-2012) was significant ($\chi^2 = 80.040$, df = 4, $p < 0.0001$).

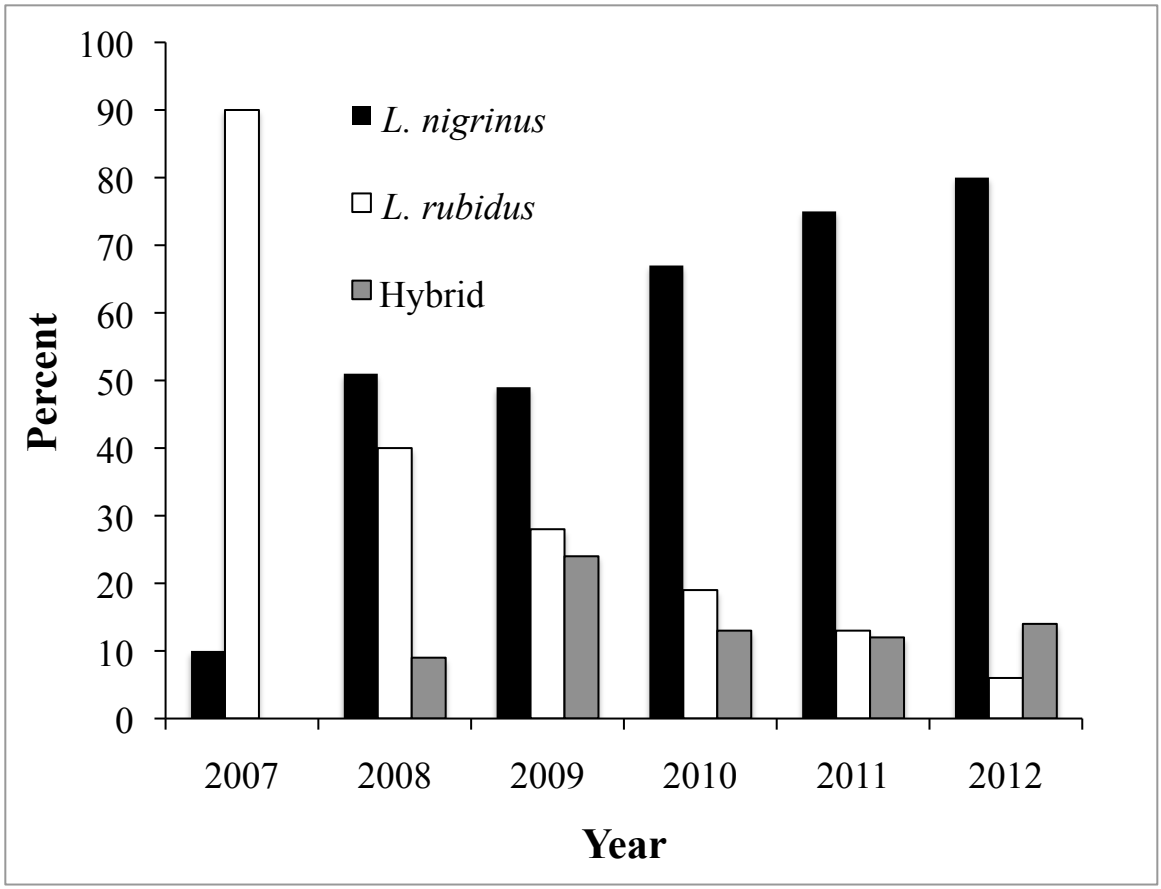

Figure 5. 3. The average percent of *L. nigrinus*, *L. rubidus*, and hybrids found on hemlock at the TN, NC, and Rothrock, PA sites from 2007-2012.

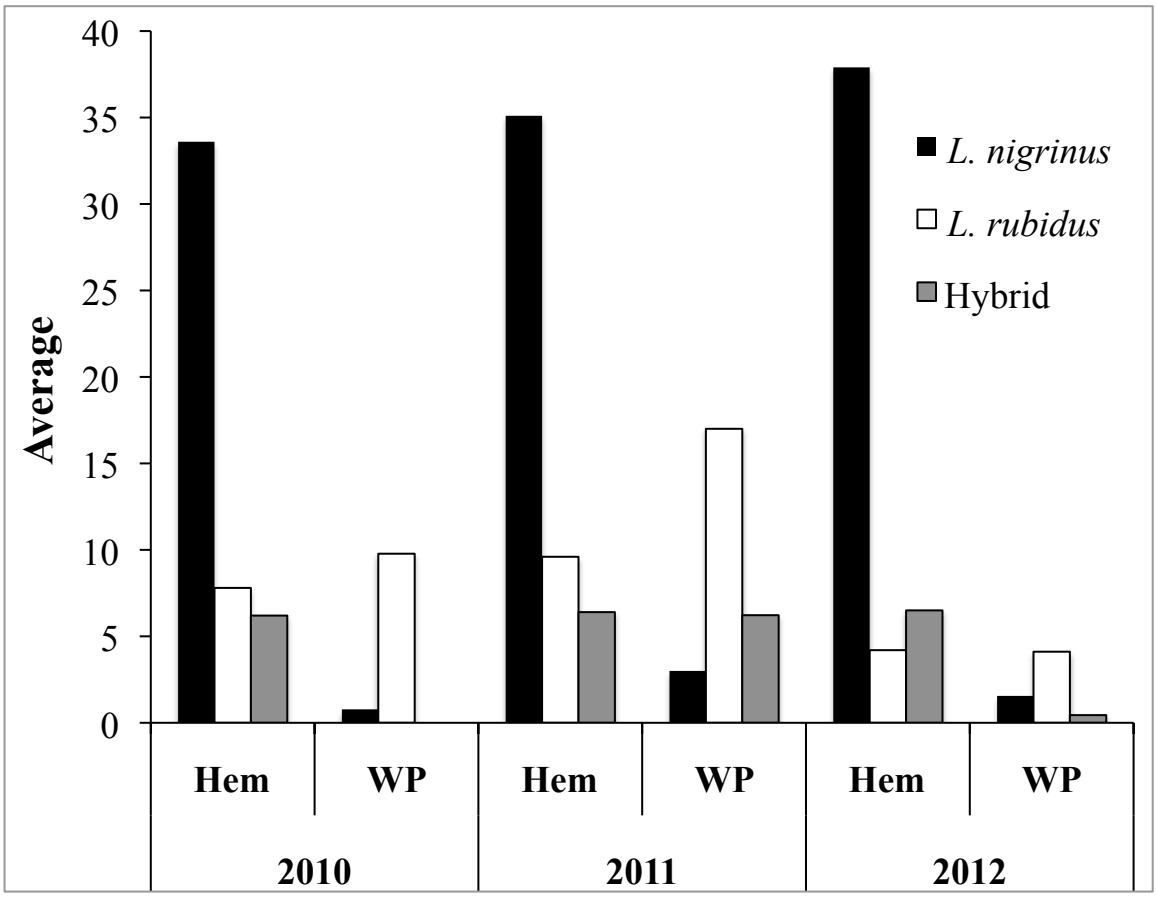

Figure 5. 4. The average number of *L. nigrinus*, *L. rubidus*, and hybrids found on hemlock vs. white pine in 2010, 2011, and 2012.

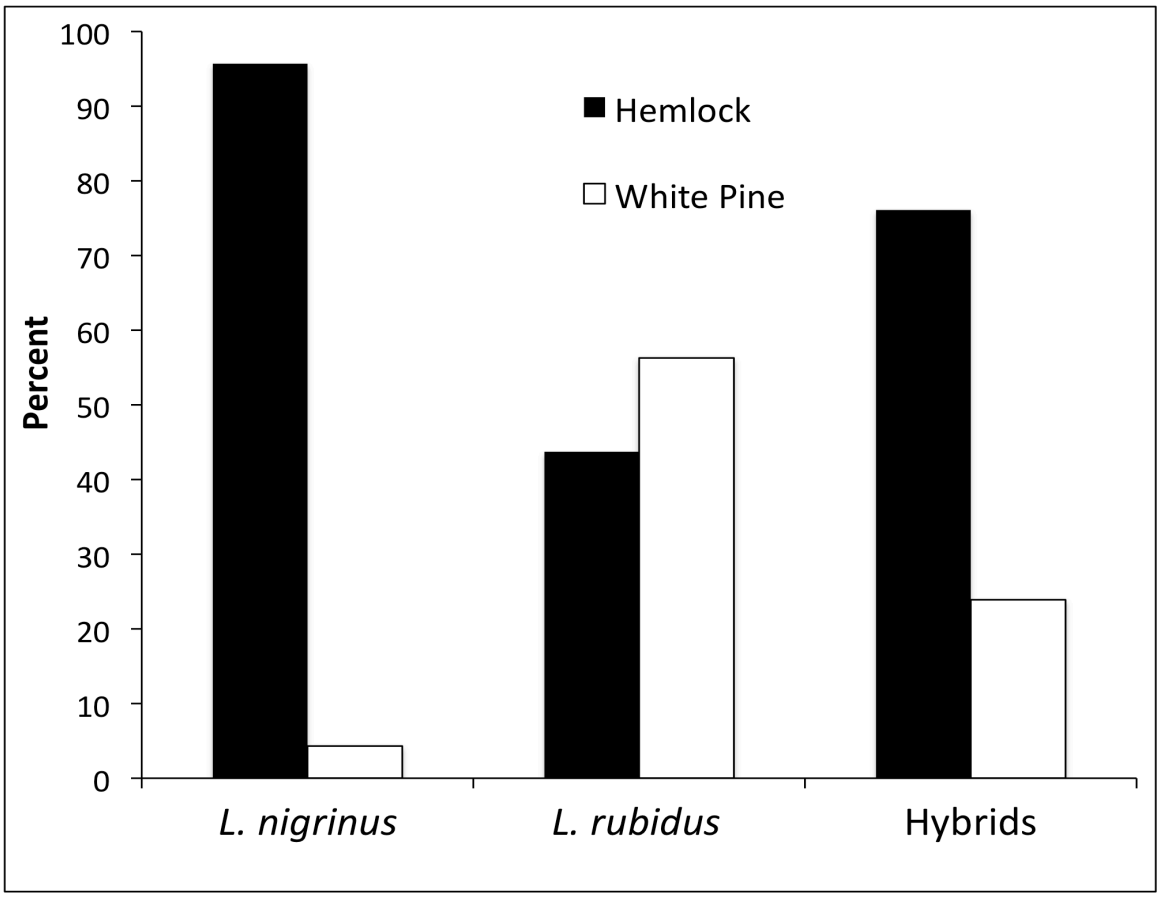

Figure 5. 5. Distribution of *L. nigrinus*, *L. rubidus*, and their hybrids found on hemlock vs. white pine from 2010-2012. Contingency analysis showed that there was a significant difference in the distribution ($\chi^2 = 550.306$, df = 2, $P < 0.0001$).

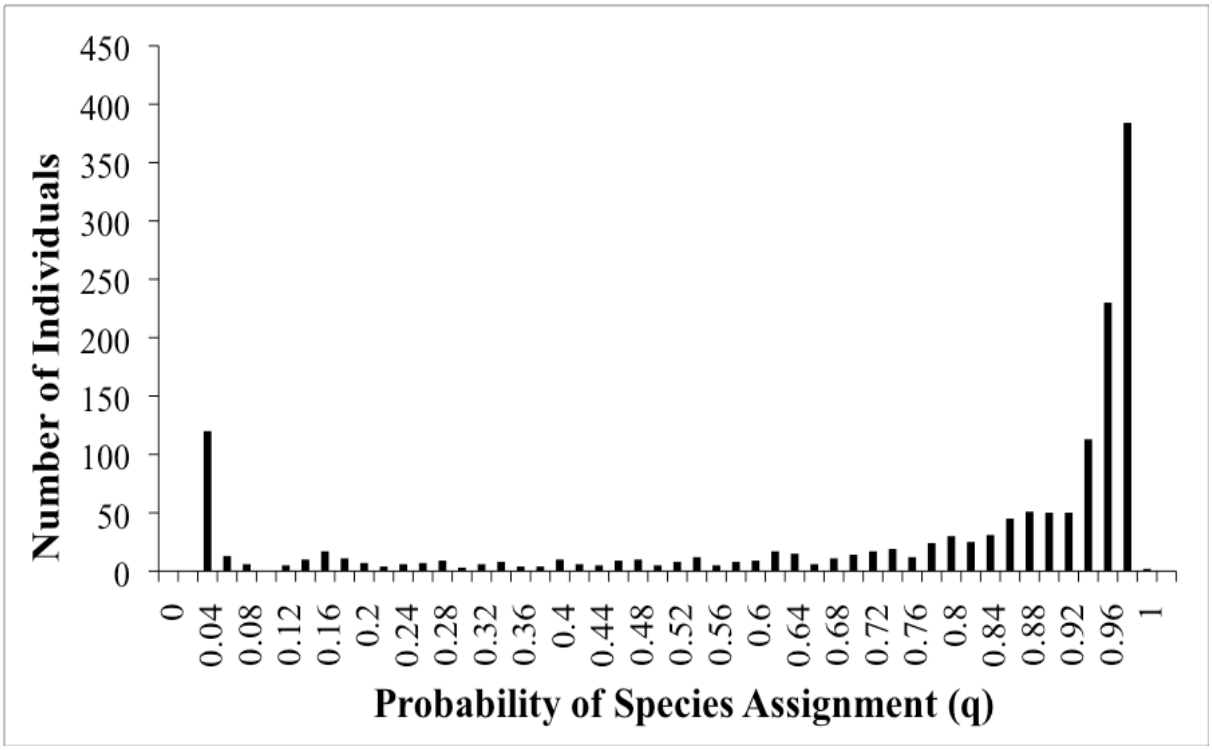

Figure 5. 6. Histogram of the probability of species assignment (q) resulting from analysis of *Laricobius* spp. collected on hemlock. A value of zero represents pure *L. rubidus*; a value of one represents pure *L. nigrinus*; .50 represents an F_1 hybrid.

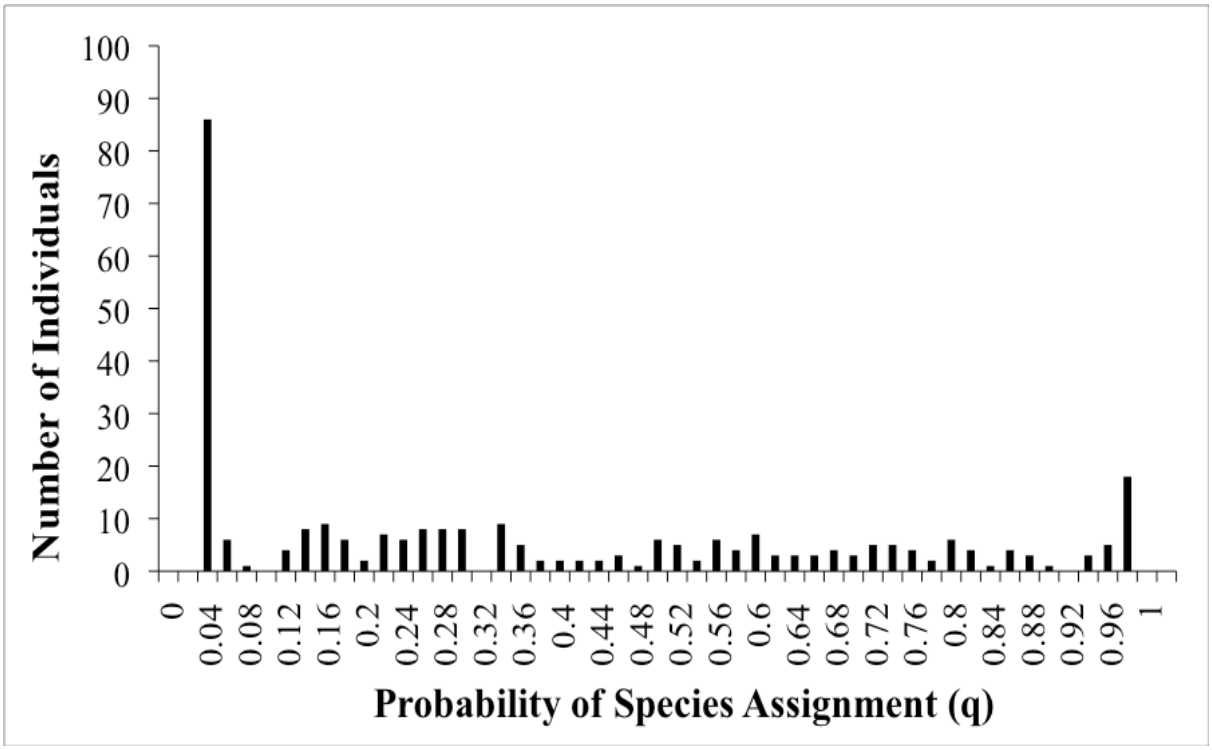

Figure 5. 7. Histogram of the probability of species assignment (q) resulting from analysis of *Laricobius* spp. collected on white pine. A value of zero represents pure *L. rubidus*; a value of one represents pure *L. nigrinus*; .50 represents an F_1 hybrid.

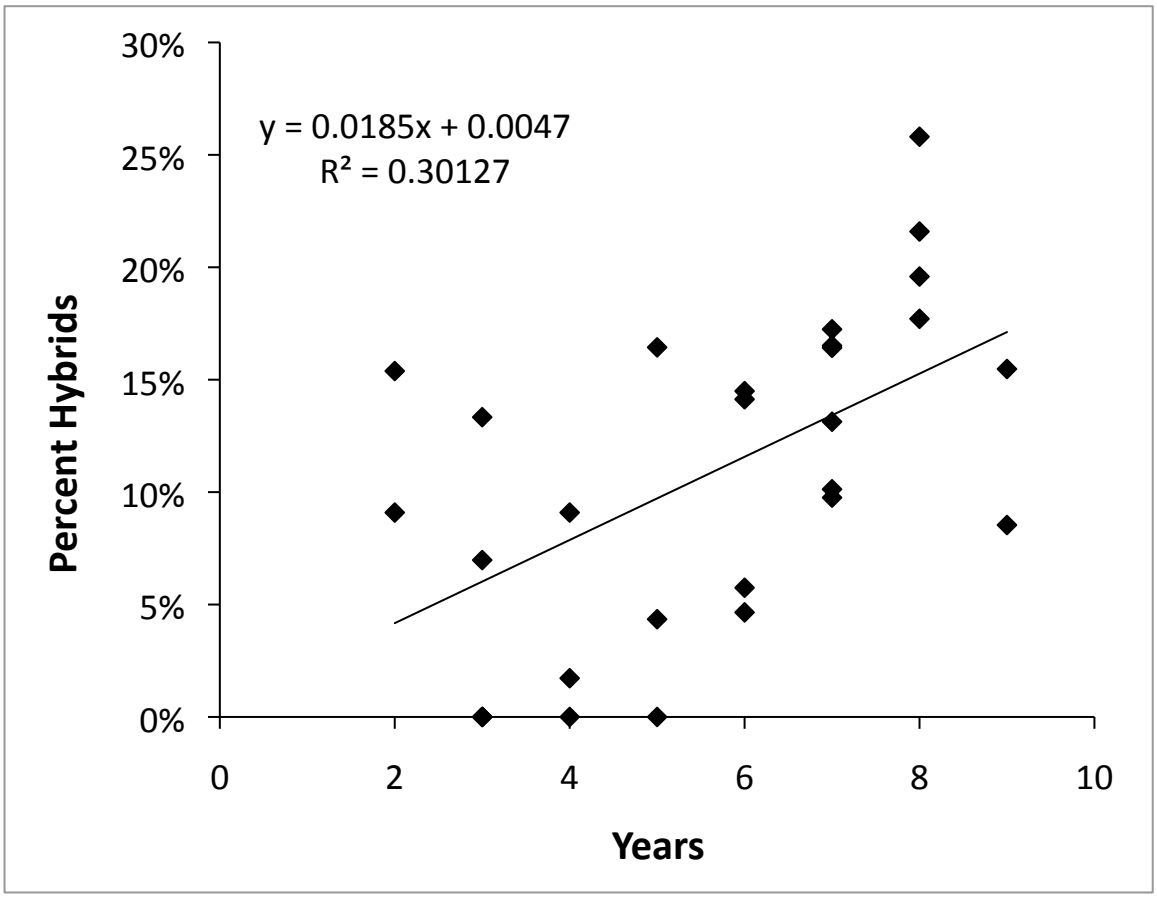

Figure 5. 8. Relationship between percent hybrids and the number of years *L. nigrinus* had been present at 10 sites from 2010-2012.

Table 5. 1. Ten sites used to determine if hybridization between *L. nigrinus* and *L. rubidus* is occurring, how it is changing over time, the host preference of hybrids in a natural setting, and the site factors that may be driving hybridization.

			UTM Coordinates (WGS84)		
State	Forest/Park	Site Name	Zone	Easting	Northing
TN	Great Smoky Mtn. Natl. Park	Laurel Creek	17S	0250526	3943370
NC	Pisgah Natl. Forest	Middle Creek	17S	0389830	3961853
VA	Jefferson Natl. Forest	Burns Creek	17S	0363081	4087612
VA	Jefferson Natl. Forest	Devil's Fork	17S	0354579	4076103
VA	Jefferson Natl. Forest	North Fork	17S	0542872	4144202
MD	Rocky Gap State Park	Rocky Gap	17S	0699934	4397022
PA	Bald Eagle State Forest	Bear Run	18T	0308873	4530115
PA	Bald Eagle State Forest	Poe Valley	18T	0293943	4522334
PA	Rothrock State Forest	Rothrock	18T	0268595	4504040
PA	Rothrock State Forest	Treatser Valley	18T	0299124	4518831

Table 5. 2. Results of a mixed regression analysis examining the relationship between the site factors listed and the percent of *Laricobius nigrinus* x *L. rubidus* hybrids collected at 10 sites from 2010-2012.

Site Factors	nDF	F Ratio	Prob > F
Number of years *L. nigrinus* was present at site	1	11.2100	0.0025
# of *L. nigrinus* released	1	0.8130	0.3754
White pine trees/acre	1	1.8820	0.1818
Hemlock basal area/acre	1	0.0000	0.9866
Latitude	1	0.0010	0.9772
Longitude	1	0.0170	0.8968

Chapter 6. DNA barcode diversity and a PCR-RFLP assay to distinguish **Laricobius osakensis** *from* **Laricobius naganoensis** *(Coleoptera: Derodontidae), predators of hemlock woolly adelgid*

6.1 Introduction

Laricobius osakensis Shiyake and Montgomery was imported into the U.S. under quarantine from Japan in 2006 for study as a potential biological control agent of the invasive hemlock woolly adelgid (*Adelges tsugae* Annand). *Laricobius osakensis* has been collected in Japan in association with the same lineage of hemlock woolly adelgid that is found in the eastern U.S. (Avise 2000, Havill et al. 2006). Since *L. osakensis* may be well adapted to this particular lineage, its use as a biological control agent is promising (Lamb et al. 2011).

It has been recommended that biological control practitioners increase the probability of adaptation and success of a biological control agent by releasing specimens that exhibit as much genetic variation as possible into the new region (Phillips et al. 2008, Szűcs et al. 2012). The amount of genetic variation in the pool of *L. osakensis* released for biological control is of particular interest because hemlock woolly adelgid is established across a broad latitudinal range, from Maine to Georgia. Therefore, a biological control agent with the potential of adapting to the climates within this range would be advantageous.

Laricobius osakensis was granted release from quarantine in 2010 by the USDA, Animal and Plant Health Inspection Service (APHIS). However, in the fall of 2011 it was discovered that the colony, from field-collected beetles in Japan in 2010, was contaminated with the presence of

another Japanese species, *Laricobius naganoensis* Leschen. APHIS states in its PPQ 526 permit that insects shipped from abroad must not contain unauthorized species; therefore the presence of *L. naganoensis* within the *L. osakensis* colony resulted in the placement of the *L. osakensis* colony back into quarantine.

Laricobius naganoensis is a recently discovered species that was collected from high altitude areas of Nagano Prefecture where it is sympatric with *L. osakensis* (Leschen 2011). The morphological similarity between the two species and the considerable variation within *L. osakensis* make it difficult to differentiate these species, especially the females. *Laricobius naganoensis* is distinguished from *L. osakensis* by having pale tibiae, more vivid orange-red coloration of the elytra, and an acute median lobe of the male genitalia (Leschen 2011).

Subsequent to the discovery of *L. naganoensis* contamination in the colony, a purification protocol was established whereby the colony was divided into groups of approximately 20 adult beetles whose progeny were kept separate. Once oviposition was completed for the season, it was necessary to identify the parents to determine if there was contamination in each group. The progeny from contaminated groups would be destroyed, and pure groups would be retained. As *Laricobius osakensis* was to be released in the fall of 2012, a quick, simple, and inexpensive assay was needed to identify the parental species within the colony.

With limited time in which to test for the presence of *L. naganoensis* (June-September) before release of its progeny in the field, a non-lethal testing method was developed, allowing genetic

analysis to be performed before adults died in May/June. This involved searching for a potential source of DNA conducive to non-lethal DNA barcoding.

6.2 Methods

Specimens used for RFLP were collected from 2006 through 2011 (Table 6.1). Twelve *L. naganoensis* specimens were collected from Japan and one *L. naganoensis* was a lab-reared F_1. *Laricobius osakensis* specimens included 121 individuals collected in Japan, as well as 260 lab-reared F_1 individuals. Specimens were preserved in 95-100% ethanol. The following methods were used to process all but 93 of the 260 F_1 *L. osakensis*, which were treated using standard DNA barcoding methods (Ivanova et al. 2006). Beetle thoraces, legs, and second wings were removed and DNA was extracted using the DNA IQ extraction kit (Promega, Madison, WI) or the DNAeasy kit (Qiagen Inc., Valencia, CA). Heads, elytra, and genitalia were saved as vouchers and deposited in Yale University's Peabody Museum of Natural History.

Partial cytochrome oxidase subunit I (COI) was amplified for the 290 specimens using forward primer LepF1 and reverse primer LepR1 (Hebert et al. 2004). PCR was performed in 30 µL reactions containing 3.0 µL 10X PCR Buffer, 2.4 µL dNTPs (10 mM), 4.8 µL $MgCl_2$ (25 mM), 1.0 µL BSA (10 mg/ml), 1.0 µL of each primer (10 mM), 0.3 µL Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1.0 µL DNA template. Thermocycling conditions 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) or Exo-AP (New England Biolabs, Ipswich, MA, USA). Sequencing reactions were performed using the BigDye Terminator kit (Applied

Biosystems, Foster City, CA) and analyzed on an Applied BioSystems 3730xl automated sequencer. Sequences were aligned using the SeqMan Pro program of Lasergene 8.0 software (DNASTAR; http://www.dnastar.com). All new sequences generated from this study were deposited in GenBank (Table 6.1).

Restriction sites within *L. osakensis* and *L. naganoensis* sequences were examined using Biology WorkBench 3.2 (Subramaniam 1998) or Geneious 5.6.5 (Drummond et al. 2011), resulting in the selection of three diagnostic restriction enzymes: AluI, MboII, and BclI. Ten individuals of each species were used to validate polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) assays using these three enzymes. COI was amplified using the PCR conditions described above. Twelve and a half microliters of PCR product were added to each of the three RFLP reactions containing 0.5 µL of AluI and 1.5 µL of 10X Buffer 4, 0.5 µL of MboII and 1.5 µL of 10X Buffer 4 or 0.5 µL of BclI and 1.5 µL of 10X Buffer 3 (New England Biolabs, Ipswich, MA). Incubation was performed at 37 °C for 4 h for AluI and MboII, and at 50 °C for 4 h for BclI, followed by gel electrophoresis and visualization on a 2% agarose gel.

Dried *L. osakensis* specimens were used to determine if DNA could be amplified using non-lethal sampling for DNA extraction. A single antenna and a single tarsus were removed from five and two dead specimens respectively, to be tested as possible non-lethal DNA sources. PCR reactions contained 6.25 µL of 10% D- (+)-trehalose dihydrate (Fluka Analytical), 1.25 µL of 10X PCR buffer, 0.625 µL $MgCl_2$ (50 mM), 0.0625 µL of each 10 µM primer (LepF1, LepR1, LCO1490, HCO2198 (Folmer et al. 1994)) , 0.0625 µL of dNTPs (10 mM), 0.060 µL of 5 U/µL

PlatinumTaq DNA Polymerase (Invitrogen) and 2.0 μL of DNA template for a total reaction volume of 12.5 μL. Thermal cycling conditions were 94 °C for 1 min, followed by 5 cycles of 94 °C for 40 s, 45 °C for 40 s, 72 °C for 1 min, then 35 cycles of 94 °C for 40 s, 51 °C for 40 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min.

Sequence divergence (p-distance) was calculated within and between species using PAUP* (Swofford 2003). Diagnostic sites that distinguish the two species were determined by examining sequences in Mesquite 2.75 (Maddison and Maddison 2011). Separate haplotype networks for Japanese wild-caught *L. osakensis* and *L. naganoensis* were reconstructed using the statistical parsimony method of Templeton *et al*. (1992) using the software TCS 1.21 (Crandall et al. 1994, Crandall 1994, Clement et al. 2000). Haplotype diversity, nucleotide diversity, Tajima's D, and Fu's F_S were calculated for wild *L. osakensis* using DnaSP 5.10 (Librado and Rozas 2009). These parameters were not calculated for *L. naganoensis* due to the low sample size.

6.3 Results

Laricobius COI sequences were 702 bp long (658 with primers removed) and their alignment required no gaps. Sequence divergence within *L. osakensis* ranged from 0-2.12% with a mean of 0.74% for wild-collected specimens and a range of 0-1.97% with a mean of 0.79% for F_1 colony-collected specimens. P-distance within *L. naganoensis* ranged from 0-2.12% with a mean of 1.05%. P-distance between *L. osakensis* and *L. naganoensis* ranged from 7.33-9.45% with a mean of 8.35%. There are 17 diagnostic nucleotide sites separating the two species, all of which are pure diagnostics with fixed differences between species (Table 6.2). The COI network for

wild-caught *L. osakensis* exhibited very high haplotype diversity with 90 unique haplotypes in the 121 individuals sampled (Figure 6.1). Haplotype diversity (H) was 0.989, and nucleotide diversity was 0.0074. Tajima's D, and Fu's F_S were -2.309 and -138.282, respectively, and both were significantly different from neutral expectations ($p<0.01$). The COI network for *L. naganoensis* exhibited high haplotype diversity as well, with 12 unique haplotypes of the 13 individuals sampled (Figure 6.1).

The three enzymes correctly identified all samples of *L. osakensis* and *L. naganoensis*. Digestion by AluI produced three fragment patterns for *L. naganoensis* (Table 6.3, Figure 6.2). The sequence from one of the 13 *L. naganoensis* specimens resulted in the 3rd pattern consisting of eight fragments. PCR amplification to visualize this RFLP pattern failed and is not shown in Figure 6.2; however the fragment pattern should be clearly distinguishable from *L. osakensis*. Digestion by AluI produced ten patterns for *L. osakensis* (Table 6.3, Figure 6.2). Digestion by MboII produced one pattern for *L. naganoensis* and three patterns for *L. osakensis* (Table 6.3, Figure 6.3). Digestion by BclI produced one pattern for *L. naganoensis* and three patterns for *L. osakensis* (Table 6.3, Figure 6.3).

Of the two potentially non-lethal treatments tested, sequences were successfully recovered from all five antennae and from two of five tarsae. A second experiment using 20 *L. osakensis* antennae was completed to further test the success rate of this method. All 20 antennae produced full-length 658 bp barcodes that matched *L. osakensis*.

6.4 Discussion

Laricobius naganoensis and *L. osakensis* are not closely related (p-distance = 8.35%). This is encouraging for biological control of hemlock woolly adelgid because it is unlikely that the two species could hybridize, an event that has occurred in recent biological control efforts involving other *Laricobius* spp. (Havill et al. 2012). In addition, the many different *L. osakensis* haplotypes suggest a high adaptive potential, which is an advantageous quality for a biological control agent. Colony maintenance and augmentation should seek to maintain this high diversity.

Recent population expansion is implied by high haplotype diversity, low nucleotide diversity, negative values for both Tajima's D test and Fu's F_s test, as well as the haplotype network showing low levels of sequence divergence and a high frequency of unique mutations (Avise 2000, Halliburton 2004). Recent population expansion following the last glacial period is a pattern that has been observed in several organisms in Japan and throughout the northern hemisphere (Hewitt 2000, Liu et al. 2006, Kawamoto et al. 2007). Climatic conditions during the last glaciation reduced the available habitat for many species through contraction of forests (Tsukada 1983, Hewitt 2000). Following this period, evidence suggests that the distributional range of many organisms expanded along with available habitat (Hewitt 2000, Liu et al. 2006, Kawamoto et al. 2007).

Without the proper permits *L. naganoensis* cannot be released legally in the U.S. Therefore, distinguishing between *L. osakensis* and *L. naganoensis* is important for quarantine facilities, universities, state, and/or federal agencies that are currently, or will in the future, be importing *L. osakensis* from Japan for biological control of hemlock woolly adelgid. The RFLP assay

developed here is less expensive and less time consuming than sequencing and the tools needed for this assay are available in most labs.

Our results show that a single antenna from a *Laricobius* specimen is sufficient for recovery of full-length 658 bp barcodes. Several samples consisted of less than an entire antenna; one consisted of only two segments, suggesting that *Laricobius* antennae are DNA rich and an excellent potential tissue source for non-lethal barcoding. Future experiments using live specimens will be necessary to determine if the removal of a single antenna will impact beetle survival and reproduction in the lab.

The extensive mitochondrial variation within *L. osakensis* and the low number of *L. naganoensis* samples available for this study suggest that we have not sampled all of the variation within these species. Continued collections of both species in Japan and genotyping of any new stock added to colonies will be necessary to fully characterize the diversity within both species.

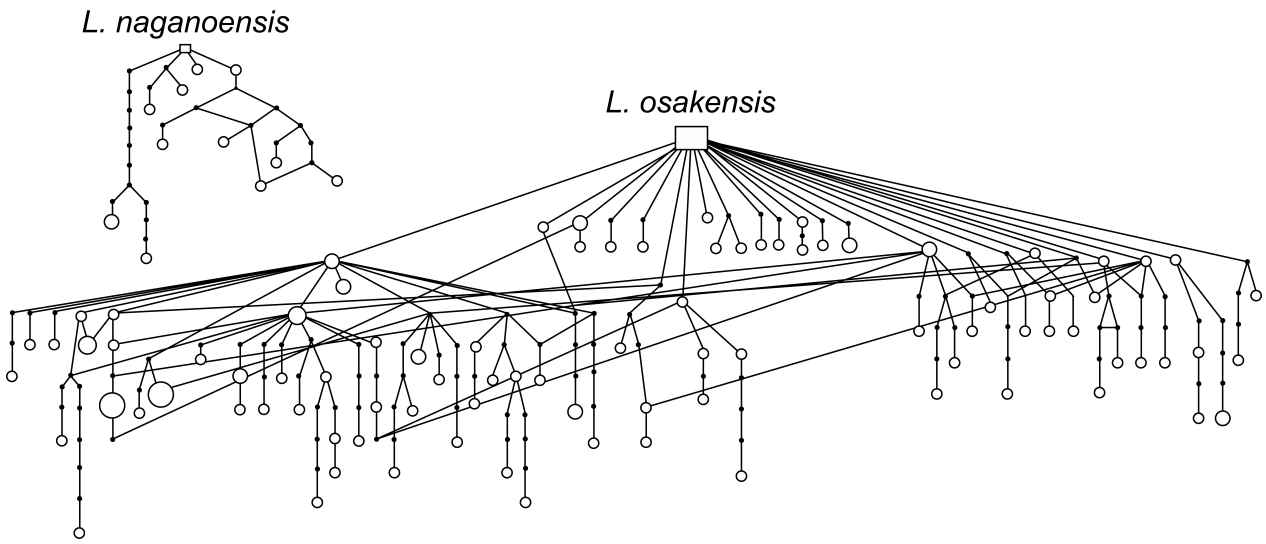

Figure 6. 1. TCS network showing relationships among COI haplotypes for *L. naganoensis* (12 unique haplotypes) and wild-caught *L. osakensis* samples (90 unique haplotypes). The squares denote the inferred ancestral haplotypes. The size of each shape is proportional to the frequency of the haplotype. Small black dots represent unsampled intermediate haplotypes.

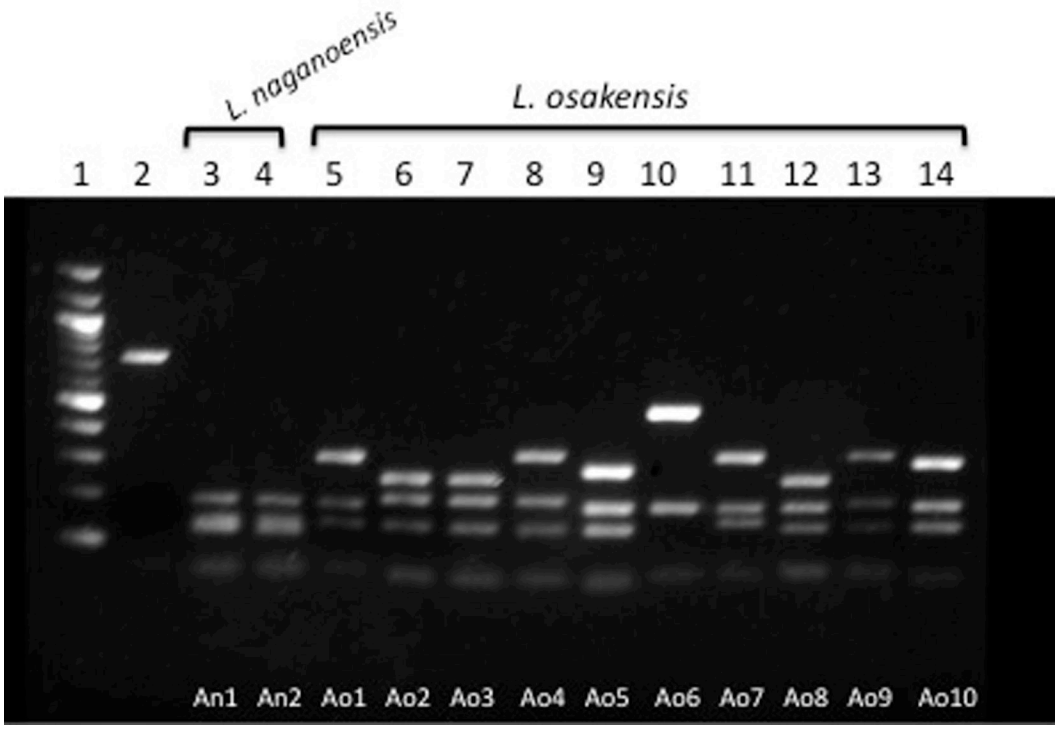

Figure 6. 2. Agarose gel electrophoreses of *L. naganoensis* and *L. osakensis* COI PCR products digested with AluI. Lane one is a 100 bp ladder (New England Biolabs, Ipswich, MA) and lane two is an undigested control.

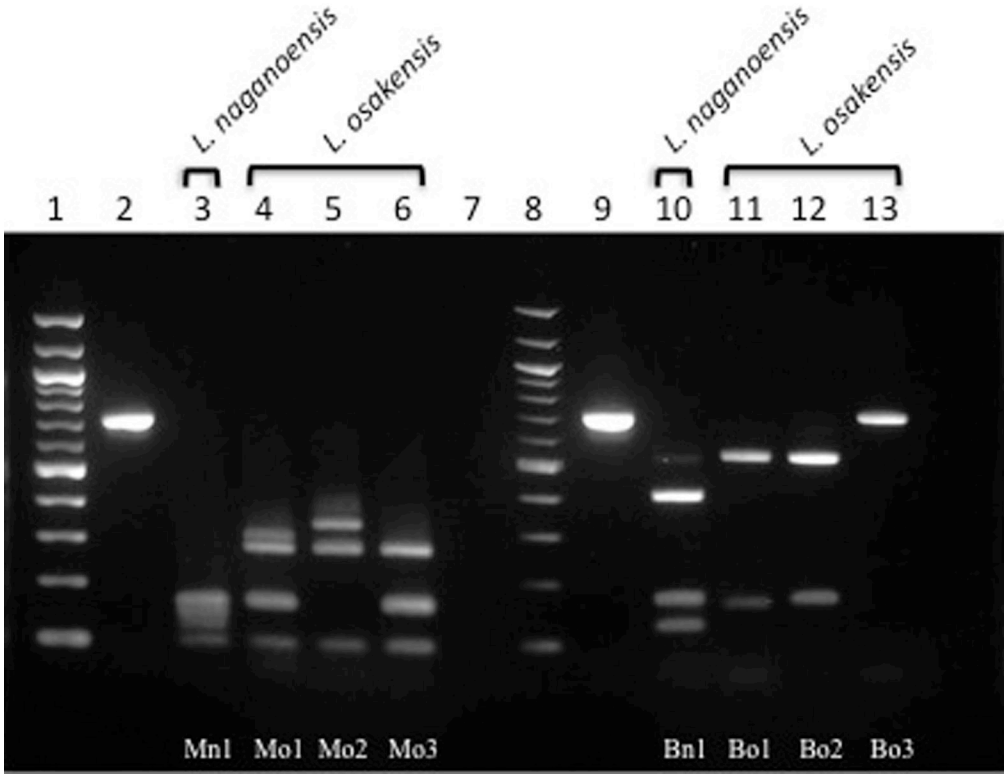

Figure 6. 3. Agarose gel electrophoreses of *L. naganoensis* and *L. osakensis* COI PCR products digested with MboII (lanes 3-6) and BclI (lanes 10-13). Lanes one and eight are 100 bp ladder (New England Biolabs, Ipswich, MA) and lanes two and nine are undigested controls.

Table 6. 1. Specimen accession numbers and collection information for *Laricobius osakensis* (*Lo*) and *L. naganoensis* (*Lnag*).

Genbank Accession No.	# of specimens	Species	Collection Information
JX871945	1	*Lnag*	Japan; Nagano Prefecture; Fujimi; Mt. Nyukasa; 1 June 2008; Coll. N.P. Havill, S. Shiyake, A. Lamb
JX872066 and JX872067	2	*Lnag*	Japan; Nagano Prefecture; Matsumoto; Mt. Norikura;15 October 2008; S. Shiyake, A. Lamb
JX872068-JX872070	3	*Lnag*	Japan; Nagano Prefecture; Ootaki; Mt. Ontake; 29 October 2009; A. Lamb, S. Shiyake
JX872071	1	*Lnag*	Japan; Nagano Prefecture; Yamanouchi; Shiga-kogen; 31 October 2010; A. Lam, S. Shiyake, C. Jubb
JX872072-JX872075	4	*Lnag*	Japan; Gunma Prefecture; Katashina; Mt. Nikko-Shirane; Marunuma-Kogen Ski Hills; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX871946	1	*Lnag*	Virgina Tech Department of Entomology Insect Rearing Facility; October 2010; M. Fischer
KC182379	1	*Lnag*	Virginia Tech Department of Entomology Insect Rearing Facility; December 2011; C. Jubb
HM803301-HM803307	7	*Lo*	Japan; Osaka Prefecture; November 2006; A. Lamb
HM803466	1	*Lo*	Japan; Hyogo Prefecture; Kobe; Arima Onsen; 7 January 2008; S. Shiyake, A. Lamb
HM803464 and HM803465	2	*Lo*	Japan; Kochi Prefecture, Tosa-Yamada; Hokigamine Forest Park; 6 January 2008; S. Shiyake, A. Lamb
HM803461	1	*Lo*	Japan; Hyogo Prefecture; Kobe; Kobe Municipal Arboretum; 9 January 2008; S. Shiyake, A. Lamb
HM803463	1	*Lo*	Japan; Osaka Prefecture; Takatsuki; Nakahata; 9 January 2008; S. Shiyake, A. Lamb
HM803467	1	*Lo*	Japan; Hyogo Prefecture; Kobe; Kobe Municipal Arboretum; 14 January 2008, A. Lamb
HM803462	1	*Lo*	Japan; Nara Prefecture; Nara Park; Wakakusa-Yama; 18 January 2008; A. Lamb
JX872076 and JX872077	2	*Lo*	Japan; Miyazaki Prefecture; Gokase; Gokase Ski Hills; 26 May 2008, N.P. Havill, S. Shiyake, A. Lamb

Table 6.1 Continued

Genbank Accession No.	# of specimens	Species	Collection Information
JX872078 and JX872079	2	Lo	Japan; Nagano Prefecture, Fujimi, Mt. Nyukasa, 1 June 2008, N.P. Havill, S. Shiyake, A. Lamb
JX872080 and JX872081	2	Lo	Japan; Yamanashi Prefecture, Hokuto; Yokote; Yokote Shrine, 2 June 2008; N.P. Havill, S. Shiyake, A. Lamb
HM803616	1	Lo	Japan; Nagano Prefecture, Shigakogen, Yamanouchi, 6 May 2009, S. Shiyake, A. Lamb
HM803617	1	Lo	Japan, Nagano Prefecture, Mt. Norikura, Matsumoto, 4 April 2009, S. Shiyake, A. Lamb
HM803618	1	Lo	Japan; Tochigi Prefecture; Nikko; Konsei Pass; 3 April 2009; S. Shiyake, A. Lamb
HM803681 and HM803682	2	Lo	Japan; Nagano Prefecture; Mt. Norikura; Matsumoto; 28 October 2009; A. Lamb, S. Shiyake
HM803683	1	Lo	Japan; Tochigi Prefecture; Nikko; Konsei Pass; 1 November 2009; A. Lamb, S. Shiyake
HM803684	1	Lo	Japan, Nagano Prefecture, Yamanouchi; Shiga-kogan; 30 October 2009; A. Lamb, S. Shiyake
HM803685	1	Lo	Japan, Tochigi Prefecture; Nikko; Nikko-Yumoto Spa; 31 October 2009; A. Lamb, S. Shiyake
JX872082-JX872084	3	Lo	Japan; Gunma Prefecture; Katashina; Mt. Nikko-Shirane; Marunuma-Kogen Ski Hill; 31 October 2009; S. Shiyake, A. Lamb
JX872085 and JX872086	2	Lo	Japan; Nagano Prefecture; Yamanouchi; Shiga-kogen; 30 October 2009; S. Shiyake, A. Lamb
JX871947-JX871960	19	Lo	Virginia Tech Department of Entomology Insect Rearing Facility; December 2009; M. Fischer, N. Morris

Table 6.1 Continued

Genbank Accession No.	# of specimens	Species	Collection Information
JX871961-JX872041	81	Lo	Virginia Tech Department of Entomology Insect Rearing Facility; October 2010; M. Fischer, P. Thomasson
JX872042-JX872065	24	Lo	Japan; October 2010; A. Lamb, S. Shiyake, C. Jubb
JX872087-JX872111	25	Lo	Japan; Nagano Prefecture; Yamanouchi; Shiga-kogen; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX872112-JX872130	19	Lo	Japan; Gunma Prefecture; Katashina; Mt. Nikko-Shirane; Marunuma-Kogen Ski Hills; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX872131-JX872157	27	Lo	Japan; Tochigi Prefecture; Nikko; Yumoto Spa; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX872215-JX872222	8	Lo	Virginia Tech Department of Entomology Insect Rearing Facility; October 2011; M. Fischer, M. Cassell
JX872211-JX872214	4	Lo	Virginia Tech Department of Entomology Insect Rearing Facility; 28 October 2011; M. Fischer
JX872158-JX872210, KC182379-KC182473	53	Lo	Virginia Tech Department of Entomology Insect Rearing Facility; December 2011; C. Jubb

Table 6. 2. Diagnostic characters to distinguish *L. naganoensis* from *L. osakensis*. Results are based on 702 base pairs of the mitochondrial COI gene sequenced from 381 *L. osakensis* individuals and 13 *L. naganoensis* individuals. Nucleotide position is based on the entire amplified region with primer sequences included.

Nucleotide Position	*L. naganoensis* nt	*L. osakensis* nt
29	T	C
74	T	C
81	T	C
86	A	T
131	T	C
302	C	T
347	T	A
362	T	A
500	A	T
503	A	T
510	C	T
512	T	A
567	C	T
573	C	T
582	C	T
593	T	A
683	C	T

Table 6. 3. Fragment patterns produced by the restriction enzymes AluI, MboII, and BclI for *Laricobius naganoensis* and *L. osakensis*.

Enzyme	Species	Pattern ID	# of samples	Fragment Pattern (base pairs)
AluI	*L. naganoensis*	An1	10	6,12,15,45,48,124,126,144,189
		An2	2	6,6,12,15,45,48,118,126,144,189
		An3	1	6,15,45,48,124,126,144,201
	L. osakensis	Ao1	238	6,12,48,144,189,310
		Ao2	1	6,48,60,144,201,250
		Ao3	5	6,15,45,48,144,201,250
		Ao4	16	6,48,144,201,310
		Ao5	4	6,12,36,48,189,274
		Ao6	4	6,12,48,189,454
		Ao7	1	6,48,156,189,310
		Ao8	16	6,12,48,60,144,189,250
		Ao9	2	12,48,144,195,310
		Ao10	2	6,12,15,48,144,189,295
MboII	*L. naganoensis*	Mn1	11	103,129,146,165,170
	L. osakensis	Mo1	283	103,165,170,274
		Mo2	2	103,274,334
		Mo3	3	165,170,376

Table 6.3 Continued

Enzyme	Species	ID	# of samples	Fragment Pattern
BclI	*L. naganoensis*	Bn1	11	132,175,410
	L. osakensis	Bo1	283	175,538
		Bo2	4	14,165,538
		Bo3	1	709

Chapter 7. Summary

The use of classical biological control as a tool to help manage invasive species can be very successful, but does include risks that need to be addressed prior to releasing the agent. One of these risks may be hybridization between the biological control agent and any closely related native species present at the release site/s. In the past, morphological characteristics were often used to determine if an agent being considered for release was related to any native species present. Although this method was useful, it was not overly accurate. The use of molecular methods to determine phylogenetic relationships has allowed researchers in biological control to more accurately derive relationships and determine the probability of hybridization between an agent that is to be released and any closely related native species. Future biological control projects should determine if there are any native species closely related to the agent prior to its release and if any are identified, steps should be taken to determine if hybridization or fitness costs as a result of attempted interspecific copulation occur.

The effects of hybridization should continue to be monitored for population changes among *L. nigrinus*, *L. rubidus* and their hybrids on hemlock and white pine. This information will help determine not only the effects to biological control of hemlock woolly adelgid, but also give insight into the evolutionary process as a whole.

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Appendices

Appendix A. Collecting and rearing **Laricobius rubidus**

Methods and Results

A search was initiated in the field for *L. rubidus* adults to begin a lab colony in March 2010 because the beetles were found to be active from March 24th to June 1st in a previous study (Clark and Brown 1960). After persistent searching through May 2010, only 26 adult *L. rubidus* were found. These beetles were collected at the Coal Mining Heritage Park in Blacksburg, VA and placed together in the lab to produce F_1 progeny for the following year. The colony was reared in the same fashion as *L. nigrinus* (Salom et al. 2012) except that the *L. rubidus* colony was fed both HWA and PBA. While PBA is its main host, only a limited number of PBA individuals are found on the branch tips of white pine. HWA was added to supplement their diet, since it was found to be a suitable host for *L. rubidus* in a previous study (Zilahi-Balogh et al. 2005).

F_1 adults emerged from October through December 2010. Emergence was at 20.8%, resulting in only 15 *L. rubidus* adults, six of which died before mating experiments began. The remaining *L. rubidus* colony members were mated on December 28th 2010. Crosses consisted of six F_1 Ln x Lr and two F_1 Lr x Lr. All beetles died within a week (by January 7th 2011), producing no eggs.

Because such a low number of *L. rubidus* were collected in the spring of 2010, the search for *L. rubidus* adults began in October the following season. Five *L. rubidus* were collected in early December and during the third week of February 2011, 101 were collected from Dismal Falls, VA. Sixty-one of the wild-caught *L. rubidus* were used to begin a new colony in the spring of

2011. In order to determine if diet had an effect on survival, the colony was divided into thirds and one third was fed PBA, another, HWA and PBA, and the last was fed HWA only.

Between November and December 2011, 43 *L. rubidus* adult progeny emerged (13.6% total emergence of adults from prepupae). The highest percent emergence was of progeny from funnels containing PBA-infested white pine (Table A.1).

Table A. 1. The number of *L. rubidus* prepupae and adults and percent emergence of adults that developed on hemlock woolly adelgid (HWA) and/or pine bark adelgid (PBA) in 2011.

	Prepupae	Adults	% Emergence
PBA	138	16	11.59
HWA	74	4	5.41
HWA & PBA	64	2	3.13

Discussion

It appears that the life cycle of *L. rubidus* in VA may be different from that reported in the previous study in New Brunswick, Canada (Clark and Brown 1960). While Clark and Brown (1960) suggested that *L. rubidus* hibernates during the month of December, we collected adults in December 2010 and 2011 in VA. Additionally, not many *L. rubidus* adults were found from March through May in VA, contrary to Clark and Brown's (1960) findings. Many beetles were found the following two years in February (see Chapter 3); 101 within two days of each other in 2011, suggesting that this may be a period of flight/dispersal for this species in VA. A study is

currently underway to determine the phenology of *L. rubidus* in southwestern VA. The information gathered from this study should help with collection in the field.

Future studies may need to determine the optimal rearing conditions for *L. rubidus*. *Laricobius rubidus* progeny developed in a similar time frame to that of *L. nigrinus*; eggs were oviposited in early spring, larvae developed through mid-spring, and prepupae dropped through late spring with adults developing in the soil, aestivating over the summer months, and emerging in the fall. For this reason we expected that the rearing procedures developed for *L. nigrinus* would be successful for rearing *L. rubidus*. Perhaps some necessary aspect for successfully rearing *L. rubidus* is not present in the *L. nigrinus* protocol. For instance, although Zilahi-Balogh et al. (2005) concluded that HWA is a suitable host for *L. rubidus*, it is possible that *L. rubidus* progeny may not be as fit after feeding on this adelgid. In 2011, the largest percent emergence of *L. rubidus* adult progeny came from the funnels that contained only PBA, although emergence was still quite low. Another possible reason that we failed to rear *L. rubidus* successfully may be due to the presence of microsporidia. Microsporidia has been shown to cause mortality of *Laricobius* beetles in the lab previously (Solter et al. 2011). Our beetles were not tested for microsporidia, therefore we do not know if they were infested.

Rather than attempting to rear an *L. rubidus* colony in the lab through their entire life cycle, *L. rubidus* larvae could be collected from the field and reared to the prepupal and adult stage. Another option may be to collect adults from the soil as they emerge in the field.

Appendix B. Backcrosses

Sometimes hybrids in the F_1 generation exhibit hybrid vigor as a result of the masking of mildly deleterious recessive mutants (Burton et al. 2006). This "hybrid vigor" may be short lived because when F_1 hybrids are crossed, the F_2 progeny can exhibit reduced fitness (Burke and Arnold 2001, Burton et al. 2006). Shaw and Wilkinson (1980) found significant differences in hybrid viability among Australian grasshoppers in the genus *Caledia*. In the F_1 generation, hybrid progeny were completely fertile and viable relative to offspring from the parental control crosses, but the F_2 generation was completely inviable and backcrosses produced only 50% viable B_1 offspring (Shaw and Wilkinson 1980). Experimentation through the second generation of *L. nigrinus* x *L. rubidus* hybrids would result in a more complete understanding of how hybridization could affect biological control of the HWA. Sterile hybrid progeny in the F_2 generation could result in lower reproductive output of beetles and reduce their potential for controlling adelgid populations. Studies of the second generation of *L. nigrinus* x *L. rubidus* hybrids would result in a more complete understanding of how hybridization could affect biological control of HWA.

To determine the fitness of Ln x Lr hybrid progeny beyond the F_1 generation, a backcrossing experiment was attempted in the spring of 2012. The beetles used in this experiment were progeny from the previous years no-choice lab mating experiment (see Chapter 3). Only progeny from Ln x Lr and Ln x Ln crosses were used, as there was no Lr x Lr progeny available.

In January 2012, 24 Ln x Lr progeny (F_1 hybrids) were crossed with one another, 14 Ln x Lr progeny (F_1 hybrids) were crossed with 14 Ln x Ln progeny, and 24 Ln x Ln progeny were

crossed with one another. Additionally, 58 beetles that emerged from larvae collected at Rocky Gap, MD where hybridization is known to be occurring, were mated with one another. The fitness measurements and rearing protocol for the backcross experiment were the same as those described for the no-choice lab mating experiments (see Chapter 3). All adults used for backcrossing were identified through genetic analysis following experimentation.

The no-choice lab mating experiment to determine the fitness of backcrosses was not very successful. Of the mating pairs, many turned out to be ♀♀ pairs (Table B. 1). Of the ♂♀ backcrosses (4 Hybrid x *L. nigrinus* and 3 Hybrid x *L. rubidus*), none produced eggs. Only the five ♂♀ Ln x Ln pairs produced eggs (Table B. 1), of which only one developed to the prepupal stage and this prepupa did not emerge into an adult.

Table B. 1. The number of ♂♀ and ♀♀ mating pairs (n) of *L. nigrinus* x *L. rubidus* hybrid progeny (hybrid x hybrid), backcrossed hybrid progeny (hybrid x *L. nigrinus* and hybrid x *L. rubidus*), and *L. nigrinus* (Ln x Ln), and the total number of eggs produced by ♂♀ and ♀♀ crosses (Eggs).

	n		Eggs	
	♂♀	♀♀	♂♀	♀♀
Hybrid x Hybrid	0	2	0	25
Hybrid x *L. nigrinus*	4	11	0	34
Hybrid x *L. rubidus*	3	0	0	0
Ln x Ln	5	7	23	33

Of the adult beetles used from the Rocky Gap, MD site, all were found to be *L. nigrinus* following genetic analysis. These *L. nigrinus* crosses produced 72 eggs, 28 prepupae, and 12 adults.

Appendix C. Number of Laricobius spp. collected from 2010-2012

Table C. 1. The number of *L. nigrinus*, *L. rubidus*, and *L. nigrinus* x *L. rubidus* hybrids collected from white pine and hemlock in 2010, 2011, and 2012 at 10 sites.

	2010		2011		2012	
	White Pine	Hemlock	White Pine	Hemlock	White Pine	Hemlock
L. nigrinus	7	336	27	351	14	379
L rubidus	88	78	153	96	37	42
Hybrids	0	62	56	64	4	65

Appendix D. Number of **Laricobius** *spp. collected from 2007-2012*

Table D. 1. The number of *L. nigrinus*, *L. rubidus*, and *L. nigrinus* x *L. rubidus* hybrids collected from hemlock in 2007 – 2012 at three sites (Laurel Creek, TN; Middle Creek, NC; Rothrock, PA).

	2007	2008	2009	2010	2011	2012
L. nigrinus	14	92	69	178	176	183
L. rubidus	142	95	41	40	30	14
Hybrids	0	22	37	29	29	33

Appendix E. Site information

Table E. 1. The latitude, longitude, elevation, biased (measurements taken at adelgid-infested tree points) and unbiased (measurements taken at systematic sample points) proportion of hemlock basal area/ acre (Hem BA/Acre), white pine basal area per acre (WP BA/Acre), hemlock trees per acre (HEM TPA), and white pine trees per acre (WPA TPA) at each site, year of first *Laricobius nigrinus* (Ln) release at each site, and the total number of *L. nigrinus* (Ln) released at each site as of 2012.

				Biased				Unbiased					
	Latitude	Longitude	Elevation (m)	Hem BA/Acre	WP BA/Acre	HEM TPA	WP TPA	Hem BA/Acre	WP BA/Acre	HEM TPA	WP TPA	Year of Ln released	# of Ln released
Laurel Creek	35.60353	-83.7537	630.48	0.0763	0.0058	0.2774	0.3677	0.0370	0.0108	0.0294	0.0416	2004	300
Middle Creek	35.794	-82.2174	940.11	0.3658	0.0293	0.5913	0.0609	0.0281	0.0000	0.0928	0.0010	2005	600
Burns Creek	36.92606	-82.5369	646.68	0.3880	0.0606	0.4615	0.2019	0.3671	0.0000	0.1989	0.0000	2008	300
Devil's Fork	36.81988	-82.6301	514.83	0.5384	0.0000	0.7723	0.0000	0.3905	0.0000	0.1008	0.0000	2008	300
Rocky Gap	39.28085	-78.6808	234.41	0.1673	0.0338	0.4013	0.1382	0.1659	0.0237	0.0825	0.0187	2004	3476
North Fork	37.44344	-80.5146	792.42	0.2870	0.1079	0.2247	0.2379	0.1506	0.0623	0.0971	0.0950	2003	600
Bear Run	40.89871	-77.2716	394.1	0.1761	0.0756	0.2188	0.1484	0.0629	0.0521	0.1189	0.1142	2005	300
Poe Valley	40.82619	-77.4437	416.77	0.3164	0.0116	0.4608	0.1471	0.2914	0.0025	0.5139	0.0464	2008	300
Rothrock	40.65508	-77.7369	390.46	0.3029	0.0762	0.3652	0.3989	0.2721	0.0100	0.2601	0.1192	2003	897
Treatser Valley	40.79569	-77.3801	460.46	0.2734	0.1326	0.3701	0.3386	0.2346	0.0666	0.2790	0.0941	2007	300