Behavioral Ecology and Genetics of Potential Natural Enemies of Hemlock Woolly Adelgid

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BEHAVIORAL ECOLOGY AND GENETICS OF POTENTIAL NATURAL ENEMIES OF HEMLOCK WOOLLY ADELGID

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ABSTRACT

Eastern and Carolina hemlock in the eastern United States are experiencing high mortality due to the invasive non-native hemlock woolly adelgid (HWA). The most promising means of control of HWA is the importation of natural enemies from the native range of HWA for classical biological control. Prior to release, natural enemies must be tested for suitability as a control agent, including the ability to locate the target prey. Coleopteran predators, including *Scymnus coniferarum* and *Laricobius osakensis* are under consideration as a means of biological control of HWA. *Laricobius nigrinus* was released in hemlock forests in 2003. It was recently discovered to hybridize with the native *Laricobius rubidus*.

Behavioral responses of these predators to HWA and host tree foliage were observed using a 4-chambered olfactometer, and genetic analysis was used to differentiate responses of *L. nigrinus*, *L. rubidus*, and hybrids. In the olfactometer, insects are allowed to amble about the arena and respond to volatile cues from each treatment. Host foliage with and without HWA was tested, as were various comparisons of eastern versus western foliage, host versus non-host foliage, and foliage containing HWA and a congeneric feeding beetle.

Olfactometer bioassays demonstrated that foliage from hosts where prey is commonly found is preferable to foliage where prey is seldom found, and that the presence of HWA-induced volatile cues is the strongest driver of behavior, and trumps the presence of a competitor. There is evidence in the study that supports the reliability-detectability phenomenon common in parasitoid biological control agents. Hybrid individuals were found to behave similarly to released *L. nigrinus*, although in some cases intermediate behavioral traits were evident, with respect to the parental species. This study and others support the continued need for strict testing of potential biological control agents prior to release, as well as a strong impetus for the inclusion and implementation of genetic analysis as a standard component of agent evaluation.
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Chapter 1: Literature Review

Invasion Biology

Global exploration and trade have brought about numerous environmental changes on global and local scales. Humans have been accountable for transportation of species beyond their endemic borders for thousands of years, and many of these introductions have been unintentional (Millennium Ecosystem Assessment 2005, Olden 2006, Hufbauer et al. 2011).

There are a number of vectors by which biota can breach their original physical isolation barriers and establish in a new range (Olden 2006), but anthropogenic transportation is cited as one of the primary methods (Waring and O’Hara 2005, Aukema et al. 2010). Humans actively and passively transport exotic species from place to place. Anthropogenic engineering of our environment led to a homogenization of biodiversity across the globe and the potential extirpation of endemic species (Olden 2006, Lockwood and Latchininsky 2008, Hufbauer et al. 2011). Many anthropogenic influences reduce the resilience of natural ecosystems to invasions, severing the biogeographic barriers developed over evolutionary time, and causing once very distinctive ecosystems to resemble one another. The time frame also causes species to interact with one another outside of the bounds of natural evolutionary time (Olden 2006). Invasive species have ecological and evolutionary consequences on ecosystem functioning (Ludsin and Wolfe 2001, Olden 2006). In this time of environmental and climatic uncertainty, biodiversity may be one of our greatest assets (Millennium Ecosystem Assessment 2005).
Currently, there is no agreed upon definition of biological invasions (Millennium Ecosystem Assessment 2005), but generally speaking, invasions occur when species establish in a novel environment. Traits of both the organism and the ecosystem interact and lead to successful establishment of invasive species over native species (Catford et al. 2012). Two key aspects make ecosystems vulnerable to invasion- traits of the invasive species and those of the invaded region. Many invasive species are generalists, have high fecundity, rapid growth and reproduction rates, widespread dispersal mechanisms, and lack predators in the novel environment. They often can capitalize on disturbance and tolerate various environments (Elton 1958, Mack et al. 2000, Lockwood and Latchininsky 2008, Catford et al. 2012). The invasive region can also be susceptible to invasion, due to recent disturbance, a moderate climate, and low local biotic diversity (deRivera et al. 2005). These factors allow non-native species to breech the bounds of natural control and become invasive. Invasion success is defined as biotic expansion beyond the boundaries of its native range, as well as a higher population density than exists in the native range (Cincotta et al. 2009).

When left unchecked, invasive species perturb worldwide biodiversity (Sorte et al. 2012), either by outcompeting and extirpating native species, or by adding to the overall diversity (Ford et al. 2012).

High fitness in invasive species is related to the “enemy release hypothesis” (ERH) (Elton 1958, Coluatti et al. 2004, Lockwood and Latchininsky 2008). The ERH posits that a lack of natural enemies in the invasive range compared to that in the native range will allow an organism’s population to boom due to a decrease in top-down regulation (Pearson and Callaway 2005, Blair et al. 2008). Natural enemies, especially
specialists, are uncommon in the exotic’s introduced environment, so there is limited external resistance to population growth. Although, as stated above, the concrete definition of biological invasion is contested, the original definition, described by Elton (1958) in the seminal paper on invasion biology, includes a caveat concerning novel interactions with organisms with which the invader had no previous evolutionary history, implying that the ERH has roots in foundational invasion biology (Cincotta et al. 2009). Studies reviewed in Liu and Stiling (2006) suggest that although the field could benefit from more prescriptive research design, there is empirical evidence that the ERH holds in nature. Escape from natural enemies is especially relevant to those invaders that are impacted most by specialists in their native range (Liu and Stiling 2006).

**Impact of Non-native Insects in Forests**

The hallmark of forest health is the system’s ability to sustain productivity while tolerating abiotic and biotic stressors (Tkacz et al. 2008). Coevolved native biotic stressors, at natural population sizes, are important in forest ecosystem functions (Waring and O’Hara 2005, Lovett et al. 2006, Tkacz et al. 2008). However, non-native biological agents cause additional stress that ecosystems are not evolutionarily adapted to, which often results tree mortality (Lovett et al. 2006). Tree mortality alters light availability, affects microclimate, alters soil composition and pH, and can potentially cause a shift in forest composition (Waring and O’Hara 2005, Lovett et al. 2006). These consequences can be short- and long-term, and their effects tend to last longer than those of naturally occurring disturbances (Lovett et al. 2006).
In the modern era, the rate of establishment of non-native invasive biological organisms in forested ecosystems is increasing (Waring and O’Hara 2005), resulting in a serious threat to forest and ecosystem function (Lovett et al. 2006). As of 2002, at least 10 invasive insects are considered major threats in the northeastern United States (Waring and O’Hara 2005), and 14% of the 455 recognized forest pests in the United States hold “high impact” status (Aukema et al. 2010). Non-native insects are more likely to be introduced and established near trade routes (Brockerhoff et al. 2006, Aukema et al. 2010).

Risk is based on two factors: susceptibility and vulnerability (Krist et al. 2010), where susceptibility is the probability of attack based on the insect’s access to the host, and vulnerability is the probability of mortality due to attack, based on the condition of the stand and the life history strategies of the insect (Krist et al. 2010). Considerable effort has been undertaken to understand the nature of insect invasions in their relation to forest health, and to map, mitigate, and manage biotic stressors in the United States (Tkacz et al. 2008).

A projection analysis completed by a US Forest Service working group predicted that 23 million acres of forested land are at risk of upward of 25% mortality over the coming 15 years, primarily due to the factors described above (Tkacz et al. 2008, Krist et al. 2010). In short, insects, especially non-native invasives, threaten forest health and function across the globe (Brockerhoff et al. 2006). There is an extremely high rate of detection of new and newly established forest pests. In fact, the detection of high impact species is 3 times higher than previous decades (Aukema et al. 2010). It is possible that this is an artifact of improved monitoring, detection and
documentation, but eradication and management efforts lag behind the rate of the
detection.

Compared to agricultural systems, introductions in forested ecosystems are more
difficult to detect, and therefore and have more time to establish and spread
(Brockerhoff et al. 2006), which makes eradication more difficult. Silvicultural and
other management strategies must be carefully implemented, as these practices can have
a lasting impact on the residual consequences of the invasion. Some practices lead to
further perturbation (Waring and O’Hara 2005), but they could also slow the spread of
invasive insects at their boundaries, in a similar fashion to a firebreak (Waring and
O’Hara 2005). Current practices do seem to be making a difference and reducing the
overall impact of these non-native invasive insects (Brockerhoff et al. 2006). Increase
in public awareness can in turn increase the rate of detection (Aukema et al. 2010).

**Integrated Pest Management and Classical Biological Control**

Invasive species can be controlled through mechanical, chemical, and biological,
as well as a combination of these (Hatcher and Melander 2003). Mechanical removal
generally refers removing problematic individuals by hand. This may be an option for
home gardens or community parks, but does not function well on larger scales.
Chemical control can be used over a large spatial area and may provide more permanent
control than mechanical methods, however, pesticides can be harmful to ecological and
human health, bolster the development of resistant pest populations, and does not offer
consistent, long-term control (Hoddle 2004). This leaves us to consider biological
control methods as a part of an Integrated Pest Management (IPM) program or as a sole means of control.

As defined by DeBach (1974) and Caltagirone (1981), classical biological control is the “regulation of a pest population by exotic natural enemies that are imported for this purpose.” Biological control is considered a component of IPM that is meant to sustainably reduce the equilibrium density of the target organism to below an economically or ecologically acceptable threshold (DeBach 1974, McEvoy 1996, Holt and Hochberg 1997, Van Den Berg et al. 2000). This can ideally be described as “minimal interference with optimal results” (Pschorn-Walcher 1977, Howarth 1991, Pedigo and Rice 2009). When undertaken correctly, biological control is less harmful to human health than pesticides (Metcalf 1980, Howarth 1991, Messing and Wright 2006), allows for evolutionary plasticity, there is a reduced risk for ecological harm (Dawkins 1976), and is suitable for landscape scale forested systems (Delfosse 2005, Messing and Wright 2006, Pedigo and Rice 2009). Successful biological control programs will be self-sustaining over multiple predator and prey generations.

Biological control programs have clear ecological foundations, including, but not limited to, the enemy release hypothesis (Keane and Crowley 2002, Colautti et al. 2004, Pearson and Callaway 2005, Liu and Stiling 2006, Blair et al. 2008, Cincotta et al. 2009). For biological control to be intuitively feasible, we must agree that 1) one organism can control another through top-down regulation, and 2) host ranges are limited to the target species (McEvoy 1996, Browne and Withers 2002). As described by Hoddle (2004) biological control is an attempt at “community reassemblage,” where an ecological community, disrupted by the establishment of a non-native invasive
species, will be realigned through the introduction of a regulatory natural enemy. Thus, higher trophic levels should be minimally affected because they can continue to feed within the same food webs and utilize the same resources as prior to the arrival of the non-native invasive and the natural enemy.

Biological control protocols are more complicated in forest systems than agricultural systems because of greater biodiversity, increased interactions (both in number and complexity), and less intensive management. Forests hold fewer empty niches than agricultural systems, so agents often have to mitigate interactions or compete with native species to establish (Pschorn-Walcher 1977). Therefore, scientists aim for precision in their biocontrol agents, intending to release the fewest, most specific agents as possible (Gaskin et al. 2011). Predictable agents, with known behavioral patterns, very narrow host and habitat ranges (set of organisms that a predator can possibly consume and sustain its life cycle on) and well-studied life history strategies make good candidates (McEvoy 1996, Van Driesche and Hoddle 1997, Browne and Withers 2002, Pearson and Callaway 2003, Hoddle 2004, Pearson and Callaway 2005).

Ecosystems are complex, and with increased complexity, there are increased risks due to non-target effects and unforeseeable events. Non-target effects are residual and indirect effects of biological control programs that were not originally intended. Usually, this term is used to describe risks to native species and systems. Since the advent of biological control, approximately 11% of projects documented cases of non-target effects (Louda et al. 2003). The assessment of non-target effects are based on
noticeable impact on what is ecologically, economically, or culturally important in the system. It is possible that less severe or conspicuous non-targets may go unrecorded.

There are inherent risks involved in releasing non-native species meant to control other non-native species (Ewel et al. 1999), as illustrated by the effects of invasive species themselves. Biological control is, by nature, an intentional invasion, and follows many of the same foundational ecological theories on which we base invasion ecology (Fauvergue et al. 2012). With consideration for these risks, biological control programs have been reassessed for the late 20th and 21st centuries. Biological control researchers and practitioners have an increased understanding of the problems of the past, and attempt to apply interdisciplinary information and open communication of ideas and concerns to contemporary programs. Namely, our increased understanding of the systems within which we implement biological control, and the agents we release should shield against drastic non-target effects.

The difficulty in these cases is to strike a balance between the effectiveness of an agent and its specificity (Rauth et al. 2011). As outlined in Berner and Bruckart (2005), there are very specific guidelines by which a biological control agent is identified and assessed for acceptability, and most countries have specific and strict regulations for the importation of natural enemies (Louda et al. 2003, Berner and Bruckart 2005). Organisms with strong host and habitat fidelity are the best biological control candidates under these foundational guidelines because it is likely that they will not stray from the target prey species (McEvoy 1996, Hoddle 2004). So, by definition the candidates are specialist predators or parasitoids.
Insect Behavior, Volatile Cues, and Host Selection

Insect predators such as those used as biological control agents use visual, chemosensory, and tactile cues to forage for acceptable prey. Not only do predators use these cues to enhance foraging success, they specifically use them to process the presence, identity, availability, and suitability of prey using unique antennal binding sites and stimuli processing for compounds and mixtures of compounds (Wallin et al. 2011). For very host-specific feeders, which biological control candidate agents are, volatile cues can be especially important for host identification and location (Keesey et al 2012). These are described as behaviorally active compounds that they convey information that incites behavior in the recipient (Asaro et al. 2004, Narayandas et al. 2006, Yoneya et al. 2009, Yasui et al. 2011, Keesey et al. 2012).

Plant defenses are either constitutive, meaning that they are continuously functional, or they are induced by herbivore feeding (Agrawal 1998). Induced plant defenses not only work directly against the herbivore itself, but also indirectly by drawing natural enemies toward the wounded foliage in a tri-trophic system (Havill and Raffa 2000). Mechanical damage such as herbivory has morphological and chemical consequences for the host plant, causing the plant to emit long-range chemical cues, in contrast to the short-range cues that herbivores characteristically emit (Vet et al. 1991, Dannon et al. 2010, Radville et al. 2011). These defenses are comprised of chemical compounds not used in primary metabolism, and are energetically expensive to produce, called secondary metabolic products (SMPs). This suggests an active, as opposed to passive, defense against herbivores, which subsequently implies that there is
a coevolutionary relationship between the host plant and the natural enemy in the feeding guild (Havill and Raffa 2000).

Insects are known to use multiple senses or types of cues to locate prey (Mausel et al. 2010). Often, insects use olfaction to find hosts at long distances, until they are within close proximety to the host. Then, they often use an ambulatory searching behavior and visual cues to locate individual prey items (Mausel et al. 2010). Odor stimuli, a very important cue type, contain three pieces of pertinent information: an identifying component, an intensity component, and a temporal component (Wallin et al. 2012). Insects use olfactory cues to locate food and mates, and to avoid predation. Sensory cues from differing hosts elicit unique excitatory output, and it is known that the amount or quality of a cue that is required to elicit a response by a predator is reduced with increased hunger or depravation (Browne and Withers 2002).

To survive, an organism to locate a food source before energetic reserves run out, or other environmental factors kill the foraging organism (Pureswaren and Borden 2005). Therefore, predators use cues that are energetically efficient to pursue at various levels of predation. An early study suggests that beetles are highly dependent upon past rewards, which is conducive to biological control, because they can learn to find prey efficiently (Dixon 1959). Multiple steps in beetle host selection behavior drive viability, as habitat location, host location, host acceptance as a food source, and host use are necessary for survival (Wallin et al. 2011, Wallin 2012). Olfactory systems must be able to process information quickly and reliably, while being able to discern important cues across a broad chemical spread (de Bruyne and Baker 2008).
Using herbivore-induced chemical cues and host selection, we can apply research on beetle searching behavior and life history characteristics to inform decisions about biological control protocols. Ideally, we can answer the questions: “Will the introduced predator be able to locate its target prey in the wild? Will it be able to reproduce with the target prey as sustenance and within the introduced habitat? Will the predator be able to disperse along with the dispersal of the pest?”

It is crucial that potential predators are studied in great detail before implementing a biological control protocol as to reduce non-target risk and optimize the chances for successful pest suppression (Berner and Bruckart 2005). Factors of risk are correlated with predator permanency, host range, habitat, genetic plasticity, behavior, mutualisms, and the vulnerability of the release region (Howarth 1991). Some genetic and adaptive plasticity is required for initial establishment of a biological control agent, and genetic diversity strengthens a species’ ability to adapt to disturbance, but otherwise, foreseeable life history strategies are critical (Mackauer 1976). The most realistic implementation of biological control programs requires a paradigm shift, away from the assumption of no risk to an assumption of high risk. From that standpoint, specificity testing and other safeguards can reduce the perceived risk to an acceptable level, and researchers and managers will have a greater understanding of potential harms to monitor for and a notion of how to adapt to problems that may arise. In short, this is much more effective and responsible than retrospective studies (Simberloff and Stiling 1996, Delfosse 2005).

In an introduced range, novel plant odors may confuse insects, and not direct them toward prey as they would in the native range. Although this is not an exhaustive
list of factors for predation, knowledge of searching behavior and mechanisms can be applied to efforts to mitigate invasive species populations through biological control. An understanding of invertebrate predation is conducive to the design of assays and protocols that will augment the success rates for biological control programs. An acceptable method of testing host specificity and response to olfactory cues is through behavioral assays in an olfactometer (Wallin et al. 2011). Olfactometer bioassays can assess whether biological control agents are attracted to the target host plant, compared to other potential hosts in the novel range (Walter et al. 2010). Host specificity assays seek to demonstrate that a potential agent can feed, reproduce, and complete its life cycle on the host, and interactions with non-target organisms will be limited.

Model System

Tsuga canadensis (L.) Carrière (eastern hemlock) and Tsuga caroliniana Englemann (Carolina hemlock) are shade tolerant tree species that form dense stands in temperate forests in the eastern United States (Ward et al. 2004). Individual trees can grow to 25-30 meters at maturity, and grow in pure or mixed stands. Hemlocks are often dominant or co-dominant trees on the landscape. Their primary economic value lies in ornamental nursery stock, lumber, or paper pulp, and can they can also play a role in the valuation of regional tourism. The ecological value is more pronounced than the economic value. As a foundation species, hemlock creates habitat for many organisms, has the ability shape ecosystems and propagate certain forest types (Evans et al. 2011, Knoepp et al. 2011). Broad, dense canopies allow very little sunlight to reach the forest floor, and acidic needles alter the soils as they drop and accumulate, lowering
the pH, decreasing oxygen availability, and selecting for a shade-tolerant forest community composition (Evans et al. 2011). Hemlock dominated forests play a role in moderating riparian ecosystems by reducing hydrological and nutrient fluctuations (Knoepp et al. 2011). They act as riparian buffers by mitigating stream characteristics, including solute concentrations, water temperature, and large, shaded pool formation from fallen woody debris (Roberts 2009). They also retain sediment and support high stream productivity (Ellison et al. 2005).

Currently, populations of *T. canadensis* and *T. caroliniana* are at risk due to an infestation of invasive *Adelges tsugae* Annand (Hemiptera: Adelgidae) (hemlock woolly adelgid, HWA). HWA is native to Asia, including regions in China and Japan; there is supporting evidence that HWA may also be native to western North America (Havill and Footit 2007). HWA was first reported in Virginia, USA during the 1950s (McClure 1991).

Family *Adelgidae* are easily recognized by the distinctive woolly masses that form around their bodies for the majority of the year, within which they remain sessile. These woolly masses protect individuals from desiccation as well as from their natural enemies (Ward et al. 2004). Adelgids exhibit unique natural history and reproductive strategies, including cyclical parthenogenesis and a multigenerational, polymorphic life cycle (Havill and Footit 2007). They engage in minimal inter-guild interactions, and tend to inhabit regions where there is a surplus of host trees available for exploitation (Ward et al. 2004). Species in the adelgidae family are highly host specific as a result of ancient coevolutionary relationships with gymnosperms (Havill and Foottit 2007).
Significant changes in forest floor light availability were detected after HWA infestation (Ford et al. 2012).

According to Cheah et al. (2004), HWA has been named “the single greatest threat to hemlock ecosystems” of our time. Ecosystem changes due to HWA have been documented, including reduced canopy density, increased light availability, altered nitrogen cycling, accumulated woody debris, and decreased forest floor moisture. It was found that the relative importance of eastern hemlock in the ecosystem, potentially altered over time as a result of dieback from HWA, is related to the disturbance of the nitrogen cycle in eastern forests (Block et al. 2012). With increased hemlock mortality, it is expected that we will witness compounded and increasingly severe abiotic effects (Block et al. 2012). Thus, in this case, the impact of a loss of the tree species is an artifact of its original relative importance in the ecosystem prior to the infestation (Block et al. 2012).

Although HWA is active during the winter, the species is only able to feed in above-freezing temperatures, which may also limit its ability to continue spreading northward, but that is unknown at this time. As a coniferous species, there are sufficient concentrations of photosynthate in hemlock needles throughout the winter to sustain HWA feeding (Ward et al. 2004). HWA feed by inserting their mouthparts into the weak tissue around the base of a needle. This needle-like stylet bundle accesses the xylem ray and parenchyma cells directly, so the insect is able to divert phloem away from the tree. The depletion of starch inhibits new growth or regeneration, slowly killing the tree (Young et al. 1995, Broeckling and Salom 2003, Cheah et al. 2004, Ward et al. 2004). HWA infestation can cause bud abortion and drop, needle
desiccation and drop, and eventual tree death (Montgomery and Lyon 1995). There is also evidence of enzymes in the saliva of HWA that accelerates mortality and reduces the suitability of individual trees for repeated colonization after the original establishment on that individual (McClure 1991). In short, HWA overwhelms the physiology of the tree.

Due to their small size, the egg and crawler generations are easily transported via wind, wildlife, and humans by means of nursery stock, lumber, fire wood, and other methods (Ward et al. 2004). HWA uses *Picea* spp for sexual reproduction that are not endemic to the eastern US. For example, in Asia, HWA forms galls and reproduces on *Picea likiangensis* (Franch.) and *Picea torano* (K. Koch), but no suitable spruce in the invasive range means no successful sexual reproduction in the invasive region (McClure 1991). For HWA specifically, the life cycle is suited for dispersal, even though the wingless form does not exist in the invasive range. Females are capable of asexual reproduction, and the eggs hatch and aestivate over the summer period, reaching maturity by the onset of the winter season (Cheah and McClure 2000).

Populations of HWA in its native range do not kill their hosts due to host tree resistance, and the existence of natural enemies endemic to the region (Cheah and McClure 2000, Cheah et al. 2004). Surveys reveal that up to 50 species of predators, both generalists and specialists, are associated with HWA in each of its native ranges (Cheah et al. 2004, McAvoy et al. 2007, Kohler et al. 2008). This evidence suggests that both genetic resistance and the naturally occurring complex of natural enemies control HWA in its native range (Montgomery and Lyon 1995).
Several coleopteran predators from across the globe are natural enemies for the adelgidae family, and have evolved similar phenologies and behavioral patterns to best survive on their unique prey. Insect fitness is highly dependent upon coinciding phenology with the species they interact with, especially prey organisms (Yurk and Powell 2010). There are numerous desirable qualities that researchers reference when considering potential biological control agents, as described by Berner and Bruckart (2005). These qualities include: the ability to survive mass rearing in a laboratory, ability to survive natural and/or variable environmental conditions in the release region, search capacity for target prey, voracity toward prey, ability to find mates, fecundity, longevity, and persistence.

A survey of predatory species associated with HWA on hemlock in the Pacific Northwest demonstrated that species from several insect orders made up a complex of natural enemies (Kohler et al. 2008). The majority of these were coleopteran predators, with *Laricobius nigrinus* comprising of about 43% of the total feeding guild (Kohler et al. 2008).

**Genus Laricobius** (Coleoptera: Derodontidae) is a member of family Derodontidae, which is primarily composed of fungal feeders. *Laricobius* spp. differ from the rest of the family in that these species are prey-specific to adelgids, and their phenologies are highly synchronized with that of their prey (Salom et al. 2005, Zilahi-Balogh et al. 2006). Before their exploration as potential biological control agents of HWA, Derodontids held little ecological intrigue, and were not the focus of many scientific studies beyond taxonomy (Mausel et al. 2010). These species demonstrate low-risk for non-target effects because they are host-specific feeders and require low
population density to control HWA populations. According to explorations thus far, *Laricobius* spp. seem to be distributed across the northern hemisphere, coinciding with adelgids associated with conifers (Montgomery et al. 2011). Similarly to Adelgidae, *Laricobius* spp. are active during the winter, aestivate for the same period, and emerge concurrently. Temperatures and photoperiod are prominent factors for this synchrony (Ward et al. 2004, Lamb et al. 2007).

Both larvae and adults feed on life stages of HWA (Vieira et al. 2011). Females are known to lay their eggs directly within HWA ovisacs significantly more often than ovisacs of other available adelgid species. Subsequently, offspring consumed more HWA eggs than eggs of other species, and could only develop into adulthood when feeding on this prey. These are all indications of host-specificity (Zilahi-Balogh et al. 2002). *Laricobius spp.* aestivate in the soil near below the hemlock tree.

*Laricobius nigrinus* is found naturally in the Pacific north western United States, as well as British Columbia, Canada (Kohler et al. 2008). *L. nigrinus* reproduces once per year, in synchrony with the HWA lifecycle. They can be found on western hemlock (*Tsuga heterophylla*) (Kohler et al. 2008, Grubin et al. 2011). Since the early 2000s, hundreds of thousands of individuals have been released in the southern Appalachian region. In recent preliminary release studies, both F₁ and F₂ generations have been recovered, suggesting that this species is able to thrive and reproduce under eastern conditions (Salom et al. 2005). However, *L. nigrius* are difficult to lab-rear because they require large amounts of fresh HWA and proper soil conditions for aestivation.

*Laricobius rubidus* is the only species under consideration that is endemic to the eastern United States. Its native range spreads from the Washington DC metropolitan
area (38° 53’ N 72° 02’ W) northward to Newfoundland, Canada (48° 57’ N 54° 36’ W) (Zilahi-Balogh et al. 2006). The primary prey for this species is *Pineus strobii*, but it has been found on eastern hemlock feeding on HWA. Studies have shown that this species prefers to oviposit within *P. strobi* wool sacs six times more than HWA wool sacs, and this must be taken into consideration for management purposes (Story et al. 2012).

*Laricobius osakensis* is a Derodontid species native to Japan that associates with and preys on HWA on southern Japanese hemlock, *T. sieboldii* (Carriere). It was discovered as an important HWA predator in Japan in 2005, collected in 2006, and reared in quarantine for several generations. This species completes the active portion of its life cycle in its native range from mid-November to late May, and aestivates during the summer months (Lamb et al. 2012). *Laricobius osakensis* has a lower temperature threshold, a higher feeding rate, and more successful larvae development on HWA than *L. nigrinus* (Vieira et al. 2011). In exclusion studies, *L. osakensis* had a significant impact on HWA mortality in its native range. HWA was the primary food preference in choice and no choice studies (Lamb et al. 2012). Although *L. osakensis* can feed on other adelgids, it could only develop to adulthood on HWA, and females laid eggs almost exclusively on HWA ovisacs compared to other choices in choice tests (Viera et al. 2011). In 2010, a Finding of No Significant Impact (FONSI) was completed, so the species can be released from quarantine and field studies can begin.

*Scymnus (Pullus) conifererum* (Coleoptera: Coccinellidae) is endemic to the western United States and associated with adelgid-infested western white pine (*Pinus monticolla*) and western hemlock (*Tsuga heterophylla*). *Scymnus coniferarum* can
develop to adulthood on HWA, but if available, pine adelgids are preferred (Montgomery and McDonald 2010). *S. conifererum* can feed on all life stages of the adelgid (Montgomery et al. 2009, Montgomery and McDonald 2010, Montgomery and McDonald 2011). Studies are underway (including this study) to test whether eastern white pine is preferred over eastern hemlock, because it is known that western pines are preferred over western hemlock. This species is a promising biological control because it does not require specific soil conditions to complete its lifecycle, in comparison to *Laricobius* spp., and it would be possible to use Scymnus and *Laricobius* in conjunction, as their life cycles are complementary and there is no chance of hybridization.

**Potential Impact of Hybridization and Multiple Release on Biological Control**

Monitoring studies after preliminary releases of *L. nigrinus* in the eastern US indicate high numbers of hybrid individuals of released *L. nigrinus* and native *L. rubidus*. This hybridization was unexpected, and the biology of *Laricobius* hybrids is currently under study. Hybridization in this system was unexpected because there are well-documented barriers to heterospecific mating that reduce gene flow between populations and species (Mallet et al. 1998, Stouthamer et al. 2000, Dopman et al. 2009, Cheyppe-Buchmann et al. 2011, Hartke and Rosengaus 2011) that exist as both pre- and post-mating barriers. Pre-mating barriers include geographical isolation, morphologically dissimilar genitalia, or incompatible mating behaviors and/or sex pheromones. Pre-mating barriers promote mating within a species, but if they fail, post-mating barriers come into play. These include decreased, reduced viability, or decreased reproductive fitness (Dopman et al. 2009, Hartke and Rosengaus 2011).
It is not uncommon for related species to hybridize immediately after introductions, with isolation mechanisms developing over many generations (Remington 1968), but this immediate gene flow results in an unstable hybrid zone. Hybrid zones are remedied in one of three ways: evolution of mating barriers, fusion of the two taxa into a single species, or the extirpation of one of the parental species (Harrison 1983, Harrison 1986, Wainger and Mazzotta 2011). There is substantial evidence that hybridization reduces fitness from generation to generation, causing a phenomenon known as “hybrid breakdown” (Dopman et al. 2009). In many systems, hybridization results in a sterile F₁ generation (Ardeh et al. 2004, Nonacs 2006, Hartke and Rosengaus 2011), because the genotypes of parental generations drive the fecundity and viability of the offspring (Remington 1968).

If hybrid individuals are viable and fecund, ecological aspects of hybridization must be considered, especially within the context of biological control. Host-specificity can be lost because hybrids are known to use new host organisms (Hora et al. 2005) and can also displace native species (Yara et al. 2010). In a biological control study exploring effects of hybridization of flea beetles used in, hybridization produced both host-specific and non-specific genotypes, and the F₂ generation was found to be an ineffective control agent (Szűcs et al. 2011). Reciprocal crossings produced generations with higher fecundity and fitness than either parental lineage (Szűcs et al. 2012).

Selection pressure against hybridization can exist, but selection pressure can also reinforce hybrid adaptations. This can cause speciation, or hybrids can swamp the gene pool, causing displacement or undue competition with native and/or parental species (Harrison 1986, Mallet et al. 1998, Nonacs 2006, Yara et al. 2010). Genetic
mixing could be crucial to the novel organism’s survival in the introduced range (Szűcs et al. 2012). For example, hybridization had increased larval developmental rates, foraging behaviors, and response to food stimuli of Drosophila (Del Pino and Godoy-Herrera 2000).

Predictable behavioral responses to stimuli are very important to biological control protocols, and hybridization reduces predictability. When hybridization occurs in species meant to be biological control agents, initially they can be quite beneficial but as generations continue, they become less effective due to loss of specificity or developmental failings (Stouthamer et al. 2000). However, if both parental species are found to be highly host specific, it is unlikely that hybrid individuals will have host preferences beyond those of the parental species, or express a non-specific phenotype (Szűcs et al. 2012). Hybrids demonstrate intermediate behavioral, feeding, and developmental traits, to those of their parental species (Howard 1993, Szűcs et al. 2012). Therefore, proper risk assessment involving potential hybridization and effects of such hybridization is imperative. Behavioral assays using field collected specimens that may or may not be hybrids can elucidate whether hybridization will have an impact on biological control efforts. Hybridization occurs in many other insect systems, and studies on those systems from which we can gain some insight exist, but the lack of *Laricobius*-specific information is an area for exploration.

Intuitively, multiple releases of different species for complementary biological control would be compounding beneficial, as each species should have a negative impact on the intended prey. If the biology of two species in close proximity with one another is understood and they can coexist successfully, multiple release can be a good
biological control strategy. For this to be the case there has to be no risk for hybridization (Ardeh et al. 2004). Interaction strength between the released species must be taken into account, because population dynamics can force predators onto other host species (Pearson and Callaway 2003). Secondary or subsequent release strategies can be beneficial if carefully considered and implemented, but may not be as safe or effective, as genetic mixing and hybridization can lead to changes in fitness or behavior, or loss of host-specificity (Szűcs et al. 2011). Mixed species release strategies can also lead to intraguild interactions, including competition for prey, interference, avoidance, or cannibalism. Predators can interact with each other through direct competition or intraguild use of the same resources, and it is possible that the overall community structure can be altered with each additional introduction.

Feeding guilds can be natural, synthetic, or restructured, and by definition, biological control undertakings are examples of unnatural systems (Ehler 1992). The interactions between guild members is extremely important to the success of biological control, and should be considered as part of a risk assessment in the pre-release testing of an candidate agents or complexes of agents (Ehler 1992). Analysis of potential guild interactions can improve or enhance the overall natural enemy complex (Ehler 1992), and provide much needed information to biological control practitioners.
Chapter 2: Behavioral response of the hemlock woolly adelgid predator, *Scymnus (Pullus) coniferarum* (Coleoptera: Coccinelidae) to host foliage odors in a multi-chambered olfactometer

Abstract

*Scymnus coniferarum* is a native insect to western North America. It is a specialist feeder on aphids and adelgids on conifers, but a generalist among these species. It was a prominent generalist predator collected from infested western hemlock in a survey of natural enemies associated with hemlock woolly adelgid. We tested the orientation behavior of adult *S. coniferarum* to foliage from hemlock woolly adelgid host trees, including eastern hemlock, western hemlock, two other conifers associated with and without adelgid species in a multi-chambered olfactometer. These laboratory bioassays are meant to inform the future testing and release of *S. coniferarum*. *Scymnus coniferarum* that were starved for six hours, and those that were tested six days after collection were most responsive to treatments. Beetles were responsive in the olfactometer, but were not selective in their preference for one host foliage type over another. They were found to prefer host foliage that is commonly fed upon by adelgids in comparison to host foliage that is rarely considered a host for adelgid species. This study suggests that *S. coniferarum* may locate and feed on HWA in the novel environment, but it may not be more likely to find HWA on eastern hemlock than other adelgid or aphid species on other host conifers in the novel environment.

Introduction

Hemlock woolly adelgid (*Adelges tsugae* Annand, HWA) is a non-native invasive forest insect, native to Asia and western United States (Havill and Foottit
that was introduced from Asia to the southern Appalachian region of the US in the mid-20th century. Eastern hemlock (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Englelmann) are susceptible to HWA infestation, and usually die within four to 10 years of infestation (Montgomery and Lyon 2005). A loss of hemlock on the landscape will have biotic and abiotic effects, including alteration of nitrogen cycling, increased light availability, changes in pH, accumulation of woody debris, and a turnover of forest composition (Evans et al. 2011, Knoepp et al. 2011, Block et al. 2012).

Populations of HWA in its native range are kept at below-pest levels in part by genetic resistance of the host tree, but also by a suite of predators (Cheah and McClure 2000, Montgomery and Lyon 2005, Kohler et al. 2008). The lack of natural enemies in the invasive range allows for population growth without top-down regulation through predator-prey interactions. This is known as the enemy release hypothesis (Keane and Crowley 2002, Coulatti et al. 2004, Liu and Stiling 2006), whereby invasive species are able to undergo significant population growth due to a lack of specialist natural enemies in the invasive range.

The premise of biological control is to introduce natural enemies from the native range of the pest species to reduce the population size of the non-native species and to establish an equilibrium of predator-prey populations that keeps pest population below an ecologically damaging threshold (DeBach 1974, Caltagirone 1981, Hoddle 2004). In a survey of predators associated with HWA in western North America, Kohler et al. (2008) discovered over 50 species in 13 insect families that could comprise a predatory
guild. Some of these are considered generalist predators and/or generalists of adelgid and aphid species, including *Scymnus (Pullus) coniferarum* (Crotch).

*Scymnus coniferarum* was collected from one site during summer months and is thought to be a summer predator of HWA (McDonald 2010). *Scymnus coniferarum* may be a promising candidate for an additional biological control of HWA because it has complementary phenology with HWA and is active during the summer when the other predators of HWA aestivate (McDonald 2010). This species is endemic to the western United States and prefers pine bark adelgid on western white pine (Montgomery and McDonald 2010), but it is also associated with HWA on western hemlock, as well as adelgids on other pines, firs, and spruces, woolly apple aphid and citrus mealybug (McDonald 2010). In pre-release testing, *S. coniferarum* was found to feed on all life stages of HWA and complete its lifecycle on HWA, even though it has characteristics of a generalist (Montgomery et al. 2009, Montgomery and McDonald 2010, Montgomery and McDonald 2011).

To test potential agents’ suitability for biological control, several host range and specificity tests are undertaken. To discern whether an agent can locate the intended host in the environment, we tested its response to olfactory stimuli from western hemlock, eastern hemlock, western white pine, and ponderosa pine. Insects use volatile cues emitted from prey, host foliage, or a combination, as well as unique antennal binding sites for these cues, to process information, including location, identification, suitability, and palatability of hosts (Lucas 2001, de Bruyne and Baker 2008, Wallin et al. 2011, Wallin 2012, Keesey et al. 2012). These compounds can be behaviorally active cues that incite a behavioral response in the insect forager (Asaro et al. 2004,
Yoneya et al. 2009, Keesey et al. 2012). Often, volatile cues from the prey are too limited to be detected in the environment, especially at long distances (Vet et al. 1991, Wallin et al. 2011). As a proxy, insects can use volatile cues from host plants, which are highly detectable in the environment, partially because of the sheer surface area for cues to be released. Cues from host foliage are not affirmative indications of prey presence or ability, but they are more beneficial than foraging at random (Vet et al. 1991, Mausel et al. 2010).

Olfactometer bioassays can assess whether biological control agents are attracted to the target host plant compared to other potential hosts in the novel range (Walter et al. 2010). The objective of this study was to evaluate the behavioral responses of adult S. coniferarum to several host foliage stimuli, both from the native and novel ranges. We also used the opportunity to inform the olfactometer protocols for S. coniferarum, as this species had not been tested in an olfactometer previously, and specifics of the laboratory bioassays are slightly unique for each species tested.

**Materials and Methods**

Behavioral bioassays were conducted to test the ambulatory response of adult predatory beetles to prey and foliage from several coniferous host species. Bioassays were conducted in 2011-2013. *Scymnus coniferarum* individuals used in these bioassays were collected off of western hemlock, western white pine, and eastern hemlock. All beetles tested were randomly chosen from the test group, tested once, and killed. Several three-and four-way choice experiments were undertaken to better understand the ambulatory behavior of *S. coniferarum.*
Olfactory responses were measured using the same four-chambered olfactometer arena (Analytical Research Systems, #OLFM-4-C-2440PE, Gainesville, FL, 30x30x3 cm) that was used in Arsenault et al. in prep, and Wallin et al. 2011. Briefly, it consisted of three parts: the base with the air output, the intermediate part that delimited the walking chamber with four air inputs and a 9-mm circular central opening to introduce insects and attach the vacuum. There are four possible chambers to place host foliage. Chambers attached to arms receiving air but without test foliage were regarded as blanks or control chambers. Four flow-meters (Brooks Instrument, Hatfield, PA) controlled airflow at a rate of 0.12 Mpa into the glass chambers containing the test foliage and carried volatiles into the olfactometer. The vacuum maintained the integrity of the volatile field while removing the volatiles at the bottom of the central arena.

For each experiment described below, one adult *S. coniferarum* was placed into the center of the assay arena, equidistant from the entrance from all four olfactometer arms. Each individual was assayed for ten minutes or until it made a choice. The assay arena was divided into four equal sized fields and a 9-cm central field. A choice consisted of a beetle leaving the central field and crossing into the delineated field boundary for more than one minute. The final position at the end of the behavioral assay was recorded, as well as the time required for the beetle to choose a field. If a beetle remained in the central field for more than six minutes without moving toward an arm, the behavior was recorded as “no choice.” If the beetle attempted to crawl into the arm, it was recorded as the final choice, and the beetle was removed from the arena. The position of each source chamber containing different host foliage or beetle type was randomly positioned at the time of the assay, and re-randomized for every individual
assayed. Host foliage was replaced every hour to minimize chemical compositional changes over time.

Foliage from western tree species used in the *S. coniferarum* bioassays was collected from trees at the Oregon State University Peavy Arboretum located approximately 1.3 km north of Corvallis, OR. Foliage used in each experiment described below was clipped, wrapped in damp paper towels and parafilm, and placed into tightly sealed plastic bags. Foliage was shipped on ice and stored no longer than 48 hours at 2-3 C until used in the assay. Uninfested eastern hemlock and eastern white pine foliage was collected in Burlington, VT and treated similarly to shipped foliage. Foliage segments used were approximately 5cm long and clipped from the distal end of branches.

*Statistical Analysis*

Insects’ final positions in the olfactometer were analyzed using the Cochran Q test and post-hoc tests for preference in a randomized block design (Experiments 1-5) (Zar 1999). Comparisons of response times (Experiments 2, 3, and 5) were completed using one-way analysis of variance (ANOVA). Analyses of differences in choice distributions based on insect collection origin were completed using the likelihood ratio statistic and Fischer’s exact test (Experiment 5). All statistical analyses were carried out using SPSS software package (IBM Corp. version 20 for Mac, released 2011).

**Experiment 1: Does *S. coniferarum* respond to odors from hemlock woolly adelgid host trees?** Three-way choice bioassay. We observed and recorded the response of *S. coniferarum* collected from western hemlock in an olfactometer to host foliage. Air
flow passed through the chambers and over each foliage treatment: western hemlock, and eastern hemlock, and an empty chamber considered the control. Each treatment was randomly attached to the arms of the olfactometer. Individuals were starved for four hours prior to bioassays.

**Experiment 2: Does the period of starvation prior to bioassays affect the ambulatory response of *S. coniferarum***? Three-way choice bioassay. We observed and recorded the responses of *S. coniferarum* starved for 6, 12, 18, or 24 hours. The host plant treatments, western hemlock and eastern hemlock, and blank control were assigned to the chambers and randomly attached to arms of the olfactometer. This assay was conducted in June 2011. Time spent walking in the center field before making a choice was recorded, and grouped dependent on the starvation period prior to the assays.

**Experiment 3: Does the amount of time between collection off of host trees and conducting the behavioral bioassays affect the ambulatory response of *S. coniferarum***? Three-way choice bioassay. We observed and recorded the responses of *S. coniferarum* that were assayed either 3, 4, 6, 7, 9 or 11 days after their collection off of western white pine. Beetles were starved for 4 hours prior to the behavioral assay. The host plant treatments, western hemlock and eastern hemlock, and a blank control were placed into the chambers and randomly attached to the arms of the olfactometer. Each time frame was replicated between nine and 22 times depending on the number collected from the field.
Experiment 4: Do *Scymnus coniferarum* reared or collected from eastern hemlock respond to odors from eastern or western tree species? Three-way choice bioassay. We observed and recorded the responses of *S. coniferarum* reared from egg to adult on hemlock woolly adelgid-infested eastern hemlock in the laboratory or collected in the field as adults from hemlock woolly adelgid-infested western hemlock. *Scymnus coniferarum* reared for two generations on eastern hemlock infested with hemlock woolly adelgid in the laboratory were sent overnight from the Beneficial Insects Rearing Facility at Virginia Polytechnic Institute. Again, populations were kept separate but were randomly selected for each trial. The treatment and insect were rerandomized for each trial. Fifteen individuals from each population were assayed. Three groups of bioassays were completed, each comprised of fifteen individuals. Treatments included eastern hemlock, a blank control, and one of the following: western hemlock, or ponderosa pine.

Experiment 5: Do individuals collected from western white pine and western hemlock respond to host foliage, and does *S. coniferarum* collected from each source tree respond differently to host foliage? Four-way choice bioassay. We observed and recorded the responses of *S. coniferarum* collected from both western hemlock near Corvallis, OR and western white pine near Boise, ID to compare behavior based on collection origin. Similar to the previous assays, individuals from each population were bioassayed once. Treatments included foliage from eastern hemlock,
western hemlock, western white pine, and a blank control using the methodology described above.

Results

Experiment 1: Does *Scymnus coniferarum* exhibit a preference for odors from host tree foliage? *Scymnus coniferarum* did not prefer host tree foliage over the blank control or center field, and there is no indication that this behavior is non-random (Table 1) (Corchran Q statistic, $X^2=1.204$, $p=0.752$). *Scymnus coniferarum* did not prefer volatiles emitted from eastern versus western hemlock in a pairwise post-hoc test (Corchran Q, $X^2=1.000$, $p=0.317$). *Scymnus coniferarum* did not prefer eastern hemlock or western hemlock over the blank control or the center field in post-hoc tests (Cochran Q, $X^2=0.412$, $p=0.814$, and $X^2=0.615$, $p=0.735$, respectively).

Experiment 2: Does the starvation period affect the ambulatory response of *S. coniferarum*? *Scymnus coniferarum* did not chose a stimulus field more or less quickly based on starvation period ($F=1.40$, df=3, $p=0.24$) (Table 2). There was also no observable difference among starvation periods and in mean time spent in each field (Table 2). *Scymnus coniferarum* chose to walk in stimulus fields containing foliage rather than remain in the center field in the six and 12-hour starvation groups (Cochran Q statistic, $F=4.666$, $p=0.198$, $F=4.667$, $p=0.198$, respectively) (Table 2). Individuals starved for 18 hours remained in the center field more than walking toward a stimulus field (Cochran Q statistic, $X^2=16.931$, $p=0.001$). Individuals starved for 24 hours
remained in the center field more than walking towards a stimulus field, however this
difference was not significant (Table 2, Cochran Q statistic, $X^2 = 1.296, p=0.730$).

**Experiment 3: Does the amount of time elapsed since collection affect the
ambulatory response of *S. coniferarum*?** Insects responded differently depending on
the time elapsed between collection and bioassay (Table 3). *Scymnus coniferarum* spent
the lowest mean and overall time required to choose a stimulus field six days after
collection ($F=3.35, df=5, p=0.008$). Regardless of time elapsed between collection date
and bioassay date, individuals were just as likely to walk in the center field as to choose
a stimulus field (Table 3).

**Experiment 4: Does *S. coniferarum* collected from eastern hemlock prefer western
host foliage?** In multiple three-way choice bioassays (Table 4), individuals did not
prefer eastern hemlock, blank control, remaining in the center field, or western white
pine (Cochran Q test, $X^2 = 3.933, p=0.269$), western hemlock (Cochran Q test,
$X^2=0.733, p=0.865$) or ponderosa pine (Cochran Q test, $X^2=5.000, p=0.172$). It is
possible that these beetles were walking randomly about the arena. In post-hoc
comparisons, insects neither choose eastern hemlock nor western white pine (Cochran
Q test, $X^2=1.000, p=0.317$), nor between eastern hemlock and western hemlock
(Cochran Q test, $X^2=0.111, p=0.739$). *Scymnus coniferarum* individuals did
significantly prefer eastern hemlock over ponderosa pine (Cochran Q, $X^2=4.500$,
p=0.034).
Experiment 5: Does the collection origin of *S. coniferarum* individuals influence the host choice preference? In this set of bioassays, *S. coniferarum* walked non-randomly in the olfactometer, as *S. coniferarum* collected from western white pine had an overall preference for stimulus fields other than the western white pine field (Table 5, experiment 5a, Cochran’s Q, $X^2=10.696$, $p=0.030$), and walked to and remained in the western hemlock stimulus field over western white pine (Cochran Q, $X^2=9.000$, $p=0.003$), as well as the blank control over western white pine (Cochran Q, $X^2=7.000$, $p=0.008$). But they did not walk to eastern hemlock over western white pine (Cochran Q, $X^2=3.000$, $p=0.083$). *Scymnus coniferarum* collected from western hemlock did not choose any specific stimulus field (Table 5, experiment 5b, Cochran Q, $X^2=7.667$, $p=0.105$), nor did they chose western hemlock, eastern hemlock, or western white pine more often than the others (Cochran Q, $X^2=3.294$, $p=0.193$). Individuals collected from both western hemlock and western white pine were more likely to choose a stimulus field than to walk in the center field without making a choice. The percent of individuals that walked in the center field without making a choice were 4% and 18%, respectively.

Direct comparison of final positions for *S. coniferarum* using the original sample sizes demonstrated no effect of collection origin on ambulatory response for any stimulus field (Table 5, experiment 5c). Likelihood ratio chi-square test demonstrated that there was no directional association between the source of *S. coniferarum* and choices made ($X^2=5.70$, $p=0.341$). The distribution of choice proportions was not found to be dependent upon the collection origin either (Fischer exact test, $p=0.417$).
There was no significant difference in time spent walking in the center field before making a choice between populations collected from each tree origin (Table 5, experiment 5c, ANOVA, F=0.159, p=0.693).

**Discussion**

*Scymnus coniferarum* did not appear to walk to or remain in any stimulus field containing host foliage over any other, as in nearly all experiments, choices were relatively evenly distributed. Since *S. coniferarum* is found associated with several aphid and adelgid prey on a number of coniferous host trees (McDonald et al. 2010, Montgomery and McDonald 2010, Montgomery et al. 2011), it is possible that their sensory reception for host cues is less specialized than that of a predator with a more limited host range (Agrawal 1998, Lucas 2001, McCormick et al. 2012), and thus can be attracted to coniferous host foliage of many types. The objective in experiment one was to determine whether *S. coniferarum* would prefer eastern white pine to eastern hemlock odors. In its native range, *S. coniferarum* was found to be associated with and feeding on adelgids on western hemlock, western white pine, other pine species, firs, and spruces, as well as with woolly apple aphid and citrus mealybug (McDonald et al. 2010, Montgomery and McDonald 2010, Montgomery et al. 2011).

To be able to properly assess *S. coniferarum* as a biological control agent, laboratory protocols need to be developed that yield the most informative and applicable results. In experiments two and three, we tested the protocols used in the olfactometer bioassays to determine which bioassay protocols elicited the best response in *S. coniferarum*. The results here demonstrate that an intermediate starvation period
of approximately 12 hours increased the response to stimuli (Table 2). Individuals collected six days prior to bioassays responded in the olfactometer most quickly, and none of these individuals remained in the center field (Table 3). It is possible that insects held in an artificial environment for too long could lose their overall vigor and response to stimuli. Conversely, insects bioassayed soon after collection would be less likely to forage because they could still be satiated from a prior foraging event. Our results demonstrate that an intermediate number of days between collection and bioassay are sufficient for a response in the olfactometer, which will further inform future collections and shipments for laboratory testing. An intermediate level of food deprivation encourages foraging, because individuals are not satiated, but they also are not conserving the last of their energy reserves (Bond 1980, Henaut et al. 2002), therefore, they have an impetus to find food, as well as enough energy to amble about and forage extensively.

*Scymnus coniferarum* was active in the olfactometer, but in a three-way bioassay completed in experiment four, choices were evenly distributed, and no stimulus field was especially attractive, and walking behavior may have been random. Host foliage from the western United States (native range) and eastern United States (novel range) were selected as the treatments in these bioassays to compare behavior based on host foliage *S. coniferarum* is exposed to in native and novel environments. Often, *S. coniferarum* demonstrated a similar response in direct comparisons between foliage from the east and the west in the olfactometer, with the exception of a preference for eastern hemlock over ponderosa pine.
In experiment five, we tested whether collection origin affected the behavioral response of *S. coniferarum*. There were no behavioral differences between *S. coniferarum* collected from western hemlock and *S. coniferarum* collected from western white pine. Individuals from both populations were responsive in the olfactometer and hemlock species were preferred over the other options. This result is a good sign for biological control in the future. Pines and hemlocks are ubiquitous and often co-dominant in the introduced environment, and the ability to discern the cues from each other and alight on the hemlock will help predators forage more efficiently and find the intended prey in the introduced environment.

*Scymnus coniferarum*’s preference for eastern hemlock over ponderosa pine may be due to the rarity of finding prey on ponderosa pine. Adelgids are known to have a coevolutionary relationship with their gymnosperm hosts (Havill and Foottit 2007), and natural enemies use this relationship to find their prey (Dixon 1959, Vet et al. 1991, Havill and Raffa 2000). Although pine bark adelgid is occasionally found on ponderosa pine, this species is not one of its primary hosts. Surveys for *S. coniferarum* in the western United States revealed high population density associated with pine adelgids and hemlock woolly adelgid on western white pine, lodgepole pine, Monterey pine, and western hemlock (Montgomery and McDonald 2010, Montgomery et al. 2011). Using both instinct and associative learning, natural enemies of adelgids can be more certain of prey availability on eastern hemlock as opposed to ponderosa pine.
Conclusions

Behavioral bioassays are intended to give researchers insight into the behavior of insects and subsequently inform management, so it is important for the specimens to react and respond to cues in the olfactometer. For the most responsive and informative olfactometer bioassays, and to inform optimal host finding in the field releases, the data suggests that *S. coniferarum* individuals should be starved for 6-12 hours and tested or released within six days of collection. Our results demonstrate that *S. coniferarum*, a known predator of adelgid species in its native range, is most attracted to foliage of species that are well-documented as habitat for either hemlock woolly adelgid or pine bark adelgids. Collection origin did not have a strong effect on *S. coniferarum* response and eastern hemlock was just as attractive as western foliage species, which indicates that *S. coniferarum* will be able to locate its prey’s host. *Scymnus coniferarum* is attracted to adelgid and aphid host foliage, but it is a generalist among its prey, it will not necessarily prefer HWA on eastern hemlock, which is the intended prey in this biological control effort.

References


Tables

Table 1[Chapter 2, Table 1] Ambulatory responses of *S. coniferarum* to eastern hemlock, western hemlock, and a blank control in 3 way choice bioassays, as well as post-hoc multiple comparisons for specific treatments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stimulus Field</th>
<th>Proportion of Choice</th>
<th>$X^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-way choice</td>
<td>Eastern hemlock</td>
<td>0.20</td>
<td>1.204</td>
<td>0.752</td>
</tr>
<tr>
<td></td>
<td>Western Hemlock</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-hoc tests:</td>
<td>EH/Blank/Center</td>
<td></td>
<td>0.412</td>
<td>0.814</td>
</tr>
<tr>
<td></td>
<td>WH/Blank/Center</td>
<td></td>
<td>0.615</td>
<td>0.735</td>
</tr>
<tr>
<td></td>
<td>EH/WH</td>
<td></td>
<td>1.000</td>
<td>0.317</td>
</tr>
<tr>
<td></td>
<td>Blank/Center</td>
<td></td>
<td>0.167</td>
<td>0.683</td>
</tr>
</tbody>
</table>
Table 2 [Chapter 2, Table 2] Proportion of individuals choosing each stimulus field, and preference for stimulus fields, based on the length of time they were starved prior to bioassays.

<table>
<thead>
<tr>
<th>Starvation Time</th>
<th>N</th>
<th>Mean Response Time ± SE (seconds)</th>
<th>Proportion of individuals choosing each stimulus field</th>
<th>( \chi^2 )</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tsuga heterophylla</td>
<td>Tsuga canadensis</td>
<td>Blank Control</td>
</tr>
<tr>
<td>6 hours</td>
<td>30</td>
<td>374.4±43.6</td>
<td>0.30</td>
<td>0.37</td>
<td>0.23</td>
</tr>
<tr>
<td>12 hours</td>
<td>12</td>
<td>266.2±69.0</td>
<td>0.25</td>
<td>0.50</td>
<td>0.08</td>
</tr>
<tr>
<td>18 hours</td>
<td>29</td>
<td>433.3±44.4</td>
<td>0.10</td>
<td>0.28</td>
<td>0.07</td>
</tr>
<tr>
<td>24 hours</td>
<td>27</td>
<td>387.9±46.0</td>
<td>0.26</td>
<td>0.19</td>
<td>0.22</td>
</tr>
</tbody>
</table>

\( P \) value=0.247
Table 3 [Chapter 2, Table 3] Proportion of individuals choosing each stimulus field, and preference for stimulus fields, based on the length of time elapsed between the day of collection and the day of bioassays.

<table>
<thead>
<tr>
<th>Elapsed Time</th>
<th>N</th>
<th>Mean Response Time ± SE (seconds)</th>
<th>Proportion of individuals choosing each stimulus field</th>
<th>X²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tsuga heterophylla</td>
<td>Tsuga canadensis</td>
<td>Blank Control</td>
</tr>
<tr>
<td>3 days</td>
<td>10</td>
<td>333.1±62.7</td>
<td>0.10</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>4 days</td>
<td>22</td>
<td>429.1±42.3</td>
<td>0.28</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>6 days</td>
<td>9</td>
<td>142.4±66.1*</td>
<td>0.56</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>7 days</td>
<td>18</td>
<td>316.8±46.7</td>
<td>0.44</td>
<td>0.28</td>
<td>0.06</td>
</tr>
<tr>
<td>9 days</td>
<td>22</td>
<td>262.5±42.3</td>
<td>0.36</td>
<td>0.23</td>
<td>0.18</td>
</tr>
<tr>
<td>11 days</td>
<td>17</td>
<td>369.5±33.4</td>
<td>0.35</td>
<td>0.18</td>
<td>0.12</td>
</tr>
</tbody>
</table>

P value=0.008*
Table 4 [Chapter 2, Table 4] Ambulatory response of *Scymnus coniferarum* collected from eastern hemlock to eastern hemlock and western host foliage in olfactometer bioassays. Post-hoc multiple comparisons for eastern hemlock, compared to western host foliage, including western white pine, ponderosa pine, and western hemlock.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Final position</th>
<th>N</th>
<th>Proportion of Choice</th>
<th>Post-hoc Comparison</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
<td>0.269</td>
</tr>
<tr>
<td></td>
<td>Eastern hemlock</td>
<td>1</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>6</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>5</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Western white pine</td>
<td>3</td>
<td>0.20</td>
<td>Eastern hemlock and western white pine</td>
<td>0.317</td>
</tr>
</tbody>
</table>

| 4b         | Overall        |   |                      |                     | 0.172   |
|            | Eastern hemlock| 7 | 0.47                 |                     |         |
|            | Blank          | 3 | 0.20                 |                     |         |
|            | Center field   | 4 | 0.26                 |                     |         |
|            | Ponderosa pine | 1 | 0.07                 |                     |         |
|            |                |   |                      | Eastern hemlock and ponderosa pine | 0.034* |

| 4c         | Overall        |   |                      |                     | 0.865   |
|            | Eastern hemlock| 5 | 0.33                 |                     |         |
|            | Blank          | 3 | 0.20                 |                     |         |
|            | Center field   | 3 | 0.20                 |                     |         |
|            | Western hemlock| 4 | 0.26                 |                     |         |
|            |                |   |                      | Eastern hemlock and western hemlock | 0.739 |
Table 5 (Chapter 2, Table 5) Ambulatory responses of *S. coniferarum* individuals collected from either western white pine or western hemlock. Direct comparisons of choices and time spent before making a choice for each collection origin.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>N</th>
<th>Stimulus Field</th>
<th>Proportion</th>
<th>$X^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a:</td>
<td>22</td>
<td>Eastern hemlock</td>
<td>0.39</td>
<td>10.696</td>
<td>0.030*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western hemlock</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western white pine</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blank control</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Center field</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-hoc tests</td>
<td></td>
<td>WWP/WH</td>
<td>0.39</td>
<td>9.000</td>
<td>0.003*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WWP/WH/EH</td>
<td>0.25</td>
<td>10.500</td>
<td>0.005*</td>
</tr>
<tr>
<td>5b:</td>
<td>24</td>
<td>Eastern hemlock</td>
<td>0.38</td>
<td>7.667</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western hemlock</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western white pine</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blank control</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Center field</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-hoc tests</td>
<td></td>
<td>WWP/WH/EH</td>
<td>0.38</td>
<td>3.294</td>
<td>0.193</td>
</tr>
<tr>
<td>5c:</td>
<td></td>
<td>Eastern hemlock</td>
<td>0.50</td>
<td>0.500</td>
<td>0.480</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western hemlock</td>
<td>0.00</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western white pine</td>
<td>0.25</td>
<td>3.000</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blank control</td>
<td>0.04</td>
<td>0.077</td>
<td>0.782</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Center field</td>
<td>0.13</td>
<td>1.000</td>
<td>0.317</td>
</tr>
<tr>
<td>5d:</td>
<td>24</td>
<td>Western hemlock</td>
<td>275.8±34.9</td>
<td>275.8±34.9</td>
<td>0.693</td>
</tr>
<tr>
<td>Mean response time</td>
<td>22</td>
<td>Western white pine</td>
<td>256.5±33.5</td>
<td>256.5±33.5</td>
<td></td>
</tr>
</tbody>
</table>
Ch. 3: Behavioral responses of *Laricobius osakensis* (Coleoptera:Derodontidae) to hemlock woolly adelgid and host tree odors in an olfactometer, and a comparison of the ambulatory response of *Laricobius nigrinus* x *Laricobius rubidus* hybrids to parental species, *Laricobius nigrinus* and *Laricobius rubidus* (Coleoptera:Derodontidae)

Abstract

*Laricobius* spp. were considered as potential biological control agents early in the efforts to control hemlock woolly adelgid. Surveys in the native ranges of hemlock woolly adelgid resulted in the discovery of several *Laricobius* species, including *Laricobius nigrinus* in western North America and *Laricobius osakensis* in Japan. *Laricobius nigrinus* was released in HWA’s invasive range beginning in 2003, and recently, hybridization was recognized between *L. nigrinus* and native *L. rubidus*. Hybridization could affect behaviors tested prior to release. Behavioral bioassays of these species can demonstrate whether biological control agents can locate prey in the environment, and whether they have a preference for the volatile cues of the intended prey. Olfactometer bioassays were used to test behaviors of these species in response to host odors of HWA infested eastern hemlock, eastern hemlock, and eastern white pine. In all cases, predators were most attracted to HWA induced host odors from infested foliage, and LnxLr hybrids behaved similarly to *L. nigrinus*. *Laricobius osakensis* was the most responsive of the species tested, and significantly preferred HWA infested eastern hemlock foliage to other host odors.
Introduction

Unlike other members of the family Derodontidae, which are fungal feeders, \textit{Laricobius} spp. are adelgid specialists, and their phenologies and life histories are highly synchronized with their hosts (Salom et al. 2005, Zilahi-Balogh et al. 2006). \textit{Laricobius} spp. are found throughout the northern hemisphere where adelgids are associated with conifers (Montgomery et al. 2011). Early efforts in biological control against hemlock woolly adelgid (\textit{Adelges tsugae} Annand, HWA), \textit{Laricobius} spp. were recognized as potential natural enemies for importation, due to their coevolutionary history with adelgids, as well as high density on adelgid-infested conifers (Zilahi-Balogh et al. 2003, Kohler et al. 2008, Leschen 2011). Explorations in the native ranges of HWA resulted in the discovery of several \textit{Laricobius} spp. on hemlocks in Asia and western North America.

\textit{Laricobius osakensis} Shiyake and Montgomery (Lo) is a recently described species that is endemic to Japan and preys on HWA associated with southern Japanese hemlock (\textit{Tsuga sieboldii} (Carriere)) (Montgomery et al. 2011). It was discovered as an important HWA predator in Japan in 2005, collected in 2006, and reared in quarantine for several generations (Lamb et al. 2012). \textit{Laricobius osakensis} has a lower temperature threshold, a higher feeding rate, and more successful larval development on HWA than congeneric species already considered for biological control of HWA (Vieira et al. 2011). \textit{Laricobius osakensis} had a significant impact on HWA mortality in its native range, can complete its life cycle on HWA, and is not able to develop on non-adelgid hosts. In 2010, a Finding of No Significant Impact (FONSI) was completed, so the species can be released from quarantine and field studies can begin.
*Laricobius nigrinus* Fender (Ln) is an HWA-specific predator native to the northwestern United States and British Columbia, and can be found associated with HWA on western hemlock (*Tsuga heterophylla*) (Zilahi-Balogh et al. 2003, Kohler et al. 2008, Grubin et al. 2009, Mausel et al. 2010). In its native range, the laboratory setting, and preliminary release studies, Ln fed voraciously on HWA, oviposited and completed its development on HWA, and was very host-specific (Mausel et al. 2012). In 2000, the federal government granted a Finding of No Significant Impact (FONSI) for Ln, making this species eligible for release in the invasive range. Since the first releases in 2003, over 100,000 individuals have been released at several sites across the invasive range (Mausel et al. 2012).

*Laricobius rubidus* LeConte (Lr) is endemic to the eastern United States (Leschen 2011). Its native range spreads from the Washington DC metropolitan area northward to Newfoundland, Canada (Zilahi-Balogh et al. 2006). The primary prey for this species is pine bark adelgid (*Pineus strobi*) on eastern white pine (*Pinus strobus*) but it has been found on eastern hemlock feeding on HWA. *Laricobius rubidus* and Ln are closely related, and only recently diverged evolutionarily (Montgomery et al. 2011, Havill et al. 2012). After the release of Ln, post-release monitoring studies indicated individuals with intermediate traits, as well as observations of Ln and Lr mating in the environment (Mausel et al. 2008). Mating between Ln and Lr results in hybridization, which was an unexpected consequence of the biological control efforts of HWA.

In the context of a biological control program, there is uncertainty surrounding the potential behavioral differences between hybrids and parental species. *Laricobius nigrinus* underwent considerable pre-release testing for host specificity and host
location prior to release (Zilahi-Balogh et al. 2003; Flowers et al. 2007; Mausel et al. 2010; Wallin et al. 2011) but in some systems, hybridization is known to cause unpredictability, a loss of host specificity (Stouthamer et al. 2000; Hora et al. 2005) and reduced effectiveness of the biological control agent (Szucs et al. 2012). These consequences could have a strong influence on the biological control efforts of HWA in the future.

Introductions of nonnative species can have large impacts on the genetics of native species through hybridization and introgression (i.e. gene flow) (Mallet 1998; Mooney and Cleland 2001). It is not uncommon for related species to hybridize immediately after introductions, with isolation mechanisms developing over many generations (Remington 1968), but this immediate gene flow creates an unstable hybrid zone. Hybrid zones are remedied in one of three ways: evolution of mating barriers, fusion of the two taxa into a single species, or the extirpation of one of the parental species (Harrison 1983; Harrison 1986; Wainger and Mazzotta 2011). There is substantial evidence that hybridization reduces fitness from generation to generation, causing a phenomenon known as “hybrid breakdown” (Dopman et al. 2009). Hybridization between Ln and Lr could impact hybrid incompatibility or hybrid vigor. Each single species might lack the necessary variation to colonize or compete in a new niche, but such variation could be maintained on the novel host and become mobilized by hybridization.

The behavior of biological control agents, with respect to host location, host range, and host specificity, is important to the success of a biological control program (Wallin 2012), and is often tested and observed as part of a suite of pre-release
assessments. Hybridization, as described above, reduces the predictability of organisms, which would subsequently impact the efficacy of the biological control program, so it is important to understand the behavior of the hybrid individuals, in addition to the behavior of the parental species.

Insect behavior is driven by volatile cues released by many trophic levels in the environment (Wallin 2012). Insects can use volatile cues to identify and locate suitable food items and habitats, avoid predation or risk, and find mates (Wallin et al. 2011, Keesey et al. 2012). Host plants emit cues, as do prey organisms. Host plants can also emit herbivore-induced cues as part of an induced defense complex (Agrawal 1998, Havill and Raffa 2000, McCormick 2012). Odors from the varying trophic levels convey information to contribute to prey foraging (Mausel et al. 2010). Cues from host foliage are accessible at long ranges and are highly detectable in the environment, whereas the often small size of prey means that prey emit less detectable, but more reliable cues, as prey-specific volatile cues are a clear indication of prey availability at the site (Vet et al. 1991). One way to overcome the reliability-detectability problem described by Vet et al. (1991) is to use multitrophic herbivore induced volatile cues, where are both long-range and specific indication of herbivore feeding (Havill and Raffa 2000, Keesey et al. 2012).

Olfactometer bioassays can offer insight into insect behaviors through observations of preference for stimuli, and subsequently assess the responses of potential biological control agents to volatile cues from target hosts compared to cues from other potential hosts in the novel environment (Walter et al. 2010). The first objective of this study was to evaluate the responses of laboratory-reared Lo to potential
host odors, including eastern hemlock, eastern white pine, and eastern hemlock infested with HWA, that it will be exposed to upon release, to determine whether this species will be attracted to HWA in the environment. The second objective was to evaluate and compare the responses of Ln, Lr and LnxLr hybrids to host odors, including eastern hemlock and eastern hemlock infested with HWA, the primary host of Ln, and eastern white pine, the primary host tree of Lr to determine to what extent the behavior of hybrid individuals follows that of either of the parental species.

Materials and Methods

Insect and Foliage Collection

Behavioral bioassays were conducted to test the ambulatory response of adult predatory beetles to prey and foliage from several coniferous host species. Bioassays were conducted from 2011-2013. We tested adult Ln, Lr, and LnxLr hybrids that were wild-caught near Asheville, NC and Banner Elk, NC in spring 2011, fall 2011, and fall 2012, and in 2013 we tested adult Lo that were reared in quarantine at the Beneficial Insects Rearing Facility at Virginia Polytechnic Institute. All beetles tested were randomly chosen from the test group and tested once. Wild-caught beetles were killed after bioassays and stored in individual vials for genetic analysis, whereas lab-reared Lo individuals were returned to Biological Rearing Facility at Virginia Polytechnic Institute within twelve hours of completing the bioassays.

Responses of individual Laricobius spp to test foliage were measured similarly to Arsenault et al. (in prep) and Wallin et al. 2011 in a four-chambered olfactometer arena (Analytical Reseach Systems, #OLFM-4-C-2440PE, Gainesville, FL, 30x30x3
cm). The arena was comprised of the base with air output, the intermediate part that delimited the walking chamber with four air inputs and a 9-mm circular central opening to introduce insects and attach the vacuum. Treatments, in this case host foliage, were placed in glass chambers that can then be attached to the arms of the arena. There were up to four possible chambers for treatments. Four flow-meters (Brooks Instrument, Hatfield, PA) controlled airflow at a rate of 0.12 Mpa into the glass chambers containing the test material, or an empty chamber regarded as a “blank” or control field, and carried volatiles into the olfactometer. Volatiles were removed from the arena through the vacuum in the center, which maintains the integrity of the air fields.

Beetles were starved 24-25 hours prior to bioassays to increase their responsiveness to treatments (Wallin et al. 2011). For each experiment described below, a single individual was placed into the center of the assay arena, equidistant from the entrance from all four olfactometer arms. The assay arena was divided into four equal sized fields and a 9-cm central field. A choice consisted of a beetle leaving the central field and crossing into the delineated field boundary for more than one minute. The final position at the end of the behavioral assay was recorded, as well as the time required for the beetle to choose a field. The maximum time a beetle was allowed to walk in the arena was 600 seconds, and at this time the beetle was removed from the arena. If the beetle attempted to crawl into the arm, the treatment held in the chamber on that arm was considered the final choice, and the beetle was removed from the arena. If a beetle remained in the central field up to ten minutes without choosing an arm, the behavior was recorded as “no choice.” The position of each source chamber containing different prey host material was randomly positioned prior to the bioassay for each
individual. Host foliage was replaced every hour to ensure that chemical compositional changes over time did not confound the results.

HWA infested hemlock foliage was shipped from the same collection location as the beetles for each respective bioassay. Uninfested eastern hemlock and eastern white pine foliage was collected in South Burlington, VT and treated similarly to shipped foliage. Foliage used in each experiment described below was clipped, wrapped in damp paper towels and parafilm, and sealed tightly in plastic bags and kept on ice and stored no longer than 48 hours at 2-3 C until used in the assay.

**Genetic Analysis**

Due to similar morphologies, field collected species of *Laricobius* spp cannot be distinguished visually, therefore bioassays using these individuals were species-blind at the time of the experiments. Following the behavioral assays, beetles were individually placed in labeled vials with 95% ethanol. The behavioral responses were sorted by species after genetic analysis. *Laricobius* spp. were determined using methods described in Havill et al. 2012 at the USFS Northern Research Station, Hamden CT in 2012 and 2013. Tissue was removed under a dissecting microscope, and DNA was extracted from the dissections using the Promega IQ DNA protocol and amplified using Promega GoTaq Flexi Polymerase PCR system. Then genotypes were analyzed using STRUCTURE and NEW HYBRIDS programs at Yale University based on six microsatellite loci (Molecular Ecology Resources Primer Development Consortium 2010) and compared to a catalogue of known individuals for each species, using a percent of similarity to the individuals in the catalogue.

**Statistical Analysis**
Insects’ final positions in the olfactometer were analyzed using the Corchran Q test and post-hoc tests for preference in a randomized block design (Zar 1999). Comparisons of response times were completed using one-way analysis of variance (ANOVA). In analyses regarding response times, individuals that remained in the center field without making a choice were considered to use the maximum time (600s) to make a choice. All statistical analyses were carried out using SPSS software package (IBM Corp. version 20 for Mac, released 2011).

Experiment 1: What was the species and hybrid distribution Laricobius spp populations collected from eastern hemlock and eastern white pine in 2011 and 2012? After olfactometer bioassays and subsequent genetic analysis, the distribution of Ln, Lr, and LnxLr hybrid individuals within each population was determined and compared by collection date and species for each insect collection using a Chi-square analysis.

Behavioral Bioassays

Experiment 2: Do Laricobius species respond to odors from hemlock woolly adelgid or host trees? Three-and four-way choice bioassays. We observed and recorded the responses of field collected Laricobius spp. to host foliage in 3- and 4-way choice bioassays in an olfactometer. These bioassays were completed in the spring of 2011, fall of 2011, and fall of 2012. The host foliage treatments included eastern hemlock, eastern white pine, and a blank control in the three-way choice bioassays, or additionally included eastern hemlock infested with hemlock woolly adelgid in the four-
way choice bioassays. Host foliage was randomly placed in each chamber and attached to an arm of the olfactometer. Individual beetles were starved for 24 hours prior to bioassays. According to the methodology described above, final positions and time required for individuals to make a choice were recorded and analyzed across collection origin, species, and collection date after the completion of the genetic analysis.

Experiment 3: Does laboratory-reared *Laricobius osakensis* respond to, or prefer, odors from foliage? Four-way choice bioassay. *Laricobius osakensis* individuals were laboratory reared to the F$_2$ generation at the Beneficial Insects Rearing Facility at Virginia Polytechnic Institute. Two shipments of 50 individuals each packed on ice and shipped overnight to the Forest Service laboratory in South Burlington, VT, on ice and off of food in December 2012. They were starved for 18-24 hours. Bioassays were completed as described above. The host plant treatments consisted of HWA-infested eastern hemlock, uninfested eastern hemlock, eastern white pine, and a blank control. Final positions and the time required to make a choice were recorded. Immediately following the bioassays, Lo individuals were returned to containers containing HWA-infested eastern hemlock, and shipped overnight to the Beneficial Insects Rearing Facility at Virginia Polytechnic Institute.

Results

Experiment 1: Did the species distribution of individuals in *Laricobius* spp populations collected from eastern hemlock and eastern white pine in 2011 and 2012 change? The percent of LnxLr hybrid individuals collected from eastern hemlock
and eastern white pine in 2011 and 2012 ranged between 8.8-28.3% on eastern hemlock, and between 13.3-18.5% on eastern white pine (Table 1). Overall, the percent of LnxLr hybrid individuals ranged from 8.8-28.3%, and was nearly 20% higher in the sample collected in fall of 2011 compared to that in 2012.

The proportion of hybrids did not trend toward an increase or decrease over time. Although the proportion of hybrids was not consistent through time, chi-square analysis of the distribution individuals, blocked by species and compared by the time of year collected, demonstrated that the relative frequencies of Ln, Lr, and LnxLr hybrids were not significantly different (F=0.8704, p=0.4855). When blocked by the time of year collected (fall vs. spring), the number of Ln are significantly higher than the number of Lr or LnxLr hybrids in collections from eastern hemlock (F=14.1417, p=0.0154). In each season, the majority of individuals collected from each tree origin were as predicted with more Ln collected off eastern hemlock and Lr collected off eastern white pine, respectively, than from the alternate tree species.

**Experiment 2:** Do *Laricobius* spp respond to odors from hemlock woolly adelgid or host trees? Three- and four-way choice bioassays. Collection origin did not affect the amount of time it took for individuals to respond to volatiles in the olfactometer (F=1.23, p=0.267, Table 2). Individuals collected and bioassayed in the spring of 2011 responded on average 168 seconds slower than those collected and bioassayed in either the fall of 2011 or 2012 (F=34.28, p<0.001, Table 2). Over all host tree species and dates of collection, Ln responded on average 76 seconds more quickly than either Lr or LnxLr hybrids (F=5.24, p=0.006, Table 2).
Bioassays with *Laricobius* spp responded to eastern hemlock and eastern white pine in the olfactometer, but they remained in the center field most often, regardless of species or collection origin (Corchran Q test, $X^2 = 60.359$, $p < 0.001$, Table 3). In post-hoc tests we removed the center field from the analysis, did not see a preference for any host foliage (Corchran Q test, $X^2 = 0.080$, $p = 0.961$, Table 3). Individuals collected from eastern white pine and eastern hemlock were more likely to remain in the center field than to chose a stimulus field (Corchran Q test, $X^2 = 38.148$, $p < 0.001$), as did all individuals collected from eastern hemlock (Corchran Q test, $X^2 = 22.471$, $p < 0.001$).

*Laricobius rubidus* individuals collected from eastern hemlock or eastern white pine remained in the center field (Corchran Q test, $X^2 = 27.231$, $p < 0.001$), both when considered separately and as a group. *Laricobius nigrinus* individuals collected from eastern hemlock and eastern white pine remained in the center field (Corchran Q test, $X^2 = 20.146$, $p < 0.001$) when considered as a group, as did Ln only collected from eastern hemlock (Corchran Q test, $X^2 = 18.704$, $p < 0.001$). LnxLr hybrids collected from eastern hemlock did not remain in the center field and chose host foliage (Corchran Q test, $X^2 = 7.333$, $p = 0.062$). However, LnxLr hybrids collected from eastern white pine remained in the center field (Corchran Q test, $X^2 = 10.800$, $p = 0.013$). In post-hoc tests, we removed the center field as an option, and *Laricobius* spp. did not choose any stimulus field more than any other (Table 2). None of the fields were completely neglected, and individuals were just as likely to choose any host treatments.

In four-way choice bioassays completed in fall of 2011 and 2012, *Laricobius* spp. individuals moved freely about the olfactometer and responded to host odors. Overall, no species collected from eastern hemlock preferred any field over the others,
so there is no indication that these individuals were choosing fields non-randomly, but Lr collected from eastern white pine in 2012 chose the field containing white pine to other fields (Corchran Q test, $X^2=28.150$, $p<0.001$). In both 2011 and 2012, Ln collected from eastern hemlock walked toward the field containing HWA-infested foliage more than any other field, proportionally (33% and 28%, respectively). Overall, individuals did not prefer any stimulus field or the central field (2011: Corchran Q test, $X^2=2.869$, $p=0.580$, 2012: Corchran Q test, $X^2=6.270$, 0.180). In 2011, LnxLr hybrid attraction to HWA infested eastern hemlock, eastern white pine and the blank control was relatively even in 2011 (27%, 33%, 27%, respectively), resulting in no overall preference for any field (Corchran Q test, $X^2=7.349$, $p=0.119$). In 2012, LnxLr hybrid individuals did not choose eastern white pine at all in the bioassay, and chose eastern hemlock more than any other field (50% of choices). Otherwise, choices were evenly distributed, with no field preferred over any other (Corchran Q test, $X^2=4.000$, $p=0.406$). Small sample size in this category accounts for the lack of statistical preference, even though there is a proportional divergence in choices.

Multiple post-hoc comparisons allow for within-experiment analysis limited to two or more stimulus fields. Post-hoc tests demonstrated that for most species, origins, and collection dates, there was no significant preference for any stimulus field or center field (Table 5). Laricobius nigrinus collected from eastern hemlock did not prefer any field over any other. LnxLr hybrids collected from eastern hemlock chose all fields containing host odors including the blank control more than remaining in the center field. These insects were more likely to choose a field than to walk in the center of the
arena. *Laricobius rubidus* collected from eastern white pine chose eastern white pine over eastern hemlock with and without HWA, or the blank control.

**Experiment 3: Does Laricobius osakensis respond to or prefer odors from hemlock woolly adelgid or host foliage?** *Laricobius osakensis* responded to prey and host tree odors in the olfactometer (Table 6). There was a significant difference in overall walking behavior, and choices were non-random between eastern hemlock with HWA (32%), uninfested eastern hemlock (25%), eastern white pine (19%), the blank control (13%), or remaining in the center field (Corchran Q test, $X^2=13.02$, $p=0.011$). Post-hoc multiple comparison tests show no significant difference in preference between eastern white pine, blank, and center field (11%), (Table 6, Corchran Q test, $X^2=2.263$, $p=0.323$). No significant difference exists between eastern hemlock, eastern hemlock with HWA, and eastern white pine (Corchran Q test, $X^2=2.716$, $p=0.257$). No significant difference in preference exists between eastern hemlock with HWA or without HWA (Corchran Q test, $X^2=0.720$, $p=0.396$), and these were the most common preferences. A significant difference in preference does exist between eastern hemlock with HWA and blank and center fields (Corchran Q test, $X^2=12.531$, $p=0.002$), and eastern hemlock compared to blank and center fields (Corchran Q test, $X^2= 6.186$, $p=0.045$).

**Discussion**

Overall, *Laricobius* spp intended as biological control agents were responsive in the olfactometer, and attracted to hosts and host foliage. *Laricobius nigrinus*, LnXLr hybrids and lab-reared Lo behaved similarly to each other. In Wallin et al. 2011, Ln was
not able to detect HWA alone in the olfactometer, but our results show that proportionally, host foliage infested with HWA is a pervasive choice for all three of these species. This result is different than what was reported in Wallin et al. 2011, where HWA remained inconspicuous to Ln. Many predators use odors from the host plant to locate their prey, rather than odors from the prey (Lima and Dill 1990; Dicke 1999; Cortesero et al. 2000; Gingras et al. 2002). These results suggest that Laricobius spp are responding to plants that are being damaged by HWA and therefore increasing reliability of prey location in the field. The changes in behavior may be a result of conditioning due to the prolonged association of Laricobius spp with HWA on eastern hemlock. This behavior is promising for a biological control agent, because it indicates that individuals discern cues and behave accordingly, because biological control agents are, by definition, meant to be host specific, efficient, and have a strong predatory impact on the target prey (Asaro et al. 2004; de Bruyne and Baker 2008; Yoneya et al. 2009; Keesey et al. 2012).

Fluctuations in hybridization rates are not uncommon recently after introduction (Howard 1993). The proportion of LnxLr hybrids in the Laricobius spp. samples was variable over time, and intermediate in number. There was some indication in early monitoring that hybridization rates were increasing over time (Havill et al. 2012), but although hybridization rates ranged from 8-28%, there was no trend during the 2 years of sampling (Table 1). Evidence suggests that habitat features may be the strongest factor. For example, the proportion of hybrids in a particular environment may inflate or deflate compared the parental species due to localized environmental changes or conditions (Howard 1993) which does not mean that hybrids are more or less viable or
fecund than the parental species, but rather, is an artifact of beneficial traits relative to specific locations, not a comment on the adaptiveness of hybrids overall.

The extent to which *Laricobius* spp adults migrate between stands would also affect the rate of interbreeding. At release sites, Ln was found to be common within 300 m of the original release trees by the fourth generation, and the dispersal distance increased with each generation (Davis et al. 2010; Davis et al. 2011). *Laricobius nigrinus* may disperse further since McDonald (2010) recovered Ln from at least 1.6 km from the release area. In other North Carolina release areas, the hybridization rate was 6%, which was lower than in our samples, and there were also considerably more Lr collected off of eastern hemlock in these sites (Havill et al. 2012).

The proximity of hemlock and white pine may affect the rate and incidence of contact and subsequent interbreeding between the species. For instance, the proportion of Lr collected from eastern hemlock was lower when eastern white pine, their primary hosts, were sparse or absent. The collection sites in Banner Elk, NC were primarily characterized by planted hemlock hedgerows, and well as ornamental trees in suburban neighborhoods (Mayfield, personal communication, May 16, 2013). Eastern white pine was present on the landscape and in nearby areas, but hemlocks on which our *Laricobius* spp. specimens were collected were in homogenous stands or stood alone.

Ecological aspects of hybridization should be considered within the context of biological control. Host-specificity can be lost, as insect hybrids are known to accept new host organisms (Hora et al. 2005) and can also displace native species (Yara et al. 2010). Hybridization can produce both host-specific and non-specific genotypes, and the F2 generation was found to be an ineffective control agent (Szűcs et al. 2011).
There are often both specific and non-specific phenotypes in the F₂ generation (Szűcs et al. 2012). Reciprocal crossings can even produce generations with higher fecundity and fitness than either parental lineage (Szűcs et al. 2012). If populations on different hosts are not reproductively isolated, selection will not eliminate mechanisms of host adaptation that are needed on the new hosts. This is costly to maintain and will likely maintain a higher population of hybrids.

Our results demonstrate that LnxLr hybrids collected on eastern hemlock responded to eastern hemlock with and without HWA and eastern white pine similarly to Ln on eastern hemlock. Laricobius spp. in bioassays containing HWA infested foliage responded more quickly and more often than those in bioassays containing only hemlock foliage without HWA (Table 2). Including HWA in the bioassay may be a driver for Laricobius spp. response in the olfactometer. The stimulus field containing HWA infested foliage was consistently chosen by Ln and often chosen by LnxLr hybrids, although hybrids also chose other foliage fields just as often.

Laricobius osakensis responded just as well in the olfactometer as any other Laricobius species. Even though these individuals were lab-reared and did not have exposure to HWA in the environment previously, they responded more quickly to odors in the olfactometer, and consistently chose foliage fields. Proportionally, odors from eastern hemlock infested with HWA and uninfested eastern hemlock were the most attractive. Our results show that it is likely that Lo will be readily able to locate HWA infested hemlock in the environment upon release. Experimentation in the native range and in the laboratory shows that Lo is highly synchronous with HWA, a voracious HWA predator, and can only develop to adulthood on HWA (Lamb et al. 2012). With
consideration for these factors, as well as the host-location behavior documented here, we believe that Lo is a good candidate agent for biological control of HWA, and will contribute to the control program upon release.

Conclusions

The occurrence of hybridization in a population intended to be a biological control agent should be closely monitored for future changes, but these bioassays demonstrate that LnxLr hybrid individuals have a similar level of attraction to HWA, and behave similarly, to imported Ln. These results also demonstrate that Lo should be able to locate prey in the novel environment using volatile cues from the prey and host foliage. All Laricobius spp considered here, possibly with the exception of native Lr, recognize and respond well to host odors, and should be able to contribute to the biological control effort against HWA in the environment.

References


to volative organic compounds identified from chestnut reproductive plant tissue. Environmental Entomology. 41, 933-940.


Table 6 [Chapter 3, Table 1] Proportion of Ln, Lr, and LnxLr hybrids, collected from eastern hemlock or eastern white pine in spring and fall of 2011, and fall of 2012. Insects off of eastern hemlock were collected near Banner Elk, NC, and those off eastern white pine were collected near Asheville, NC.

<table>
<thead>
<tr>
<th>Season</th>
<th>Eastern hemlock</th>
<th>Eastern white pine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ln</td>
<td>Lr</td>
</tr>
<tr>
<td>Spring 2011</td>
<td>79.4%</td>
<td>8.8%</td>
</tr>
<tr>
<td>Fall 2011</td>
<td>67.9%</td>
<td>3.8%</td>
</tr>
<tr>
<td>Fall 2012</td>
<td>92.0%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>
Table 7 [Chapter 3, Table 2] Mean time (and standard error) taken to choose a stimulus odor field by collection origin, collection date, and species. Comparisons were completed using a one-way ANOVA for each category. Asterisks indicate significant differences in preference, with significance when p<0.05.

<table>
<thead>
<tr>
<th>Comparison Factor</th>
<th>N</th>
<th>Mean ± SE (s)</th>
<th>F Ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection origin</td>
<td>1.23</td>
<td>319.8±14.3</td>
<td>0.267</td>
<td></td>
</tr>
<tr>
<td>Eastern hemlock</td>
<td>393</td>
<td>299.5±11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eastern white pine</td>
<td>253</td>
<td>247.4±17.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collection date</td>
<td>34.28</td>
<td>281.7±11.8</td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Spring 2011</td>
<td>156</td>
<td>431.9±17.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall 2011</td>
<td>160</td>
<td>258.5±22.0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall 2012</td>
<td>156</td>
<td>318.5±14.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>5.24</td>
<td>351.3±18.5</td>
<td>0.006*</td>
<td></td>
</tr>
</tbody>
</table>

* Loricobius nigrinus
* Loricobius rubidus
* LnXLr hybrids

69
Table 8 [Chapter 3, Table 3] Ambulatory responses of Laricobius spp. in 3 way choice experiments. All post-hoc experiments include eastern hemlock, eastern white pine, and blank control stimulus fields. Multiple post-hoc comparisons enable direct comparison between two or more stimulus fields (or the center field) to demonstrate preference, after overall preference is determined. Asterisks indicate significant differences in preference, with significance when p<0.05.

<table>
<thead>
<tr>
<th>Sub-experiment</th>
<th>Stimulus field</th>
<th>N</th>
<th>(X^2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) all Laricobius spp. from all tree origins</td>
<td>E. hemlock</td>
<td>25</td>
<td>60.359</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>81</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-hoc comparison</td>
<td>0.080</td>
<td>0.961</td>
<td></td>
</tr>
<tr>
<td>b) all Laricobius rubidus</td>
<td>E. hemlock</td>
<td>10</td>
<td>27.231</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-hoc comparison</td>
<td>1.652</td>
<td>0.438</td>
<td></td>
</tr>
<tr>
<td>c) all Laricobius nigrinus</td>
<td>E. hemlock</td>
<td>13</td>
<td>20.146</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-hoc comparison</td>
<td>0.318</td>
<td>0.853</td>
<td></td>
</tr>
<tr>
<td>d) all LnxLr hybrids</td>
<td>E. hemlock</td>
<td>2</td>
<td>17.636</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-hoc comparison</td>
<td>0.250</td>
<td>0.882</td>
<td></td>
</tr>
<tr>
<td>e) all Laricobius spp. collected from E. white pine</td>
<td>E. hemlock</td>
<td>9</td>
<td>38.148</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-hoc comparison</td>
<td>1.143</td>
<td>0.565</td>
<td></td>
</tr>
<tr>
<td>f) Laricobius rubidus from E. white pine</td>
<td>E. hemlock</td>
<td>8</td>
<td>25.930</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-hoc comparison</td>
<td>1.333</td>
<td>0.513</td>
<td></td>
</tr>
<tr>
<td>g) LnxLr hybrids from E. white pine</td>
<td>E. hemlock</td>
<td>1</td>
<td>10.800</td>
<td>0.013*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-hoc comparison</td>
<td>0.000</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>h) all Laricobius spp. from E. hemlock</td>
<td>E. hemlock</td>
<td>16</td>
<td>22.471</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>E. white pine</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank control</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Center field</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-hoc comparison</td>
<td>0.617</td>
<td>0.712</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>-------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

i) *Laricobius nigrinus* from *E. hemlock*  
- E. hemlock: 13, 18.704, <0.001*  
- E. white pine: 15  
- Blank control: 16  
- Center field: 37  
- Post-hoc comparison: 0.318, 0.853

j) LnxLr hybrids from *E. hemlock*  
- E. hemlock: 1, 7.333, 0.062  
- E. white pine: 2  
- Blank control: 2  
- Center field: 7  
- Post-hoc comparison: 0.250, 0.882

Table 3 continued from page 70
Table 9 [Chapter 3, Table 4] Relative proportions of each species choosing each stimulus field, based on collection origin and collection year. Cells marked n/a signify sample sizes that are too small for relevant statistics. *Laricobius* spp were not collected from eastern white pine for 4-way choice bioassays in the fall of 2011. Asterisks indicate significant differences in preference, with significance when p<0.05.

<table>
<thead>
<tr>
<th>Stimulus field</th>
<th>Eastern hemlock</th>
<th>Eastern white pine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Year</td>
<td>2011</td>
</tr>
<tr>
<td>Species (N)</td>
<td>Ln (99)</td>
<td>Lr (43)</td>
</tr>
<tr>
<td>Eastern hemlock with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWA</td>
<td>0.33</td>
<td>n/a</td>
</tr>
<tr>
<td>Eastern hemlock</td>
<td>0.19</td>
<td>n/a</td>
</tr>
<tr>
<td>Eastern white pine</td>
<td>0.14</td>
<td>n/a</td>
</tr>
<tr>
<td>Blank control</td>
<td>0.14</td>
<td>n/a</td>
</tr>
<tr>
<td>Center field</td>
<td>0.19</td>
<td>n/a</td>
</tr>
<tr>
<td>X²</td>
<td>2.869</td>
<td>n/a</td>
</tr>
<tr>
<td>P value</td>
<td>0.580</td>
<td>n/a</td>
</tr>
</tbody>
</table>

<0.001*
Table 10 [Chapter 3, Table 5] Relevant post-hoc comparisons for ambulatory responses of *Laricobius* spp individuals in a four-chambered olfactometer. Multiple post-hoc comparisons enable direct comparison between two or more stimulus fields (or the center field) to demonstrate preference, after overall preference is determined. Asterisks indicate significant differences in preference, with significance when p<0.05.

<table>
<thead>
<tr>
<th>Year</th>
<th>Collection Origin</th>
<th>Species</th>
<th>Post-hoc comparison</th>
<th>(X^2)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>Eastern hemlock</td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine, Blank</td>
<td>2.800</td>
<td>0.423</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine</td>
<td>1.625</td>
<td>0.444</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, E. hemlock</td>
<td>0.783</td>
<td>0.376</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lm</em></td>
<td>E. hemlock, Center field</td>
<td>1.089</td>
<td>0.297</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine</td>
<td>1.750</td>
<td>0.417</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock w/HWA, E. hemlock</td>
<td>1.636</td>
<td>0.201</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock, E. white pine</td>
<td>0.667</td>
<td>0.414</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock, Center field</td>
<td>7.118</td>
<td>0.008*</td>
</tr>
<tr>
<td>2012</td>
<td>Eastern hemlock</td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine, Blank control</td>
<td>6.276</td>
<td>0.099</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine</td>
<td>3.102</td>
<td>0.212</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, E. hemlock</td>
<td>0.421</td>
<td>0.516</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, E. white pine</td>
<td>3.125</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lm</em></td>
<td>E. hemlock w/HWA, Center field</td>
<td>0.676</td>
<td>0.411</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine</td>
<td>3.500</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock w/HWA, E. hemlock, Center field</td>
<td>1.600</td>
<td>0.449</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock w/HWA, E. hemlock</td>
<td>1.000</td>
<td>0.317</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock w/HWA, E. white pine</td>
<td>1.000</td>
<td>0.317</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>LmLr</em></td>
<td>E. hemlock, E. white pine</td>
<td>3.000</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>Eastern white pine</td>
<td><em>Ls</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine, Blank control</td>
<td>8.388</td>
<td>0.039*</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ls</em></td>
<td>E. hemlock w/HWA, E. hemlock, E. white pine</td>
<td>7.942</td>
<td>0.019*</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ls</em></td>
<td>E. hemlock w/HWA, E. hemlock</td>
<td>1.143</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ls</em></td>
<td>E. hemlock w/HWA, E. white pine</td>
<td>2.848</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ls</em></td>
<td>E. hemlock, E. white pine</td>
<td>7.451</td>
<td>0.006*</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ls</em></td>
<td>E. white pine, Blank control</td>
<td>3.282</td>
<td>0.070</td>
</tr>
</tbody>
</table>
Table 11 [Chapter 3, Table 5] Ambulatory responses of laboratory-reared Lo in 4 way choice bioassay to host foliage with and without HWA. Multiple post-hoc comparisons enable direct comparison between two or more stimulus fields (or the center field) to demonstrate preference, after overall preference is determined. Asterisks indicate significant differences in preference, with significance when p<0.05.

<table>
<thead>
<tr>
<th>Stimulus Field</th>
<th>Proportion</th>
<th>X^2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eastern hemlock with HWA</td>
<td>0.32</td>
<td>13.023</td>
<td>0.011*</td>
</tr>
<tr>
<td>Eastern hemlock</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eastern white pine</td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank control</td>
<td>0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Center field</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Response time ± SE (s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. osakensis 4-way choice (N=89)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-hoc tests:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EH w HWA/Blank/Center</td>
<td>12.531</td>
<td>0.002*</td>
<td></td>
</tr>
<tr>
<td>EH/Blank/Center</td>
<td>6.186</td>
<td>0.045*</td>
<td></td>
</tr>
<tr>
<td>EH w HWA/EH</td>
<td>0.720</td>
<td>0.396</td>
<td></td>
</tr>
<tr>
<td>EH w HWA/EH/EWP</td>
<td>2.716</td>
<td>0.257</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4: Ambulatory response of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), a hemlock woolly adelgid predator, to host odors and conspecific feeding beetles in a four-chambered olfactometer

Abstract

Classical biological control efforts face challenges that include the ability of natural enemies to regulate the intended prey, sustain their population size, and distribute themselves on the landscape. These characteristics are dependent on interactions with the pest, as well as with conspecifics. Interactions between insects and their environments are often mediated by unique and specific volatile cues that can elicit behaviors. Behavioral assays are used to identify and describe responses to cues. Using olfactometer bioassays, we evaluated the ambulatory response of adult *Laricobius nigrinus*, a predator of hemlock woolly adelgid (HWA), to volatiles of eastern hemlock foliage, foliage infested with HWA, and HWA-infested foliage with a feeding conspecific. *Laricobius nigrinus* was attracted to the treatments in a hierarchical fashion, with the strongest preference for HWA infested foliage, then for uninfested foliage, and least attracted to HWA infested foliage with a conspecific beetle feeding on HWA. This study supports that predators forage in a manner that proffers the optimal and most efficient energetic rate of return. Specific cues indicating available and unexploited food sources are dependable, and likely distribution of biological control agents across the host range.
Introduction

Behavioral interactions between insects and their environments are often mediated by volatile cues. Plant-produced chemical cues induced by herbivore activity are often more effective at attracting predators than cues produced by the herbivore alone (Dicke and van Loon 2000). The presence of herbivore-induced plant volatiles makes foraging by predators more efficient than undirected hunting (Yoneya et al. 2009, Dannon et al. 2010), presumably because they indicate the presence of prey.

Olfactory cues are not only used across trophic levels, but are also important within feeding guilds. Predators interact with each other through direct or indirect competition for prey. Conspecific olfactory cues also exist, and impact individual foraging behaviors (Flowers et al. 2007). Just as it is common for predators to find prey using olfactory cues, they can also use cues to avoid intraguild competition or aggression (Janssen et al. 1995, Cakmak et al. 2006), and evidence suggests that avoidance is a common response to the reception of a conspecific cue from a particular location (Janssen et al. 1995, Stout and Goulson 2001, Gnanvoussou et al. 2003). In several insect orders, foragers are known to concede to previous or superior feeders, and visit new patches or trees accordingly (Gnanvossou et al. 2003).

Optimal foraging strategies dictate gaining the highest possible energy intake while avoiding competition, predation risk, and wasted foraging effort in sites where food is not available, yielding the highest possible net energy gain (Charnov 1976). The ability to recognize congers feeding at a site, through volatile cues for example, aids predators in efficiently locating available prey while avoiding competition or prey depletion.
Understanding these interactions may be important to the implementation of a biological control program in which a natural enemy complex is released and established to control a target pest (Flowers et al. 2007). Because biological control releases are expensive and time consuming, information about predator interactions can inform the optimal release density of agents on the landscape and increase the efficiency and effectiveness of the program.

Eastern hemlock (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Engelmann) in the United States are currently suffering high rates of mortality due to hemlock woolly adelgid (*Adelges tsugae* Annand, HWA), an invasive insect. Classical biological control practices are being implemented in the region, to exert top-down control on HWA populations (DeBach 1974, McEvoy 1996, McDonald 2010). *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), is an adelgid predator associated with HWA on western hemlock (*Tsuga heterophylla*) in the northwestern United States (Kohler et al. 2008). It is highly synchronized with HWA, feeds voraciously on all life stages, oviposits within HWA ovisacs, and develops to adulthood on HWA (Zilahi-Balogh et al 2002). This predator is considered a promising candidate agent for biological control of HWA, and over 100,000 individuals have been released in the southern part of the invasive range of HWA in the U.S. since 2003 (Mausel et al. 2012).

Previous behavioral bioassays indicate that *Laricobius nigrinus* detects and responds to volatiles released from HWA-infested and uninfested host foliage (Wallin et al. 2011, A. Arsenault unpublished data), suggesting use of stimuli from several trophic levels, including multitrophic induced olfactory cues to locate prey in the
environment. In this study, using a four-chambered olfactometer, we examined orientation behavior of *L. nigrinus* to eastern hemlock, eastern hemlock infested with HWA, infested eastern hemlock with a feeding beetle on HWA, and an empty chamber used as a blank control. The objective of this study was to determine whether field-collected *L. nigrinus* responded to host and prey odors in an olfactometer, and to observe whether adding a conspecific individual feeding on HWA on host foliage would alter the orientation preferences of *L. nigrinus*.

**Materials and Methods**

*Olfactometer Bioassays*

In 2011 and 2012, *Laricobius nigrinus*. individuals were collected in the vicinity of Banner Elk, NC (36.165643°N, -81.872118°W), where releases of this predator have been made for biological control of HWA since 2003 and where field populations of the beetle are now relatively abundant. Behavioral bioassays were used to test the ambulatory responses of adult *L. nigrinus* to various stimuli in a four-chambered olfactometer (Analytical Research Systems, #OLFM-4-C-2440PE, Gainesville, FL). The arena was comprised of the base with vacuum air output, an intermediate section that encompassed the walking chamber as well as four air input arms, and a 9mm circular central opening for the introduction of insects. Four flow meters (Brooks Instrument, Hatfield, PA) controlled airflow through the glass chambers and into the arena at a rate of 0.12 Mpa. Volatiles were removed from the arena through the vacuum in the center, which maintained the integrity of the four air fields.
Responses of individual *L. nigrinus* to treatments were measured using methodology similar to Wallin et al. 2011. For this bioassay, the host treatments placed in the glass chambers included an empty chamber used as a blank control, eastern hemlock foliage, eastern hemlock foliage infested with hemlock woolly adelgid, and eastern hemlock foliage with *L. nigrinus* feeding on HWA. Hereafter, the latter treatment will be called the feeding beetle treatment. Beetles for the feeding beetle treatments were randomly selected from the pool of possible individuals and starved for 24 hours. In the feeding beetle treatment, a beetle was placed on a piece of HWA-infested foliage and allowed to settle and begin feeding prior to placement of the foliage in the chamber. Eastern hemlock foliage infested with HWA was obtained from trees near the *Laricobius nigrinus* collection sites in North Carolina, whereas uninfested eastern hemlock foliage was collected in South Burlington, VT (44.4669° N, 73.1714° W).

Treatments were placed into the glass chambers, and then randomly attached to one of the olfactometer’s arms. Chambers were randomly reassigned to a new arm for each replicate. Foliage was replaced every hour. Foliage containing the feeding beetle was replaced, the feeding beetle was removed from the foliage, and stored in a vial with 95% ethanol, and a new individual was introduced to a new HWA infested branch.

To test the ambulatory response of the beetle to the treatments, beetles were starved for 24-25 hours prior to bioassays. A single individual was selected at random from the pool of possible individuals and placed in the center of the arena, equidistant from the entrance of each arm. Individuals were allowed to walk about the arena for up to 10 minutes, and their choice was recorded. A choice consisted of an individual
crossing into the delineated field boundary for a particular arm and remaining beyond the boundary for at least one minute. The time required to make a choice was recorded, as well as its choice and final position. Bioassays were completed when either 1) a beetle remained in a field boundary for at least one minute, 2) the 10 minute time limit was reached, or 3) a beetle attempted to crawl into an arm. At that time, the individual was removed from the arena and placed in a labeled vial containing 95% ethanol for further analysis. Bioassays were conducted in the winters of 2011 and 2012. In 2011, 55 *L. nigrinus* beetles were tested, and in 2012 we observed the responses of 31 individuals.

**Statistical Analysis**

Comparisons of final positions in the olfactometer were analyzed using the Cochran Q test for a randomized block design, where each treatment is considered a block, as well as post-hoc tests for preference (Zar 1999). Analyses were completed using the SPSS statistical software package (IBM Corp. version 20 for Mac, released 2011).

**Results**

*Laricobius nigrinus* responded to odors in the olfactometer, and moved about the arena and chose fields containing stimuli in a manner that suggests that their behavior was non-random in 2011 and when the results were pooled across both years (2011: Cochran Q, $X^2=9.273$, $p=0.055$, 2012: Cochran Q, $X^2=3.032$, $p=0.552$, pooled: Cochran Q, $X^2=11.791$, $p=0.019$). In 2011, 15% of individuals remained in the center
field, while in 2012, 16% of individuals remained in the center field, ranking the center field among the least preferred options.

In 2011, 2012, and in both years combined, *L. nigrinus* chose the stimulus field containing eastern hemlock infested with HWA more often than any other field, proportionally (Table 1). When experiments are considered separately, this was a significant preference for *L. nigrinus* in 2011 and in pooled years. Post-hoc tests (Table 2) demonstrate that in pairwise comparisons, *L. nigrinus* preferred eastern hemlock infested with HWA to the feeding beetle (2011: Cochran Q, $X^2=5.538$, $p=0.019$, pooled: Cochran Q, $X^2=6.400$, $p=0.011$), the blank field (2011: Cochran Q, $X^2=4.481$, $p=0.034$, pooled: Cochran Q, $X^2=6.400$, $p=0.011$) and the center field (2011: Cochran Q, $X^2=4.481$, $p=0.034$, pooled: Cochran Q, $X^2=5.488$, $p=0.019$), but not eastern hemlock alone (2011: Cochran Q, $X^2=1.125$, $p=0.289$, 2012: Cochran Q, $X^2=0.059$, $p=0.808$, 2012: Cochran Q, $X^2=1.000$, $p=0.317$).

The stimulus field containing the feeding beetle was consistently among the least preferred option, proportionally (Table 1). In post-hoc tests (Table 2), *L. nigrinus* was significantly more likely to choose eastern hemlock with or without HWA than choose the field with the feeding beetle (pooled: Cochran Q, $X^2=6.328$, $p=0.042$). *Laricobius nigrinus* was just as likely to choose the feeding beetle as the blank control field or remain in the center field (Cochran Q, $X^2=0.054$, $p=0.973$). In pairwise comparisons, *L. nigrinus* preferred eastern hemlock with HWA to the feeding beetle, as stated above. Uninfested eastern hemlock was more attractive than the feeding beetle observationally (Table 1), but pairwise comparisons show that this is not significant (2011: Cochran Q, $X^2=1.800$, $p=0.180$, 2012: Cochran Q, $X^2=1.143$, $p=0.285$, pooled: $X^2=2.943$, $p=0.086$).
Cochran Q, $X^2=2.445$, $p=0.117$). There is no difference in preference between the feeding beetle and the blank field (2011: Cochran Q, $X^2=0.067$, $p=0.796$, 2012: Cochran Q, $X^2=0.111$, $p=0.739$, pooled: Cochran Q, $X^2=0.000$, $p=1.000$) or the feeding beetle and the center field (2011: Cochran Q, $X^2=0.067$, $p=0.796$, 2012: 0.000, $p=1.000$, pooled: 0.040, $p=0.317$) (Table 2).

**Discussion**

*Laricobius nigrinus* responded to odors in the olfactometer, choosing a stimulus field over the center field in both 2011 and 2012, proportionally. Insects consistently chose the field containing the feeding beetle among the least often, and in similar proportions to choosing the blank control or remaining in the center field. Pooling data across both experimental years indicated that odors from hemlock foliage with and without HWA were more attractive than other stimulus fields.

*Laricobius nigrinus* reliably responded to host foliage with HWA, but also responded to uninfested hemlock foliage. As described in Wallin et al. (2011), the similarity in preference between these two host treatments may be due to the low detectability of HWA on its own. This phenomenon has been described as the reliability-detectability problem (Vet et al. 1991), where the magnitude and surface area available for release of olfactory cues is much greater for the foliage than for the prey, and in the olfactometer, the cues from hemlock may overwhelm those due to HWA feeding on hemlock to some extent. However, odors from hosts alone are not necessarily a reliable indication of prey availability, so predators can use a combination of these, and, in addition, herbivore induced volatile cues, emitted by the host when foliage is wounded through feeding (Agrawal 1998, Dicke and Van Loon 2000, Havill
and Raffa 2000, Radville et al. 2011). The data presented here supports that \textit{L. nigrinus} predators, when foraging, are attracted to HWA and host odors, and may use hemlock foliage as a proxy due to low detectability of prey.

Resource allocation can be defined by mathematical constructs or energy efficiency models. As individuals become more deprived of food, they take greater risks in foraging (Pureswaren and Borden 2005). When given a choice between feeding with a conspecific or feeding alone, when there are no other factors that would mean difference in expended energy, such as distance, it may be most energy efficient for the predator to take advantage of the food source without competition. Therefore, even though \textit{L. nigrinus} is unlikely to act aggressively toward a congener, and is even less likely to participate in cannibalism, because they specialize so specifically on adelgids, avoidance of the feeding beetle is still the most energetically efficient choice. These data suggest that information about potential resource competitors can be relayed through volatile cues, as demonstrated in an olfactometer where visual cues were not applicable. Our data also suggest that although \textit{L. nigrinus} may prefer not to forage on HWA-infested foliage where a conspecific beetle is feeding, the presence of prey may be a stronger driver of behavior than the presence of a conspecific forager, as feeding beetles were not avoided completely.

Herbivore induced volatile cues were reliable, detectable, have the highest potential energetic returns, and were the most attractive to the predators, proportionally. Volatiles from host foliage are highly detectable in the environment; however, they are not a strong indication of prey presence. Volatiles that signify feeding congeners already at the site could suggest competition, a slower intake rate, or prey depletion at
the site. Therefore, this option would likely be the least energetically efficient choice, when faced with the options presented in this bioassay. There may be a graduated attraction or aversion to odors from foliage, prey, and conspecifics, where volatiles from each type are attractive because they could indicate prey availability, yet there is varying reliability, detectability, and energetic returns for each case (Vet et al. 1991, Kennedy and Gray 1993). Our results are consistent with these theoretical indications, where odors that signify induced volatile cues are most preferred, cues that indicate host foliage but not damage induced by prey are attractive but not significantly so, and those that indicate competition are not preferred when uncontested prey is available elsewhere nearby.

References


Laricobius nigrinus Fender (Coleoptera : Derodontidae), a potential biological
control agent of the hemlock woolly adelgid, Adelges tsugae Annand
Tables
Table 12 [Chapter 4, Table 1] Ambulatory responses of *L. nigrinus* individuals to odors from host foliage, prey and conspecifics in a 4-way olfactometer over two years of bioassays. A Cochran Q test was completed for each year of data, plus the pooled data set. An asterisk indicates a significant difference in preference, *p*<0.05.

<table>
<thead>
<tr>
<th>Stimulus Field</th>
<th>Proportion of Choice by Year</th>
<th>2011 (N=55)</th>
<th>2012 (N=31)</th>
<th>Pooled years (N=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding Beetle</td>
<td></td>
<td>0.13</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>E. Hemlock with HWA</td>
<td></td>
<td>0.35</td>
<td>0.29</td>
<td>0.33</td>
</tr>
<tr>
<td>E. Hemlock</td>
<td></td>
<td>0.24</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td>Blank Control</td>
<td></td>
<td>0.15</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>Center Field</td>
<td></td>
<td>0.15</td>
<td>0.16</td>
<td>0.15</td>
</tr>
</tbody>
</table>

\[
X^2=9.273 \quad X^2=3.032 \quad X^2=11.791
\]

\[
P=0.055 \quad P=0.552 \quad P=0.019^* 
\]
Table 13 [Chapter 4, Table 2] Post-hoc comparisons, where one or more stimulus field(s) are removed from the analysis, to compare responses to host odors within an experiment. Asterisks indicate significant preference, p<0.05.

<table>
<thead>
<tr>
<th>Post-hoc Comparison</th>
<th>2011</th>
<th>2012</th>
<th>Pooled years</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X²</td>
<td>pvalue</td>
<td>X²</td>
</tr>
<tr>
<td>Feeding Beetle, E. Hemlock with HWA, E. Hemlock</td>
<td>5.538</td>
<td>0.063</td>
<td>1.182</td>
</tr>
<tr>
<td>Feeding Beetle, Blank, Center</td>
<td>0.087</td>
<td>0.957</td>
<td>0.143</td>
</tr>
<tr>
<td>E. Hemlock with HWA, Blank, Center</td>
<td>6.924</td>
<td>0.032*</td>
<td>2.333</td>
</tr>
<tr>
<td>E. Hemlock, Blank, Center</td>
<td>1.724</td>
<td>0.422</td>
<td>1.529</td>
</tr>
<tr>
<td>Feeding Beetle, E. Hemlock with HWA</td>
<td>5.538</td>
<td>0.019*</td>
<td>0.692</td>
</tr>
<tr>
<td>Feeding Beetle, E. Hemlock</td>
<td>1.800</td>
<td>0.180</td>
<td>1.143</td>
</tr>
<tr>
<td>Feeding Beetle, Blank</td>
<td>0.067</td>
<td>0.796</td>
<td>0.111</td>
</tr>
<tr>
<td>Feeding Beetle, Center</td>
<td>0.067</td>
<td>0.796</td>
<td>0.000</td>
</tr>
<tr>
<td>E. Hemlock with HWA, E. Hemlock</td>
<td>1.125</td>
<td>0.289</td>
<td>0.059</td>
</tr>
<tr>
<td>E. Hemlock with HWA, Blank</td>
<td>4.481</td>
<td>0.034*</td>
<td>1.333</td>
</tr>
<tr>
<td>E. Hemlock with HWA, Center</td>
<td>4.481</td>
<td>0.034*</td>
<td>0.692</td>
</tr>
<tr>
<td>E. Hemlock, Blank</td>
<td>1.190</td>
<td>0.275</td>
<td>1.923</td>
</tr>
<tr>
<td>E. Hemlock, Center</td>
<td>1.190</td>
<td>0.275</td>
<td>1.143</td>
</tr>
<tr>
<td>Blank, Center</td>
<td>0.000</td>
<td>1.000</td>
<td>0.111</td>
</tr>
</tbody>
</table>
Chapter 5: Opinion: A call for the addition of molecular methods and genetics as a necessary part of risk assessment for the improvement of biological control programs

Introduction

In attempting to understand biological control, risk assessment, and agent behavior for my masters thesis herein, I delved into the available literature as all graduate students do. In doing so, I began to ask questions and collect evidence concerning the steps taken to improve and safeguard biological control since its beginnings over 100 years ago, and the future steps we can take to continue to reduce the risks of biological control. As described in the summarization of the literature, thorough risk analysis is one of the foundational components of a successful biological control program. The necessary pre-release testing required for agent acceptance has evolved over time, and the requirements have become more stringent.

The inherently analytical, descriptive, and collaborative nature of biological control under government regulation, a variety of expertise, and scientific foundations results in a bevy of scientific literature published and disseminated for consumption by scientists, which gives us insight into the types of studies that currently warrant funding and publication. In 2005, Stiling and Cornelissen completed a review of biological control from 1999-2003, where they categorized the studies by the type of question they addressed. This study offers an indication of the proportion of studies of each type, and subsequently which type of study was most important during the time period (Stiling and Cornelissen 2005). The results of Stiling and Cornelissen (2005) are shown in the
table below (Table 1). This figure represents the relative proportion, and subsequently relative importance as demonstrated by funding and completion, of each type of study. Genetic and molecular studies fall under the “other” category (H).

With the intention of seeing whether the relative importance and/or prevalence of study types had changed, when considering studies published between 1999-2003 compared to those published between 2004 and the summer of 2012, I completed a similar analysis for studies completed between 2004-2012 following the methods below.

**Collection of Articles, Categorization, and Qualitative Analysis**

Using the primary literature database Web of Science, we collected references for the qualitative meta analysis. We accessed articles using the keyword search “biological control,” and restricted to years 2004-2012 (August). We only considered articles published in Annual Review of Entomology, Applied Entomology and Zoology, Journal of Applied Entomology, Biocontrol, Biological Control, Canadian Entomologist, and Environmental Entomology were considered.

Only studies pertaining to natural systems and insect-related control (either as agents, targets, or both) were considered, as well as studies concerning classical biological control only (opposed to conservation or augmentative biological control). To follow these criteria, we excluded all agriculture and aquatic studies, and control projects utilizing fungi, bacteria, viruses, nematodes, and/or Arachnida. After exclusions, this search led to 382 records.
Records were sorted into categories derived from those used in Stiling and Cornelissen (2005), and based on an initial survey of collected articles, according to the primary purpose of the research and subsequent article. For our purposes, categories are mutually exclusive, meaning that a paper was only counted in one category, even though a small number of the articles broached two or more of the topics. These papers were tallied into the primary category for the paper, based on the title and the proportion of the paper dedicated to the specified category.

**Results**

A number of categories were represented differently in this later time period, as shown in the figure below. The most notable change is the number of papers that described genetic and molecular methods. Under my study, genetics and molecular methods are the 7th most often addressed research category, compared to the previous study, where these research questions did not get their own category. They were considered part of the “other questions” category, which still ranked lower in 2005 than 2012, even though it is an aggregation of several study types. This demonstrates that through time, this type of study became more prevalent, and arguably more important to biological control.

**Argument: Benefits of adding genetics and molecular methods to biological control risk assessment**

Historically, biological control projects do not have the same rate of successful implementation compared to other “predictive” sciences (Roderick and Navajas 2003), which is discouraging and should be addressed. An example of this is the current
undertaking of addressing the post-release hybridization of *Laricobius nigrinus*, released as a biological control agent for hemlock woolly adelgid in the eastern United States, and its native relative, *Laricobius rubidus*. This hybridization was unexpected, and is the impetus for additional research and testing to determine whether this hybridization is detrimental to the biological control effort, and *L. nigrinus*'s place as a control agent against HWA. Beyond the implications of hybridization on biological control, we must also consider the example provided within the context of maintaining genetic diversity in both the native *L. rubidus* population, and the released *L. nigrinus* population. In these environmentally uncertain times, biodiversity may be one of a species’ greatest assets (Millennium Ecosystem Assessment 2005). Genetic diversity allows for tolerance toward abiotic perturbations, such as climate change, and can also reinforce a system’s biotic resistance against additional invasions and biotic perturbations (deRiviera et al. 2005).

At its advent, biological control was approached in the same “shot-gun,” or broad-spectrum style as early pesticide use (Mills and Kean 2010), where more effort went into inundating the system with agents rather than carefully selecting and testing potential agents prior to release. In 1980, this issue was recognized, as Myers and Sabath (1980) called for the development of an agent selection process that includes a scientifically rigorous experimental methodology as part of the biological control protocols. This should lead to the improvement of the predictability, and subsequent success, of the programs. Over the evolution of biological control as a component of a pest management strategy, this methodology became recognized as a risk assessment, during which potential agents undergo various tests to assess their suitability in the
novel environment. There is considerable evidence that genetics and molecular methods can offer a boon to the risk assessment of biological control agents.

Genetics began to be associated with ecology early in the history of genetics as a science, as evidenced by Sammeta and Levins (1970) stating that the two were implicitly linked and should be considered together. Genetics and ecology, in concert, were also recognized as important components to integrated pest management (IPM) prior to 1970 (Sammeta and Levins 1970). In the modern era, biological control is only feasible as a scientifically sound practice if it is implemented under the paradigm of ecologically based principles. Invasion ecology offers some insight, as well as some limits, for biological control in practice. Invasion ecology teaches us that certain life history traits, like high fecundity, tolerance for a range of abiotic conditions, and rapid growth and development are advantageous for the establishment of invasive species (Elton 1958, Mack et al. 2000, Catford et al. 2012). Since biological control programs are purposeful invasions, we should consider traits that would proffer success for the establishment of the control agent, while limiting the spread to a very specific host range. It is possible to quantify and correlate ecologically relevant traits with their genetic basis, such as fitness (Rauth et al. 2011). DNA is unaffected by environmental conditions (Gaskin et al. 2011), unlike behavior, so a purer understanding of adaptability and plasticity could be gleaned from an understanding of genetics. By understanding the propensity of an organism to adapt in new environments through a study of the breadth of its phenotypic plasticity, we gain knowledge of an agent’s ability to spread beyond the target area.
The use of genetics in biological control has clear ecological foundations. Theoretically, introducing a biological control agent is an attempt at “community reassemblage” (Hoddle 2004), so we should aim to reconstruct the food web as nearly to the original as possible. The accelerated time frame of a biological invasion causes species that were normally geographically isolated to interact with one another outside the bounds of natural evolutionary time (Ludsin and Wolfe 2001, Olden 2006), so if we could find an agent that did have a coevolutionary relationship with the target species to release, it would strengthen the ecological basis of biological control. An understanding of the taxonomic past of both the pest and the agent can inform the future of each organism and their existence together (Aebi et al. 2008): how they will react, develop, and behave during their interactions. Biological control relies on the coevolution of pest and agent over both temporal and spatial scales, and genetics play a key role in the ability of an agent to maintain this relationship and hold the pest below economic and ecological injury levels (Hufbauer and Roderick 2005). In the same vein, we can support and encourage coevolution by locating the correct biotypes of the agents during the exploration process (Davis et al. 2011).

When looking for potential biological control agents, it is crucial to find the most specific and vigorous agent possible, while avoiding all possible non-target effects, and this is the express purpose of the risk assessment. There is evidence that greater genetic diversity yields better establishment after intentional releases (Hufbauer et al. 2004, Rauth et al. 2011), and there is also a correlation with fitness (Hufbauer and Roderick 2005). Ideally, the agent population would be somewhat restricted, as we do not want it to breach the bounds of management and lose host specificity, but at the
same time, too little diversity in the released agent population would not allow for coevolution over time.

Genetic information can be very informative in biological control, when attempting to understand new pest species, when searching for potential agents, when preparing these agents for release, and when monitoring the agents after release. Resources may be available for agents to be monitored for a while after release, but depending on the specific project, many phytophagous insect releases are not monitored for long enough to truly understand the evolution of the agent after release (Hufbauer and Roderick 2005). Genetics can offer insight into potential adaptations that may occur on a timescale beyond that at which biological control programs are closely monitored (Roderick and Navajas 2003).

It is possible to assess a potential agent for suitability on both micro- and macro-scales (Aebi et al. 2008). Genetic analysis can help researchers find agents that are ecologically and environmentally relevant— for instance, those that coevolved with the introduced pest population, or those that are able to survive the climatic conditions in the introduced range (Mills and Kean 2010, Rauth et al. 2011). A catalogue, on the genetic level, of the geographic origin(s) of the pest species can direct researchers to the most relevant potential sites to explore for natural enemies (Rauth et al. 2011). This may help to narrow the search, saving both labor and economic resources that could better be allocated to a more thorough risk assessment.

Small, introduced populations, such as those implicit in biological control efforts, undergo certain genetic factors, including random processes, bottlenecks, and the founder effect. These can result in behaviors that were unexpected (Fauvergue et al. 1988).
2012), adaptations on a local scale, or lower fitness (Hufbauer and Roderick 2005). These genetic factors apply to both the invasive species and the released agent species, but they are particularly relevant to agent species that undergo further small population effects through laboratory rearing. Laboratory colonies have greatly reduced genetic diversity after several generations of rearing. Some researchers suggest utilizing genetic bottleneck experienced through quarantine and laboratory rearing in an advantageous way.

Quarantine is, by definition, artificial selection (Fauvergue et al. 2012), where the population is whittled down over time to those that can survive, reproduce, and develop in the laboratory setting, so it is important to rear under the paradigm that humans have a heavy hand in the evolution of the released population. It may be possible to take advantage of the laboratory rearing process, but we must also ensure that genetic diversity is maintained. On a similar note, new techniques are on the horizon that may be best implemented during the laboratory rearing period. It may be possible to genetically engineer potential agents for particular traits that will be beneficial to the biological control efforts in a particular region (Roderick and Navajas 2003, Hufbauer and Roderick 2005). There is also potential to genetically modify agents for higher fecundity, more phenotypic synchrony with the invasive target population, better abiotic tolerance with respect to the release areas, and other desirable traits (Hufbauer and Roderick 2005).
Conclusions and Implications

As demonstrated by the wide range and sheer number of publications related to biological control, millions of dollars and thousands of hours are dedicated to finding, testing, rearing, and establishing biological control agents for each project. A transition in project distribution is expected over time, as knowledge expands, needs change, and new questions arise. Over the past two decades, research involving genetics and molecular methods has become more prolific, which is a simple indication of their worth and importance. Looking deeper, the details above, outlining the merits of genetics as an integral part of biological control, act as an even stronger metric. In the case of *Laricobius* spp, the potential for post-release hybridization brought genetic analysis to the forefront of the efforts, because the influence of hybridization is a potential confounding variable for most projects currently undertaken. These types of analyses also eased concerns, because researchers were able to recognize that the new Japanese species under consideration, *L. osakensis*, is more distantly related to those species in the invasive range, and it is unlikely that hybridization would impact this species (Montgomery et al. 2011). In this same system, genetic analysis informed us that the strain of HWA invasive to the eastern US is endemic to Japan (Havill et al. 2006), which led to thorough exploration in the region, and the discovery of a potentially superlative candidate agent.

It is evident that adding genetics and molecular methods to the current laundry list of necessary pre- and post-release testing will add to the economic burden of biological control projects, but in the long term, genetics can aid in reducing costs related to exploration and rearing, reduce the number of agents run through the gambit
of host specificity tests, and lessen the chance of having to mitigate post-release non-target effects. It is quite possible that a cost-benefit analysis would favor the continued and expanded use of genetics and molecular methods. Either way, if we consider biological control as a part of integrated pest management (IPM), then we owe projects, by definition, the integration of all reasonably available techniques. IPM calls for scientifically based and environmentally sensitive practices, and with the knowledge and expertise we currently have available, genetic analyses reside, undoubtedly, beneath that umbrella.

References


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Tables
Table 14 [Chapter 5, Table 1] The proportion of published papers dedicated to each type of study from 1999-2003, based on the results of Stiling and Cornelisson, 2005.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Stiling and Cornelissen category</th>
<th>Proportion of studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agent efficacy</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>Oviposition/feeding/behavior</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>Host specificity and life history</td>
<td>0.11</td>
</tr>
<tr>
<td>4</td>
<td>Biotic effects</td>
<td>0.10</td>
</tr>
<tr>
<td>5</td>
<td>Abiotic effects</td>
<td>0.09</td>
</tr>
<tr>
<td>6</td>
<td>Pest management</td>
<td>0.07</td>
</tr>
<tr>
<td>7</td>
<td>Agent establishment</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
<td>Other questions</td>
<td>0.05</td>
</tr>
<tr>
<td>9</td>
<td>Natural history</td>
<td>0.05</td>
</tr>
<tr>
<td>10</td>
<td>Agent management</td>
<td>0.05</td>
</tr>
<tr>
<td>11</td>
<td>Non-target effects</td>
<td>0.04</td>
</tr>
</tbody>
</table>
### Table 15 [Chapter 5, Table 2] Descriptions of the criteria used to categorize studies for the 2012 categorization of studies.

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Agent Efficacy</td>
<td>Studies describing the impact of released biological control agents on specified target species, including the response of target populations to the release of agents- not limited to population size but also behavior and biology</td>
</tr>
<tr>
<td>2) Intraguild Interactions</td>
<td>Studies considering the effects of the release of multiple agents for the same target, or the effects of the interaction of released agents with native relatives or native species within the same feeding guild</td>
</tr>
<tr>
<td>3) Agent Biology, Behavior, Specificity</td>
<td>This complement of studies describes oviposition and predation behavior, host specificity and host range fidelity, and interaction with the environment of biological control agents</td>
</tr>
<tr>
<td>4) Target Biology and History</td>
<td>These studies describe the biology, natural history, and anecdotal history of the target species in their native and introduced ranges</td>
</tr>
<tr>
<td>5) Agent Establishment</td>
<td>Studies pertaining to the survival, reproduction, and overall viability of released agents in the geographic range of the target species</td>
</tr>
<tr>
<td>6) Agent Management, Rearing, Exploration, and Assessment</td>
<td>This category includes all studies that prepare for the release of agents by locating and observing them in their native ranges, assessing the possibility of viable laboratory rearing, making necessary preparations for release, and preliminary assessment for potential efficacy</td>
</tr>
<tr>
<td>7) Nontarget Effects</td>
<td>Any study relating to effects of agents on species other than the intended targets, both prior to and after release</td>
</tr>
<tr>
<td>8) Tritrophic or Ecosystem Level</td>
<td>Studies considering the effects of biological control on multiple levels of ecosystem function, including top down and bottom up regulation, as well as abiotic factors</td>
</tr>
<tr>
<td>9) Philosophical, Opinion, or General</td>
<td>These studies do not necessarily describe specific systems- rather, they claim generalities in biological control, and are often review articles</td>
</tr>
<tr>
<td>10) Host Considerations</td>
<td>Studies related to the effects of the producer in a tritrophic biological control system, namely, the resulting effects of a target species on its host, and the indirect effects of agent release to the target's host</td>
</tr>
<tr>
<td>11) Genetics and Molecular Methods</td>
<td>These studies consider the importance of genetics in biological control, compare populations and describe hybridization. They also consider molecular and cellular function in the overall system, including the effects of various chemical manipulations. In short, these studies are based in molecular laboratories</td>
</tr>
</tbody>
</table>
Table 16 [Chapter 5, Table 3] A comparison of ranking and proportionality in study-types, between those analyzed in Stiling and Cornelissen, 2005, and the analysis completed in 2012.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Stiling and Cornelissen 2005 Analysis</th>
<th>Proportion of studies</th>
<th>2012 Analysis</th>
<th>Proportion of studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agent efficacy</td>
<td>0.25</td>
<td>Agent management, rearing, exploration</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>Oviposition/feeding/behavior</td>
<td>0.14</td>
<td>Agent behavior</td>
<td>0.17</td>
</tr>
<tr>
<td>3</td>
<td>Host specificity and life history</td>
<td>0.11</td>
<td>Agent efficacy</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>Biotic effects</td>
<td>0.10</td>
<td>Multitrophic interactions</td>
<td>0.08</td>
</tr>
<tr>
<td>5</td>
<td>Abiotic effects</td>
<td>0.09</td>
<td>Life history of target</td>
<td>0.08</td>
</tr>
<tr>
<td>6</td>
<td>Pest management</td>
<td>0.07</td>
<td>Philosophical questions</td>
<td>0.08</td>
</tr>
<tr>
<td>7</td>
<td>Agent establishment</td>
<td>0.06</td>
<td>Genetics and molecular methods</td>
<td>0.06</td>
</tr>
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<td>8</td>
<td>Other questions</td>
<td>0.05</td>
<td>Intraguild interactions</td>
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<tr>
<td>9</td>
<td>Natural history</td>
<td>0.05</td>
<td>Agent establishment</td>
<td>0.05</td>
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<tr>
<td>10</td>
<td>Agent management</td>
<td>0.05</td>
<td>Host considerations</td>
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<tr>
<td>11</td>
<td>Non-target effects</td>
<td>0.04</td>
<td>Non-target effects</td>
<td>0.03</td>
</tr>
</tbody>
</table>
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